Handbook of
Hydrocarbon and Lipid Microbiology

Series Editors: Kenneth N. Timmis (Editor-in-Chief)
Matthias Boll · Otto Geiger · Howard Goldfine · Tino Krell
Sang Yup Lee · Terry J. McGenity · Fernando Rojo
Diana Z. Sousa · Alfons J. M. Stams · Robert J. Steffan · Heinz Wilkes

SPRINGER REFERENCE

Robert J. Steffan *Editor*

Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation

Springer

Handbook of Hydrocarbon and Lipid Microbiology

Series Editors

Kenneth N. Timmis (Editor-in-Chief)
Emeritus Professor
Institute of Microbiology
Technical University Braunschweig
Braunschweig, Germany

Matthias Boll
Institute of Biology/Microbiology
University of Freiburg
Freiburg, Germany

Otto Geiger
Centro de Ciencias Genómicas
Universidad Nacional Autónoma de México
Cuernavaca, Morelos, Mexico

Howard Goldfine
Department of Microbiology
University of Pennsylvania
Philadelphia, PA, USA

Tino Krell
Department of Environmental Protection
Estación Experimental del Zaidín
Consejo Superior de Investigaciones Científicas
Granada, Granada, Spain

Sang Yup Lee
Dept. Chem. Engineer. and BioProcess
Korea Adv. Inst. Science and Techn.
Taejon, Korea (Republic of)

Terry J. McGenity
School of Biological Sciences
University of Essex
Colchester, UK

Fernando Rojo
CSIC
Centro Nacional de Biotecnología
Madrid, Spain

Diana Z. Sousa
Laboratory of Microbiology
Wageningen University and Research
Wageningen, The Netherlands

Alfons J. M. Stams
Laboratory of Microbiology
Wageningen University and Research
Wageningen, The Netherlands

Centre of Biological Engineering
University of Minho
Braga, Portugal

Robert J. Steffan
Blue Crab Lure Company
Cape Coral, FL, USA

Heinz Wilkes
ICBM
Carl von Ossietzky University
Oldenburg, Niedersachsen, Germany

This handbook is the unique and definitive resource of current knowledge on the diverse and multifaceted aspects of microbial interactions with hydrocarbons and lipids, the microbial players, the physiological mechanisms and adaptive strategies underlying microbial life and activities at hydrophobic material:aqueous liquid interfaces, and the multitude of health, environmental and biotechnological consequences of these activities.

Scientific Advisory Board
Victor de Lorenzo, Eduardo Diaz, Otto Geiger, Ian Head, Sang Yup Lee, Terry McGenity, Colin Murrell, Balbina Nogales, Roger Prince, Juan Luis Ramos,Wilfred Röling, Eliora Ron, Burkhard Tümmler, Jan Roelof van der Meer, Willy Verstraete, Friedrich Widdel, Heinz Wilkes and Michail Yakimov.

More information about this series at http://www.springer.com/series/13884

Robert J. Steffan
Editor

Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation

With 50 Figures and 25 Tables

Editor
Robert J. Steffan
Blue Crab Lure Company
Cape Coral, FL, USA

ISBN 978-3-319-50432-2 ISBN 978-3-319-50433-9 (eBook)
ISBN 978-3-319-50434-6 (print and electronic bundle)
https://doi.org/10.1007/978-3-319-50433-9

© Springer Nature Switzerland AG 2019
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

For many decades scientists have studied the biotransformation of hydrocarbons by microorganisms. This research often begins with microcosm studies that evaluate the transformation of chemicals in simulated environments, and sometimes leads to the isolation of individual microbes that can transform the target chemical in some way. These studies then can lead to biochemical analyses to identify specific transformation reactions and the responsible enzymes, and to genetic studies to identify and analyze the underlying genetic basis of the observed biotransformations. As illustrated in some of the chapters presented in this volume, these genetic studies can even lead to deep understandings of the intricate networks of regulatory control that allow microorganisms to balance carbon and energy flow even while surviving in chemically rich hostile environments. The use of modern "omic" techniques (e.g., genomics, proteomics, metabolomics, etc.) now allows scientists to evaluate some of these regulatory and biochemical processes at the community level and even in actual environmental samples. Although some of this research has led to fundamental understandings of universal principles or mechanisms, much of the work is targeted toward understanding the fate of chemicals in nature and the development of techniques for remediating polluted environments. Together, these studies form the foundation of the fields of bioremediation and biological waste treatment which involve the purposeful application of microbial catalysts to remediate contaminated environments or treat chemical waste streams, respectively.

In this volume, we bring together international experts to discuss the state of the art in the bioremediation of hydrocarbons. The volume begins with a forward-looking introduction to the field of bioremediation that provides a path forward for the use of modern molecular and biochemical tools to improve the treatment of environmental pollutants. I then provide a first-hand perspective on the many factors that drive the selection and application of advanced technologies at actual remediation sites. After a comprehensive chapter about how actual contaminated industrial sites can be used to enhance bioremediation research, several chapters provide insight into hydrocarbon biodegradation in specific environments, including marine systems, groundwater, shale oil formations, and soils. Other chapters focus on specific hydrocarbon remediation techniques, including natural attenuation, biostimulation, bioaugmentation, co-metabolism, and surfactant enhancement. Two chapters focus on specific classes of pollutants, ether-containing hydrocarbons

(e.g., MTBE, 1,4-dioxane, etc.) and plastics, that have been in the recent forefront of research because of their recalcitrance and public interest. Another comprehensive chapter summarizes decades of research on the genetic regulation of toluene biodegradation in *Pseudomonas*, and it brings us full circle back to our introductory chapter that informs us of how modern "omic" data, built on the foundation of such landmark basic research, can improve remediation in the future. Finally, the volume ends with a very detailed chapter on the use of chemostats to generate essential kinetics data for evaluating biodegradation. This chapter should become a go-to instruction manual for this somewhat lost art that still has great potential for evaluating, understanding, and improving pollution biodegradation.

I am extremely grateful to the authors who shared their valuable time to contribute to this excellent collection of chapters on hydrocarbon biodegradation and bioremediation. For those of you that I have known through our years of interactions, I look forward to seeing you again in the not-too-distant future, and to those of you I have not yet met, I look forward to the chance to someday thank you in person for your help in this effort.

Cape Coral, FL, USA Robert J. Steffan

Acknowledgment

More than 30 years ago as a budding scientist having just received my Ph.D., I arrived at the National Institute for Biotechnology (GBF) in Braunschweig, Germany, for my postdoctoral studies under the direction of Professor Kenneth N. Timmis. We were a new group then that grew rapidly, and with that growth was the need for everyone to accept significant levels of responsibility so that the group could become established and prosper. Professor Timmis provided me with many opportunities at that time, including the chance to mentor students, manage my own small group, review manuscripts, and to help organize conferences. These opportunities and others offered by him throughout my career made me a better scientist and a more active member of the scientific community. This editorial effort may be my last chance to work with Professor Timmis in a scientific capacity as I fade into retirement. I thank Ken for the opportunity to edit this volume, and for his many years of friendship, mentoring, and collaboration.

Contents

1 **Biodegradation and Bioremediation: An Introduction** 1
Víctor de Lorenzo

2 **Developing Bioremediation Technologies for Commercial Application: An Insider's View** 21
Robert J. Steffan

3 **Utility of Industrial Experimental Sites for Developing Analytical, Monitoring, and Remediation Technologies** 33
D. L. Freedman and R. Yu

4 **Bioremediation of Marine Oil Spills** 45
Roger C. Prince and Ronald M. Atlas

5 **Oil Biodegradation in Deep Marine Basins** 71
Terry C. Hazen and Stephen M. Techtmann

6 **Biostimulation Strategies for Enhanced Bioremediation of Marine Oil Spills Including Chronic Pollution** 89
Maria Nikolopoulou and Nicolas Kalogerakis

7 **Weathered Hydrocarbon Biotransformation: Implications for Bioremediation, Analysis, and Risk Assessment** 99
S. Cipullo, K. J. Brassington, S. J. T. Pollard, and F. Coulon

8 **Role of Biosurfactants** 117
Eliora Z. Ron and E. Rosenberg

9 **Plant-Endophyte Partnerships to Assist Petroleum Hydrocarbon Remediation** 123
S. Thijs, N. Weyens, P. Gkorezis, and J. Vangronsveld

10 Removal of Hydrocarbons and Other Related Chemicals via the Rhizosphere of Plants 157
Lázaro Molina, Pieter van Dillewijn, Estrella Duque, Craig Daniels, Tino Krell, Manuel Espinosa-Urgel, María Isabel Ramos-González, Sara Rodríguez-Conde, Miguel A. Matilla, Regina Wittich, Juan Luis Ramos, and Ana Segura

11 Natural Attenuation of Hydrocarbon Compounds in Groundwater 171
Steven F. Thornton

12 In Situ Groundwater Bioremediation 197
Terry C. Hazen

13 Microbiology of Oil- and Natural Gas-Producing Shale Formations: An Overview 215
Christopher G. Struchtemeyer

14 Cometabolic Bioremediation 233
Terry C. Hazen

15 Anaerobic Biodegradation of Hydrocarbons: Metagenomics and Metabolomics 249
Lisa M. Gieg and Courtney R. A. Toth

16 Genomic Responses of *Pseudomonas putida* to Aromatic Hydrocarbons 287
Víctor de Lorenzo and Hiren Joshi

17 Biodegradation of Ether Pollutants 303
Amie McElroy and Michael Hyman

18 Plastic Biodegradation: Challenges and Opportunities 333
Nick Wierckx, Tanja Narancic, Christian Eberlein, Ren Wei, Oliver Drzyzga, Audrey Magnin, Hendrik Ballerstedt, Shane T. Kenny, Eric Pollet, Luc Avérous, Kevin E. O'Connor, Wolfgang Zimmermann, Hermann J. Heipieper, Auxiliadora Prieto, José Jiménez, and Lars M. Blank

19 Determination of Kinetic Parameters and Metabolic Modes Using the Chemostat 363
Rich Boden and Lee P. Hutt

Index 405

About the Series Editor-in-Chief

Kenneth N. Timmis
Emeritus Professor
Institute of Microbiology
Technical University Braunschweig
Braunschweig, Germany

Kenneth Timmis studied microbiology and obtained his Ph.D. at Bristol University. He undertook postdoctoral training at the Ruhr-University Bochum, Yale and Stanford, at the latter two as a Fellow of the Helen Hay Whitney Foundation. He was then appointed Head of an independent research group at the Max Planck Institute for Molecular Genetics in Berlin and subsequently Professor of Biochemistry in the University of Geneva, Faculty of Medicine. Thereafter, for almost 20 years, he was Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI), and concomitantly Professor of Microbiology in the Institute of Microbiology of the Technical University Braunschweig. He is currently Emeritus Professor in this institute.

The Editor-in-Chief has worked for more than 30 years in the area of environmental microbiology and biotechnology, has published over 400 papers in international journals, and is an ISI Highly Cited Microbiology-100 researcher. His group has worked for many years, inter alia, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, and on the ecology of hydrocarbon-degrading microbial communities, discovered the new group of marine oil-degrading hydrocarbonoclastic bacteria, initiated genome sequencing projects on bacteria that are paradigms of microbes

that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*), and pioneered the topic of experimental evolution of novel catabolic activities.

He is Fellow of the Royal Society, Member of the European Molecular Biology Organisation, Fellow of the American Academy of Microbiology, Member of the European Academy of Microbiology, and Recipient of the Erwin Schrödinger Prize. He is the founder and Editor-in-Chief of the journals *Environmental Microbiology*, *Environmental Microbiology Reports*, and *Microbial Biotechnology.*

About the Volume Editor

Dr. Robert J. Steffan received his Ph.D. in biology from the University of Louisville, where he worked on the development of molecular biological tools for monitoring genetically engineered microorganisms in the environment. His work included the first ever application of the polymerase chain reaction (PCR) for monitoring organisms in environmental samples. This early work provided a basis for much of the PCR-based environmental analysis in use today. Following his doctoral studies, he received an Alexander von Humboldt fellowship to perform postdoctoral research at the National Institute for Biotechnology (GBF) in Braunschweig, Germany. There, he worked to identify and genetically engineer biodegradation pathways in bacteria and to evaluate genetic transformation of bacteria in nature. After his postdoctoral studies, he worked in the environmental industry for more than 25 years, where he served as a technical expert to help solve real-world remediation and pollution treatment challenges, and also led a research and development group. His primary research interests were biodegradation of chlorinated solvents and ether-containing pollutants (1,4-dioxane, MTBE, BCEE, etc.), bioaugmentation for remediation, development of novel remediation strategies, and molecular biology of degradative bacteria. He directed the development and commercialization of SDC-9™, a *Dehalococcoides* sp.-containing bacterial culture that has been applied at more than 2000 sites for remediation of chlorinated solvent contaminated groundwater. In addition, he led a program that developed a novel patented combinatorial biocatalysis process that resulted in the production of several unique and patented antibiotic compounds with activity against Gram-positive microorganisms including

multidrug resistant TB. He has authored more than 100 scientific papers, reports, and book chapters, including two invited review articles in the *Annual Review of Microbiology*, and has received 11 patents. He has served as a member of the editorial boards of the *Journal of Bacteriology* and *Applied and Environmental Microbiology*, and was the recipient of the 2008 Excellence in Review Award from Environmental Science & Technology. He retired from his work in the environmental industry in 2014.

Contributors

Ronald M. Atlas Department of Biology, University of Louisville, Louisville, KY, USA

Luc Avérous BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, Strasbourg, France

Hendrik Ballerstedt Institute of Applied Microbiology (iAMB), RWTH Aachen University, Aachen, Germany

Lars M. Blank Institute of Applied Microbiology (iAMB), RWTH Aachen University, Aachen, Germany

Rich Boden School of Biological and Marine Sciences, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK

Sustainable Earth Institute, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK

K. J. Brassington School of Water, Energy and Environment, Cranfield University, Cranfield, UK

S. Cipullo School of Water, Energy and Environment, Cranfield University, Cranfield, UK

F. Coulon School of Water, Energy and Environment, Cranfield University, Cranfield, UK

Craig Daniels Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Developmental and Stem Cell Biology Program, Brain Tumour Research Centre, The Hospital for Sick Children, Toronto, ON, Canada

Víctor de Lorenzo Systems Biology Program, Centro Nacional de Biotecnología-CSIC, Madrid, Spain

Oliver Drzyzga Biological Research Center (CIB-CSIC), Madrid, Spain

Estrella Duque Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Christian Eberlein Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany

Manuel Espinosa-Urgel Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

D. L. Freedman Department of Environmental Engineering and Earth Sciences, Clemson University, Clemson, SC, USA

Lisa M. Gieg Department of Biological Sciences, University of Calgary, Calgary, AB, Canada

P. Gkorezis Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

Terry C. Hazen Department of Civil and Environmental Engineering, Department of Earth and Planetary Sciences, Department of Microbiology, Institute for Secure and Sustainable Environment, University of Tennessee, Knoxville, TN, USA

Oak Ridge National Laboratory, Oak Ridge, TN, USA

Hermann J. Heipieper Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany

Lee P. Hutt School of Biological and Marine Sciences, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK

Michael Hyman Department of Plant and Microbial Biology, North Carolina State University, Raleigh, NC, USA

José Jiménez Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK

Hiren Joshi Bhabha Atomic Research Centre, Kalpakkam, India

Nicolas Kalogerakis School of Environmental Engineering, Technical University of Crete, Chania, Greece

Shane T. Kenny Bioplastech Ltd., Nova UCD, Belfield Innovation Park, University College Dublin, Belfield, Dublin, Ireland

Tino Krell Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Audrey Magnin BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, Strasbourg, France

Miguel A. Matilla Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Amie McElroy Department of Civil, Construction, and Environmental Engineering, North Carolina State University, Raleigh, NC, USA

Lázaro Molina Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Tanja Narancic UCD Earth Institute and School of Biomolecular and Biomedical Science, University College Dublin, Belfield, Dublin, Ireland

Maria Nikolopoulou School of Environmental Engineering, Technical University of Crete, Chania, Greece

Kevin E. O'Connor UCD Earth Institute and School of Biomolecular and Biomedical Science, University College Dublin, Belfield, Dublin, Ireland

BEACON – Bioeconomy Research Centre, University College Dublin, Belfield, Dublin, Ireland

S. J. T. Pollard School of Water, Energy and Environment, Cranfield University, Cranfield, UK

Eric Pollet BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, Strasbourg, France

Auxiliadora Prieto Biological Research Center (CIB-CSIC), Madrid, Spain

Roger C. Prince ExxonMobil Biomedical Sciences, Inc., Annandale, NJ, USA

Juan Luis Ramos Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

María Isabel Ramos-González Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Sara Rodríguez-Conde Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Eliora Z. Ron Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel Aviv, Israel

E. Rosenberg Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel Aviv, Israel

Ana Segura Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Robert J. Steffan Blue Crab Lure Company, Cape Coral, FL, USA

Christopher G. Struchtemeyer Department of Biology, McNeese State University, Lake Charles, LA, USA

Stephen M. Techtmann Department of Biological Sciences, Michigan Technological University, Houghton, MI, USA

S. Thijs Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

Steven F. Thornton Groundwater Protection and Restoration Group, Department of Civil and Structural Engineering, University of Sheffield, Sheffield, UK

Courtney R. A. Toth Department of Biological Sciences, University of Calgary, Calgary, AB, Canada

Pieter van Dillewijn Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

J. Vangronsveld Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

Ren Wei Department of Microbiology and Bioprocess Technology, Institute of Biochemistry, Leipzig University, Leipzig, Germany

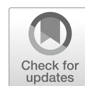
N. Weyens Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium

Nick Wierckx Institute of Applied Microbiology (iAMB), RWTH Aachen University, Aachen, Germany

Regina Wittich Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

R. Yu Department of Environmental Engineering and Earth Sciences, Clemson University, Clemson, SC, USA

Wolfgang Zimmermann Department of Microbiology and Bioprocess Technology, Institute of Biochemistry, Leipzig University, Leipzig, Germany



Biodegradation and Bioremediation: An Introduction

1

Víctor de Lorenzo

Contents

1 Introduction: Biodegradation Versus Bioremediation 2
2 What Is an Environmental Pollutant and What to Do with Them 3
3 Towards Bioremediation 3.0 6
4 Past and Current Challenges 8
5 Biodegradation and Bioremediation in the Times of Systemic Biology 9
6 Overcoming the GMO Controversy? 14
7 Research Needs 15
References 18

Abstract

The scientific interest of the fate of environmental pollutants generated by industrial and urban activities is not only about the pursuit of ways to foster the mitigation or ideally the complete removal from the afflicted sites. It also provides insights on how environmental microorganisms evolve new molecular devices for first tolerating and then catabolizing many of such otherwise toxic molecules. Along with resistance to antibiotics, emergence of fresh biodegradative routes for new compounds is one of the most conspicuous cases of contemporary biological evolution in real time. Understanding the rules of such evolution thus provides new principles for predicting – and in case accelerating – biochemical adaptation to novel chemical structures. These phenomena occur in space and time and also at very different scales depending on the nature and dimension of the pollutants at stake. The impact of contaminants that received considerable attention decades ago is decreasing in many cases owing to better industrial procedures along with growing environmental awareness and legal regulations. Alas, the last decade has

V. de Lorenzo (✉)
Systems Biology Program, Centro Nacional de Biotecnología-CSIC, Madrid, Spain
e-mail: vdlorenzo@cnb.csic.es

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_1

witnessed also the emergence of other types of contaminants (in particular greenhouse gases, plastics, and micropollutants) that threaten not just specific sites but also the functioning of the planet's homeostasis at a global scale. This state of affairs calls for new bioremediation strategies that take into account the multiscale complexity involved in possible interventions much beyond the focus on specific biodegradative pathways. Fortunately, the environmental microbiome and the possibilities of engineering it with the tools of Systems and Synthetic Biology remains the best resource to tackle the phenomenal challenge of preserving the biosphere in good shape for the future generations.

1 Introduction: Biodegradation Versus Bioremediation

The growing industrialization and urbanization of our societies along the last century has left us a heritage of emissions that, whether involving natural or synthetic molecules, have had an impact in virtually every Earth's Ecosystems. Although anthropogenic pollutants have always co-existed with us (e.g., heavy metals), the development of transportation and large-scale manufacturing has not only released quantities of xenobiotic compounds into the biosphere but has also mobilized carbon species that were otherwise trapped geologically in fossil fuels or forming part of natural compounds (Alexander 1999). Scientific studies on microbiological biodegradation of potentially complex organic molecules can be traced to the work initiated in the 1950s by I.C. Gunsalus on transformations of aromatic compounds (Gunsalus et al. 1953) and terpenoids (Conrad et al. 1965) by environmental bacteria. His pioneering research spread recognition of how microorganisms isolated from nature could run amazing reactions that were thus far thought to be possible only through organochemical methods. While the industrial applicability of transformations of this sort became clear very early, realization of the immense potential of the same microorganisms for dealing with pollutants had to wait until the conspicuous environmental crises of the 1980s (e.g., the noticeable effects of the Agent Orange, the Bhopal accident, the Exxon Valdez spill) to receive a considerable public attention – along with the onset of *green* awareness in Germany and the popularization of recombinant DNA technology in laboratories throughout the World. This created a widespread expectation on the capacity of genetic engineering to solve many of Mankind's problems, including environmental deterioration (Lindow et al. 1989).

This combination of circumstances set the motion for both the *science* of biodegradation (i.e., understanding – and ultimately refactoring – how microorganisms catabolize otherwise unpalatable environmental chemicals) and the *technology* of bioremediation (using biological agents for removing or at least alleviating pollution in given sites). Note that the number of variables in each case is quite different. One can address experimentally a biodegradation question by just setting system in which one microbial strain faces one target molecule in the controlled conditions of a Petri dish. In this case, the user can adjust the rest of the environmental conditions at will. In contrast, the physicochemical circumstances of a polluted site are generally prefixed and involve a much larger total of biological and abiotic

factors. The setups for bioremediation research thus encompass more intricate experimental formats that try to capture the principal components of the target site (e.g., in a microcosm or a mesocosm; Teuben and Verhoef 1992). In any case, the challenge in the field has typically involved going from a certain biodegradative property in a strain or a consortium (whether naturally occurring or genetically enhanced) in the Laboratory towards a full-fledged, uncontained process for removal of the pollutant from a specific location. To this end, the generic concept of *bioremediation* is often broken down in various substrategies: i.e., *bio-attenuation* (basically let the indigenous microbes of the polluted site to do the cleanup with no or minimal intervention), *bio-stimulation* (addition of limiting nutrients or electron acceptors to foster the emergence and/or activity of naturally occurring degraders), *bio-augmentation* (inoculation of microbial strains to speed up the rate of degradation of the target contaminant), or *bio-adsorption* (capture of the contaminant on the microbial biomass in a biologically innocuous form). In every case, interventions may eliminate the problem altogether (e.g., *mineralization*) or at least alleviate its effects by, e.g., transforming toxic chemicals in less harmful species (*mitigation*). Note that each of these settings can be further addressed through *intensive* interventions (at the source, typically dealing with concentrated waste) or *extensive* actions (low levels of the pollutant spread through a wide-ranging area). Finally, treatments can be engineered *ex situ* (removal of the contaminated material to a different processing site, e.g., a water or soil treatment plant) or *in situ* (application of whatever decontaminating agent in the same place where the problem occurs). There are also in-between scenarios, such as *soil farming* that may involve partial or total relocation of the polluted layers for a more effective treatment. Ideally, extensive bioremediation with biological agents becomes the method of choice when physical and chemical removal of waste fails to take toxic levels below tolerable concentrations. As a consequence, bioremediation is often pictured as the complete mineralization of a given pollutant that contaminates a large site through *in situ* bio-augmentation with a degradative strain designed or nurtured in the Laboratory for doing the job. Needless to say that such a rosy scenario hardly ever happens in reality.

2 What Is an Environmental Pollutant and What to Do with Them

Intuitively one envisages pollutants as a suite of chemicals of either natural or synthetic origin which, once released into the environment, causes the malfunction of one or more components of an otherwise balanced niche or ecosystem. There are compounds that are completely natural (e.g., oil hydrocarbons) but which have been mobilized by the chemical industry to places that they do not naturally belong to. Others are entirely human-made xenobiotics (e.g., most cloro-organic or nitro-organic molecules) that were often synthesized for the very purpose of being extremely stable. They might be released to the environment deliberately (e.g., DDT and other pesticides) or accidentally (oil spills, PCBs) in different amounts.

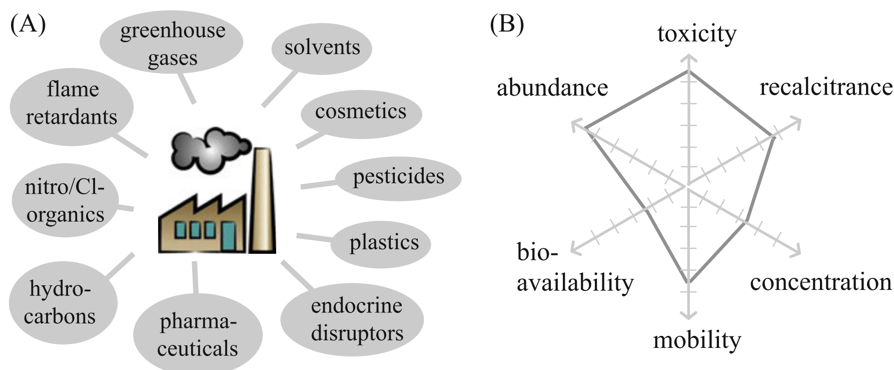


Fig. 1 **Typical anthropogenic emissions**. (**a**) Industrial and urban activities generate molecules that impact negatively the functioning of the biosphere, such as those indicated in the sketch. (**b**) The profile of an environmental pollutant is defined by the six parameters indicated, the outcome of which frames the bioremediation strategy (de Lorenzo et al. 2018)

Their detrimental effects can stem from the inherent properties of the product or may reside in the harmful qualities of their synthesis intermediates or their partial metabolism. Finally, their chemical structures might be amenable to total or limited microbial transformations under specific circumstances – or recalcitrant to biodegradation and thus persist in the afflicted sites for long periods of time. The functionalities of these molecules are very diverse (Fig. 1a), but they form part of our modern society and it is hard to imagine living without them. We leave deliberately heavy metals and metalloids aside the picture, as they raise a different type of problems and possible solutions. Also, whereas intuitively one tends to picture pollutants as structurally intricate toxic compounds (e.g., PCBs or chlorodioxins), other superficially inconspicuous molecules as CO_2, lignin, or plastics become perilous when released at high levels (Eriksen et al. 2014). By the same token, some bioactive molecules (e.g., pharmaceuticals and other so-called *micropollutants*; Schwarzenbach et al. 2006) that are typically found at low levels may have a devastating impact in the corresponding ecosystems. The status of specific chemical species as environmental pollutants goes much beyond their inherent toxicity to biological systems, but encompasses at least the six parameters pictured in Fig. 1b.

Innate toxicity of specific molecular species can be in many cases predicted through the use of various computational platforms that deliver a score on the basis of the molecular structure of the compounds of interest (Mayr et al. 2016). Alas, most of these *in silico* tools neither detect the effects of micropolymers (e.g., partially degraded plastics) in food and reproductive chains nor possible toxic synergies of chemical mixtures. Another key parameter is recalcitrance or biodegrabability – and again users may make preliminary estimations by querying the compound at stake in various biodegradation-prediction platforms (Pazos et al. 2005; Ellis et al. 2006; Hadadi et al. 2016; Wicker et al. 2016; Latino et al. 2017). One study suggested that the ability of a certain chemical to be metabolized through the merged biochemical

network of the known microbiota depends on the frequency of chemical triads (also called *chemotopes*) in the molecule under scrutiny (Gomez et al. 2007). Unfortunately, these predictions say little on the kinetics of such potential degradation, which may vary enormously between different sites. Other parameters include gross physical characteristics (abundance, concentration, and mobility). And finally there is the issue of bioavailability: the fraction of the compounds that is accessible to the biological side. This is a somewhat slippery concept that is often associated to aqueous solubility and the measure of which is claimed to be delivered by dedicated whole-cell biosensors (Harms et al. 2006; van der Meer and Belkin 2010). Unluckily, the dearth of recognized standards for quantifying bioavailability makes this parameter difficult to tackle. In any case, Fig. 1b indicates that the qualification of a molecule as an environmental pollutant is related not just to its toxicity and degradability but also to a number of physicochemical circumstances that make them a lesser or a higher matter of concern.

The fate of the pollutants amenable to bioremediation also deserves a separate comment. As mentioned above, the ideal scenario is complete removal of the harmful compounds from the contaminated sites (e.g., mineralization to CO_2 and H_2O). However, CO_2 is in itself a major issue because of its ramping levels in the atmosphere in recent decades. In this case, bioremediation also includes legitimately the efforts to capture the surplus of CO_2 and convert it into sugars (Antonovsky et al. 2017), polymers, and other useful materials, something at hand with contemporary metabolic engineering. A separate issue is raised by recalcitrant polymeric materials whether synthetic (e.g., plastics) or with a natural origin (lignin). In a first sight, it would be desirable to nurture microorganisms with a superior capacity to degrade them altogether. However, if the catabolism of large amounts of such polymers were complete, we would be converting one pollutant into another (CO_2). And if degradation is not complete, we could be aggravating the problem by generating microplastics and other microparticles (Andrady 2011) that can interfere with the trophic and reproductive chains of many organisms. In these cases, the adequate bioremediation strategy might be just the contrary: either compaction or disposal of the target materials in a biologically unavailable form and conversion into an entirely inert material. These considerations highlight the difficulty to engineer interventions that are both efficacious and safe.

The diversity and complexity associated to real polluted scenarios has often created a big gap between the *science* of biodegradation and the *technology* of bioremediation. Challenges include not just the genetic stability of the agents and the influence of the extremely variable abiotic factors that prevail in polluted sites, but also the frequent syntropy of the members of the resident microbial community for catabolism of complex molecules. Often the key environmental catalyst is not a single strain of one species but a consortium of microorganisms with different degrees of compositional complexity. Moreover, it is often the case that the bacteria that are best at degrading target compounds in nature are not the fast growers that are typically isolated in enrichment experiments. Furthermore, key degradative pathways are hosted by bacteria that cannot be cultivated: their presence and activity are revealed only through meta-genomic, meta-proteomic, and meta-transcriptomic

approaches. Finally, resistance to colonization by exogenously added bacteria and the foraging of predatory protozoa do the rest to check the action of artificially implanted strains. But obviously, most of these caveats were unknown when the field started. Unsurprisingly, the somewhat naïve propositions of the late 1980s and 1990s to rationally create biodegradative *superbugs* which, upon release, could mineralize of many of the worst environmental pollutants, collapsed altogether one decade later (Cases and de Lorenzo 2005). But along the way, a wealth of new knowledge on microbial ecology was unveiled; clear indications of the blockages for effective bioremediation were pinpointed, and much of the fundamental work on unusual catabolic enzymes was repurposed for the sake of industrial biotransformations. In view of all this, where are we now?

3 Towards Bioremediation 3.0

It would not be realistic to deny that the interest on biodegradation and bioremediation that enjoyed a big hype in the late 1980 – mostly due to the work of Timmis', Knackmuss' and Chakrabarty's Laboratories – lost much ground short after because of the failure to deliver the promise of environmental cleanup with genetically engineered microorganisms (Cases and de Lorenzo 2005). There are various follow-up developments that stem from such state of affairs. *First*, the biodegradation and bioremediation research of the 1980s–1990s suggested that one has to consider the target sites as a whole system, including the physicochemical and geological characteristics of the place, the ecology and dynamics of the native microbial community, and the catalytic potential of biological and non-biological components – including what could be called the *post-mortem* enzymatic activities of many microorganisms (French et al. 2014). While the complexity of such systems is indeed high, they offer entirely new opportunities to address them with the tools of systems biology and systems science – which were not available before. *Second*, GM strains with enhanced or entirely new catabolic activities that do well under controlled laboratory conditions generally perform poorly when released into the environment. This is connected to the long-standing biotechnological challenge of how to genetically program microorganisms to stably behave as desired. The issue of *retroactivity* (Gyorgy and Del Vecchio 2014) and *metabolic burden* (Ceroni et al. 2015) between implanted genetic constructs and the preexisting biochemical and regulatory network of the bacterial host goes much beyond the mere stabilization of the new genes by having them encoded in the chromosome (Fig. 2). Let alone that extensive inoculation technologies developed thus far have not been too successful and strains released as agents to clean up specified spots often succumb to predators and/or straight competition with other microbial inhabitants of the place. What one could call *Environmental Galenic Science* (i.e., strategies for maintaining, formulating, and delivering remedies to a sick individual in order to optimize their intake and action) has not really developed much despite the evident bottleneck that that step imposes to bioremediation as an advanced technology. *Third*, the main beneficiary of most of the advances in the field of biodegradation in the last 20 years has not been the

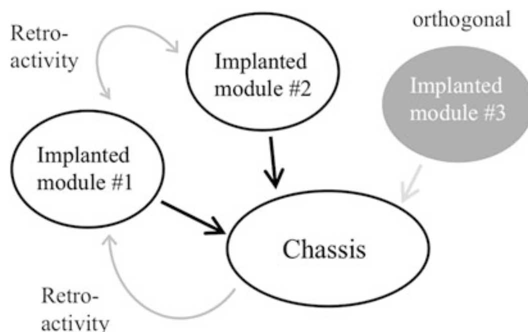


Fig. 2 The interplay between the host's regulatory and metabolic network and the genetic implants. Formalizing context-dependency for the performance of engineered genetic constructs. The preexisting physiological and metabolic host (*chassis*) of engineered devices and the corresponding constructs may mutually compete for cell resources (e.g., ribosomes, energy currency, metabolic building blocks, etc.). This creates a perturbation called *retroactivity.* In the best-case scenario, the implanted genetic modules can be made orthogonal, i.e., have little or no influence with the chassis and with other engineered devices. (Figure from de Lorenzo and Schmidt 2018)

environment, but the industrial biotechnology sector. The wealth of information on the genetic and biochemical diversity of the microbiota that thrives in polluted sites has enabled the setup of bio-based alternatives to chemical processes with whole-cell biocatalysts developed through metabolic engineering. Conspicuous examples include degradable polymers, biofuels and both bulk and fine chemicals, which are bound to take over a large portion of the current market, as oil becomes more and more scarce and expensive as the feedstock for their production (Chae et al. 2017). Furthermore, many emissions can be biologically met at the point of manufacturing, thereby preventing their eventual release and live catalysts can be integrated in zero-pollution industrial pipelines. Indirectly, the onset of biological manufacturing of more and more added-value chemicals contribute to sustainability by replacing otherwise polluting chemical processes. But the direct environmental dividends of the efforts done for two decades on molecular biodegradation and *in situ* bioremediation research have hardly materialized and the most popular technologies for in situ cleanup to this day largely involve attenuation and bio-stimulation (e.g., fertilization with N and P, injection of O_2, NO_3, etc)—bio-augmentation with microorganisms nurtured in the Laboratory still being marginal in comparative terms.

While these developments set the scene for a strong research program that builds on previous successes and failures, reality is that the current commercial value of environment-oriented biotechnology has evolved orders of magnitude below that of health-oriented, agricultural, and other demand-driven counterparts. The share of the bioremediation field within the whole landscape of modern, frontline biotechnology is minimal, a typical case of the paradox between highly relevant research from a social point of view —but low-added value— versus highly profitable alternatives in the biomedical and food sectors. Does this mean that the subject has a bleak scientific and technological future?

The limitations of what has been called Bioremediation 1.0 (based on mere trial-and-error) and Bioremediation 2.0 (fostered by recombinant DNA technology) have become clear above. But at the same time, the last decade has witnessed the emergence of novel environmental challenges along with the onset of game-changing technologies in the life sciences realm that were not there before. Excess of greenhouse gases (CO_2, CH_4, N_2O, chlorofluorocarbons, and hydrofluorocarbons) and the overwhelming abundance of microplastics in marine ecosystems have revealed themselves as the most phenomenal global challenges faced by our generation. Also, lignocellulosic waste, although not toxic by itself, has become a major unwanted residue that impacts the normal functioning of many ecosystems. This is accompanied by the global spread of pharmaceuticals (e.g., antibiotics) and the realization that many known pollutants (dioxin and dioxin-like compounds, polychlorinated biphenyls, DDT) and other pesticides, plasticizers, and flame retardants like bisphenol A behave as endocrine disruptors. Finally, a large number of new-to-nature molecular species with unusual chemical bonds (e.g., ionic liquids) has been synthesized and expected to hold many large-scale applications, but the environmental fate of which is virtually unknown (Jordan and Gathergood 2015). The onset of these new environmental concerns have gone in parallel with the onset of systems and synthetic biology, the availability of amazing volumes of *omics* data and the growing interface of life sciences research with information technologies. The scientific, methodological, and social background of biodegradation and bioremediation interests has thus changed profoundly since the field was created. But the same circumstances pave the way towards Bioremediation 3.0 (Dvorak et al. 2017).

4 Past and Current Challenges

Thirty years ago, the frontline research in the subject of biodegradation/bioremediation involved the cloning, sequencing, characterization, and – whenever possible – genetic enhancement of pathways for catabolism of certain pollutants following one's intuition and limited knowledge, i.e., mostly a trial-and-error endeavor. Now one can predict accurately the metabolic potential of one bacterium and even a complete microbial community by just looking at DNA sequences and their associated transcriptomic, proteomic, and metabolomic data. And all this without having to hold the biological materials in hand. Numerous computational platforms are available to guide the genetic assembly of new pathways (Hadadi et al. 2016; Wicker et al. 2016; Latino and Wicker 2017). The earlier romanticism of microbial ecology about going out to unusual places to collect biodegradative strains has been replaced nearly entirely by hours of bioinformatic analyses of genomes and metagenomes (and other *omes*) in front of a computer. The focus on the pollutants of interest has also changed, as large emissions of apparently innocuous molecules like CO_2 or plastics have revealed themselves as far more dangerous for the planet than intensive contamination of located sites with very toxic compounds (Eriksen et al. 2014). Instead, the most concerning environmental problems are now global and threaten the climate and the biogeological and reproductive cycles that sustain the functioning of Earth.

As discussed above, qualification of a pollutant as such has to be unfolded in a whole of parameters beyond its intrinsic toxicity (Fig. 1b): large volumes of apparently safe but highly mobile molecules might be as risky at a global scale as lower concentrations of a site-bound dangerous compound. Once released, pollutants and emissions may propagate globally beyond the point of production and become a planetary problem. The scale of the problem asks also for new ways of delivering possible remedies, e.g., bioremediation strategies aimed at propagating an enhanced CO_2 capture through the environmental microbiome and spreading plastic-degradation capacity to marine microorganisms (de Lorenzo 2017). The same applies to other globally spread pollutants like pharmaceuticals and endocrine disruptors (de Lorenzo et al. 2018).

The scenario that has therefore developed in recent years is one in which (i) many contaminants have dispersed through virtually every ecosystem, even to supposedly pristine locations; (ii) new molecules now qualify as real or potential environmental pollutants either because of their high concentrations (e.g., CO_2 and greenhouse gases) or unusual chemical structures (e.g., ionic liquids); (iii) the information available on the multiscale responses of the microbiota to environmental stresses (from enzymes to pathways to communities) is unprecedentedly large; and (iv) contemporary systems and synthetic biology allows revisiting traditional pollution problems with conceptual and material tools for handling complex systems that were unheard of time ago. Given this frame, how may the field move further?

5 Biodegradation and Bioremediation in the Times of Systemic Biology

The term *systemic* attempts to merge the two major interpretative structures that contemporary biology has set in motion to address the problem of complexity in live systems. On one hand, systems biology attempts to describe and understand quantitatively biological objects in their entirety – in contrast with the extreme reduccionism of molecular biology. On the other hand, synthetic biology faces the same biological objects through the perspective of electric and mechanical engineering in the pursuit of the relational logic between their components that makes the system work. The tenet of synthetic biology is the famous Feynman's statement that "*. . . what I cannot create I do not understand . . .*" In this respect it has been argued that synthetic biology is *the* authentic genetic engineering, as the term *engineering* is not a metaphor any longer but a veritable framework to both understand and refactor live entities (de Lorenzo and Schmidt 2018). The onset of systemic biology widens tremendously the scope and possibilities of biodegradation and bioremediation, as it allows addressing questions and entertaining intervention strategies for which there were no tools before. In fact, we can consider Bioremediation 3.0 fact the result of the encounter between the field and systemic biology (Dvorak et al. 2017). There are at least three aspects in which such an encounter may bring about entirely new angles to the subject.

The primary feature is the current ease of analyzing and genetically programming many types of microorganisms of environmental relevance such as Pseudomonads

(Nikel et al. 2014, 2016). The last few years have witnessed the emergence of a suite of theoretical and practical tools to rewrite at user's will the genome of bacteria of interest, including chemical synthesis of large chromosomal segments or even the complete genetic complement. This facilitates and accelerates studies on bottlenecks that limit catabolism of the compounds of interest, whether enzymatic, regulatory, or physiological. In some cases, it has been possible to create new enzymes from scratch through a combination of rational protein design and directed evolution approaches (Kan et al. 2016; Arnold 2017). This opens amazing opportunities to engineer agents able to cope with new types of chemicals for which nature has not yet invented a biodegradative solution. Efforts to develop new whole-cell catalysts are also assisted by computational platforms that guide the assembly of new pathways by automatically searching in databases the best enzyme and genes to deliver the activities of interest. The pioneering work of Larry Wackett with the University of Minnesota Biocatalysis and Biodegradation-Database (https://goo.gl/3x76uZ; now hosted and upgraded in EAWAG-ETH https://envipath.org/;Latino and Wicker 2017) has provided a generation of biotechnologists with a user-friendly resource for pathway prediction and assembly. More recently, the ATLAS platform (http://lcsb-databases.epfl.ch/atlas/) and its Pathway Search feature allow the user to search for all the possible routes from any substrate compound to any product (Hadadi et al. 2016). The resulting pathways involve known and novel enzymatic steps that may indicate unidentified enzymatic activities as well as providing potential targets for protein engineering to alter substrate specificity. Thereby assembled pre-pathways could then be optimized by playing *in vivo* with the wealth of biological parts available through various repositories of promoters and other regulatory components (e.g., http://parts.igem.org/). The objective of such an optimization is not so much to express the desired catalytic activity at very high levels, as to ensure that the genetic implants nest well within the broader regulatory and enzymatic network of the host. As mentioned above, the retroactivity between the genetic constructs and the genomic and biochemical chassis of the carrying bacteria (Fig. 2) is one of the frequent reasons of the instability of designer microbes and finding ways to minimize it is one of the key challenges of present-day genetic engineering. In sum, we have now a large number of tools for constructing bacteria *à la carte* – from protein engineering to whole cells. Furthermore, the adoption of CRISPR/Cas9 technology for genome editing (Aparicio et al. 2016, 2017; Choi and Lee 2016; Ricaurte et al. 2018) overcomes many of the concerns traditionally associated to transgenic microorganisms (see below). But the question remains on how to deliver biodegradative activities to a target site and how to scale this up to tackle global pollutants.

A second characteristic of Bioremediation 3.0 is the recognition of consortia rather than single species as the main agents for catabolism of most pollutants. Having one sole strain as the recipient of a complete pathway makes regulation of expression of the genes of interest the only possibility for adjusting the right dose of activity required for a specific application. In reality, it is unusual to find naturally occurring strains that can run by themselves a complex route, in particular for very novel compounds. A consortium can not only divide the catabolic labor between its various components but also let new functions to emerge that could not happen in

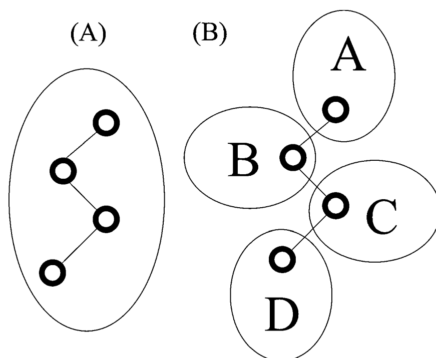


Fig. 3 The single-cell catalyst versus the catalytic consortium dilemma. (**a**) A pathway of interest may appear naturally in a given strain or can be engineered in a single host. However, the background metabolic network of such a single-compartment reactor may not be optimal for each biochemical step of the route. (**b**) An equivalent pathway could be formed by a consortium A, B, C where each member contributes to the biochemical itinerary with a separate enzymatic step. The advantages of this scenario of such a *distributed catalysis* versus the single-cell counterpart are discussed in the text

single cells (Fig. 3). For instance, one member of the community may not contribute to catabolism of the target molecule, but might eliminate an intermediate toxic side-product. Moreover, the tuning of the optimal doses of biochemical activities for each step of the degradative route can be adjusted not just by endogenous, e.g., transcriptional regulation of the pathway in one strain but also by altering community composition and the breakdown of each species in the community. Having separate catabolic steps in different bacteria also allows optimization of the biochemical background of each of them for the best performance of individual reactions. Since the enzymes and transcription factors involved in many biodegradative pathways are often promiscuous, the catabolic capacity of a consortium is often higher than the sum of their components, a phenomenon known as *ectopic metabolism* (de Lorenzo et al. 2010).

Finally, the tridimensional structure of microbial consortia makes a difference in the efficiency of the processes that they catalyze. Designing the architecture of a multi-strain agent thus becomes another point of action where systems-guided engineering can be applied for the sake of better remediating activities. A side-benefit of utilizing communities rather than single strains is that one can artificially assemble a catalyst by putting together specimens that naturally bear one or more of the steps of a pathway of interest. In this case, when brought together, the consortium displays the desired metabolic capacity (Zhou et al. 2015; Ponomarova et al. 2017). Doing this requires a deep knowledge of the metabolic networks of each of the components of the group, but it has the advantage of resulting in a biological material that is not genetically modified. The new wave of Biodegradation could thus expand the former focus on assembling pathways and constructing degradative *superbugs* towards engineering catalysts in their entirety, including consortia with a

desirable physical shape. But still, these developments do not solve the problem of delivering cleanup activities prepared in the Laboratory into the target sites.

This takes us to the final feature of systemic bioremediation: rational spreading of biodegradative activities. As mentioned above, quite in contrast with the many pathway assembly methods there are only a few advanced technologies for releasing catabolic agents to the environment – whether natural or GM – in an efficacious form beyond mere spreading of the agents in a aqueous suspension. These include inter alia formulations with plant seeds (e.g., for rhizoremediation), adsorption to corncobs (Raina et al. 2008), trapping in silicon tubing (Mertens et al. 2006), dispersal with foam and packaging in gelatin capsules (de las Heras and de Lorenzo 2011). In many instances, such carriers are combined with additives that prolong the lifespan and shelf life of the biological components. Larger-scale bioremediation interventions may combine also biological activities with some type of civil engineering (e.g., reactive barriers) or blending with electrostimulation (Mena et al. 2016) in which electrical current serves as either electron donor or acceptor of the biological process.

While these could be good solutions for specific scenarios, such inoculation methods still need to be designed on a case-by-case basis and in no instance bioaugmentation is scalable to a very large level, as required for tacking global emissions. In the meantime, data on massive horizontal gene transfer has revealed how quickly a new trait (e.g., antibiotic resistances) spreads through the entire planet provided that there is enough selective pressure (Loftie-Eaton et al. 2015). Under the right conditions, DNA seems to move easily through the entire complexity pyramid (Fig. 4), and genetic innovations that appear in one genome may disperse through the environmental microbiome in just a few years. The idea of developing DNA superdonors able to implant and propagate particular activities in a community of recipients has been considered at various times throughout the history of bioremediation (Top et al. 1998; Dejonghe et al. 2000). Alas, the genetic tools available to engineer such occurrences were very limited and the concept has not been really developed to its full potential. But, this is not the end of the notion. There is a suite of plasmid-based DNA transfer systems that are promiscuous and active enough to think along the line. Furthermore, the role of phages to propagate new genotypes in a large bacterial population is becoming increasingly evident (von Wintersdorff et al. 2016). However, expression signals vary dramatically from one host to the other, and one pathway engineered in *E. coli* may not work at all when passed to another host whether through plasmid-based or phage-based systems. Synthetic Biology allows addressing this caveat through engineering of 5′ regions of the genes of interest bearing very promiscuous (if not universal) expression signals. The problem remains, however, on how to foster propagation of preset DNA sequences in the absence of a major selective pressure. In reality, this challenge is by no means exclusive of large-scale bioremediation, but it is a general one: how to stimulate propagation of beneficial traits through a population without an exogenous force to push it. For diploid species, a sophisticated strategy called *gene drives* has been developed which allows transmission of a given genetic cargo to the progeny at frequencies above the mere Mendelian distribution of inherited traits (Champer et al. 2016). After a few

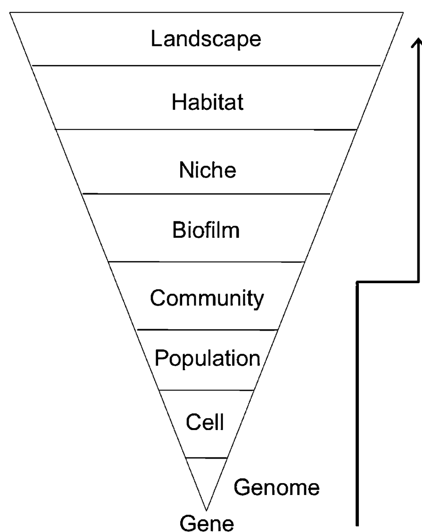


Fig. 4 **DNA propagates very quickly through the multiscale complexity pyramid**. As the studies on global spread of antibiotic resistance have shown, mutations that emerge in a single genome may spread very rapidly through a suite of horizontal gene transfer mechanisms to eventually reach out virtually every ecosystem – provided that there is a strong selective pressure to do so. We argue that Bioremediation research could learn from such mechanisms of dispersal in order to engineer dissemination of beneficial traits (e.g., CO_2 capture, degradation of microplastics, removal of micropollutants, etc.) at a global scale

reproductive cycles, this allows them to eventually spread to all members of the target population. The potential power of the technology has raised serious safety concerns, as the method could be use to precisely eliminate particular species (e.g., by propagating infertility traits; Oye et al. 2014). But by the same token, one could think on using the approach for dissemination of activities that are intrinsically beneficial for the environment, e.g., CO_2 capture, biodegradation of plastics, and mineralization of endocrine disruptors. That bacteria are generally haploids prevents doing something similar to what has been successfully attempted in yeasts and animals, but one could entertain scenarios in which cargoes engineered inside promiscuous plasmids or phages are directed to very conserved chromosomal regions of a suite of species and force recombination or else die. Whether adopting this strategy or formulating others for spreading DNA-bearing biodegradative activities (rather than degradative strains) remain one of the key challenges in the field. In this respect, while Bioremediation 2.0 was much concerned with containment of the agents engineered in the laboratory, Bioremediation 3.0 might walk in the opposite direction and explore possibilities for maximizing horizontal gene transfer. Note that discharges of greenhouse gases and microplastics in the oceans require interventions that go beyond merely decreasing emissions or acting on a limited number of sites (de Lorenzo et al. 2018). The much-debated geoengineering of planet Earth could potentially be complemented or even replaced by large-scale bioremediation strategies to capture

CO_2 and to improve the capacity of marine microorganisms to act on plastics and other globally widespread micropollutants. As before, there is a question mark on whether or not the public will accept such unprecedented actions for handling emissions, which are reminiscent of *Terraforming* (de Lorenzo 2017).

In sum: while the *science* of biodegradation will remain focused on pathways, hosts, communities, and their interplay with the physicochemical constituencies of polluted site, the *technology* of bioremediation will move forward taking stock of past failures and capitalizing on the many opportunities and tools brought up by contemporary systems and synthetic biology. This includes methods that allow assembly of new pathways and enhancement of the catabolic capacity of a large community without necessarily relying on the release of transgenic GMOs.

6 Overcoming the GMO Controversy?

Although the primary reason for the difficulties of Bioremediation 2.0 to deliver sound intervention strategies was not public acceptance, the mere proposition to release genetically for environmental cleanup ignited a major argument between pro-GMO and anti-GMO parties that continues to this day. While massive evidence indicates that the impact of recombinant microorganisms when released for bioremediation purposes is not worse than naturally occurring counterparts, a large part of the public still invokes the Black Swan[1] argument (Taleb 2007) to oppose any purposeful liberation of genetically *manipulated* agents. The first concern is about the spreading non-natural genetic information (e.g., recombinant DNA) or GM strains into a new niche where the effects might be unknown. Early in the history of Bioremediation 2.0, this question was thoroughly tackled through (i) stabilization of transgenic genes in the chromosome of the carrier bacterium (in contrast to the use of transmission-prone plasmids), (ii) active killing of the bacterium at stake once the job has been completed – or when it departs from a specific target scenario, and (iii) vigorous barriers to horizontal gene transfer with conditional suicide genetic circuits (Ramos et al. 1995). While these genetic devices increased containment by various orders of magnitude, the figures never reached a full 100%. This was due to spontaneous mutations and the activity of mobile insertion sequences, an issue hardly tractable by that time. This same question has been picked again more recently by synthetic biologists in the pursuit of certainty of containment (CoC) for highly refactored microorganisms. The favourite approach in this case involves the emancipation of one of the stop codons and its recoding to guide insertion of non-natural amino acids in the structure of essential proteins. This makes viability of the bacterium entirely dependent on addition to the medium of such chemically synthesized amino acid (Rovner et al. 2015). Although the level of containment of such strains is extraordinary compared to previous ones, they are still above CoC.

[1]The metaphor developed by Nassim Taleb to argue that the non-occurrence of a certain event thus far is not an evidence that it cannot happen.

Attempts to move the figure still further up involve altering the genetic code, bear the genetic information in a non-DNA molecule, or replace one or more of the nucleotides with chemically synthesized alternatives (Schmidt and de Lorenzo 2012, 2016). In this way, the thereby modified genetic information could not be read by any potential capturer, which could not interpret standard DNA either. Whether or not these approaches will be useful for agents to be released is unclear, because the resulting strains may have lost much of the necessary competitive fitness of naturally occurring bacteria. And in any case, it is unlikely that the anti-GMO community could accept strains that are far more engineered than the first generation of environmental recombinants. Is there a way of producing advanced and efficacious bioremediating agents or strategies that circumvent this problem and thus ease public sympathy for the technology?

In the paragraphs above, we have hinted at some approaches to this challenge. At the time of writing this article, biological systems (including microorganisms) that have their genome edited with CRISPR/Cas9 technology do not qualify as GMOs proper (Waltz 2016). This opens a window of opportunity for their application to problems that were not amenable before because of the *transgenic* tag. A second possibility is systems-guided assembly of naturally occurring strains to form a catalytic consortium, with the added advantages mentioned before readjusting of expression levels and possibilities to design their 3D architecture. Also, propagating DNA rather than GMOs could be a way to go to scale up interventions (Fig. 4). Finally, we can entertain the design of cell-free agents (Karig 2017) or DNA-free cells (Rampley et al. 2017) that capture the pathways of interest and deliver their catabolic activities but are unable to spread beyond the site of application. However, the question remains on whether we can develop new catabolic and enzymatic activities without resorting to genetic engineering.

7 Research Needs

To address how biodegradation and bioremediation research may look like in the future, it is useful to look back into some of the earlier studies, in particular what was called by the time plasmid-assisted molecular breeding (Kellogg et al. 1981), a technology that become popular before the spreading of recombinant DNA methods. The key idea was to start with a complex community of microorganisms retrieved from sites with a history of pollution by the target compound, some of which bearing plasmids with catabolic genes. Progressive selection of best growers in a chemostat eventually led to isolation of strains that incorporated in their genome the complement of genes that could afford biodegradation of an otherwise recalcitrant compound (e.g., 2,4,5-trichlorophenoxyacetic acid). The solution to the metabolic problems was thus the result of horizontal gene transfer and spontaneous mutagenesis. Although the method was well-liked for a while, it was soon replaced by more directed approaches where the user had a better control of the changes leading to a degradative phenotype. The positive side of molecular breeding was, however, that the resulting strains were not GMOs and the assembled pathways non-recombinant, thereby enabling their

immediate application if necessary. If we used today's systems terminology, we could describe the setup as a case where a given metabolic problem is embodied in a material object (the starter microbial community) and the system let to fluctuate for exploring the solution space upon application of a selective force. And the result is a physical entity (a strain) that has gone through a multi-objective optimization. For the new strain to grow, the system had to solve not just the assembly of the metabolic route proper but also its adequate nesting in the host's biochemical and regulatory network (Fig. 2). We argue that such methodology could be revisited in the times of systemic biology for generating new strains and properties that may not be amenable to forward design with the level of knowledge we have today.

While evolutionary optimization is the method of choice for fine-tuning expression parameters of pathways assembled in given hosts, its utility can be upgraded to generate new biodegradative strains and new consortia that could do ultimately better than any forward-designed alternative. The ease of DNA sequencing available today allows us to determine evolutionary itineraries in single strains and complete communities (Celiker and Gore 2014) and principles to guide further actions. Under the right selective pressure, bacteria seem to be able to invent reactions that were difficult to implement otherwise (Donnelly et al. 2018). It has been recently reported that metabolic stress resulting from faulty redox reactions generate reactive oxygen species (ROS), which in turn, accelerates diversification and solution-finding of the corresponding bacteria to overcome the metabolic bottleneck (Perez-Pantoja et al. 2013). This phenomenon, which might be at the basis of the rapid evolution of Rieske non-heme iron oxygenases (Pérez-Pantoja et al. 2016) could be exploited under a directed evolution setup to speed up discovery of enzymes able to cope with new substrates (Fig. 5). Development of microbial *activity farms* that combine the chemical computation power of naturally occurring

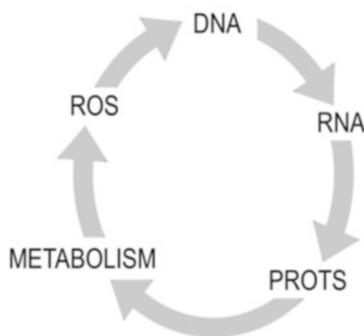


Fig. 5 **The stress-genetic innovation cycle**. The figure sketches how metabolic troubles (e.g., faulty oxidation of no-substrates or poor substrates of oxygenases) cause reactive oxygen species (ROS), which mutates DNA (as well as damaging RNA), triggers the SOS response and brings about genetic diversification, which might find a solution to the metabolic problem that originates the release of the mutagenic agent. While the ROS → DNA loop does not involve transfer of coded information, it does deliver an input that accelerates the rate of novelty production

bacteria with human-made DNA amendments could in fact result in highly evolvable systems. They could find catabolic solutions to virtually every present or future biodegradation challenge, and materialize the result in the form of mono-strains or consortia that are not GMOs.

Figure 6 shows a streamlined roadmap of bio-based approaches for tackling environmental pollution, from prevention to global-scale remediation. The process typically involves identification of new catalytic properties in a strain or a community – natural or human-designed – and it follows their utilization in prevention, monitoring, or bioremediation interventions. These diversify depending on a large number of parameters and the dimension of the problem, from local to global.

Most contributions that follow this Introductory Chapter to the volume on *biodegradation and bioremediation* bear witness of the impasse triggered by the transition between stages 2.0 and 3.0 in the field, as discussed at length above. The former emphasis on genetic engineering as the main driver of the field has been largely left aside in favor of less risky and more acceptable approaches based on sound microbial ecology, geobiochemistry, and physicochemical methods. Also, the articles reflect the effort to understand in detail what is going in the biological realm during natural attenuation of pollution with or without much human intervention (e.g., biostimulation). But also, a large share of the work herein reported capitalizes on the suite of *omics* technologies that allow a detailed follow up of responses of individual strains, community composition, and activity monitoring in very different bioremediation scenarios. In the meantime, new and acute environmental challenges have become noticeable and cannot be ignored (e.g., global greenhouse emissions, plastics, and micropollutants) while novel conceptual and material tools have arrived – in particular those of synthetic biology. A new encounter between the immense possibilities of these new fields and the pursuit of remedies for both chronic and new environmental problems is not only desirable but also unavoidable. And some of the chapters also testify that frontline technologies can open avenues for solving thus far intractable pollution puzzles.

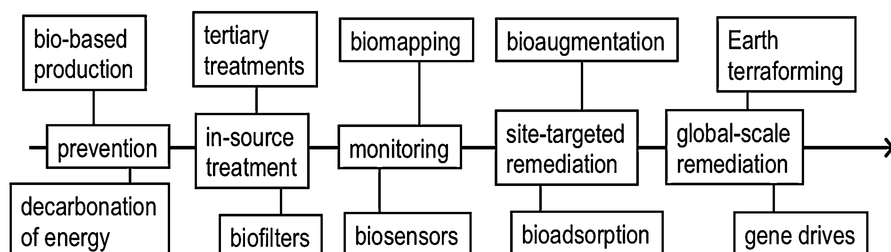


Fig. 6 Bio-based approaches to tackle environmental pollution, from prevention to global-scale remediation. The direction of the arrow indicates the increasing complexity and diversification of the technologies involved (see de Lorenzo et al. 2018 and text for explanation). Some of the items shown in the flow are addressed separately in other Volumes of this series

Acknowledgments This work was funded by the HELIOS Project of the Spanish Ministry of Economy and Competitiveness BIO 2015-66960-C3-2-R (MINECO/FEDER), ARISYS (ERC-2012-ADG-322797), EmPowerPutida (EU-H2020-BIOTEC-2014-2015-6335536), MADONNA (H2020-FET-OPEN-RIA-2017-1-766975), Contracts of the European Union, and InGEMICS-CM (B2017/BMD-3691) contract of the Comunidad de Madrid.

References

Alexander M (1999) Biodegradation and bioremediation. Academic, San Diego

Andrady AL (2011) Microplastics in the marine environment. Mar Pollut Bull 62:1596–1605

Antonovsky N, Gleizer S, Milo R (2017) Engineering carbon fixation in *E. coli:* from heterologous RuBisCO expression to the Calvin-Benson-Bassham cycle. Curr Opin Biotechnol 47:83–91

Aparicio T, Jensen SI, Nielsen AT, de Lorenzo V, Martinez-Garcia E (2016) The Ssr protein (T1E_1405) from *Pseudomonas putida* DOT-T1E enables oligonucleotide-based recombineering in platform strain *P. putida* EM42. Biotechnol J 11:1309–1319

Aparicio T, de Lorenzo V, Martinez-Garcia E (2017) CRISPR/Cas9-based counterselection boosts recombineering efficiency in *Pseudomonas putida*. Biotechnol J. https://doi.org/10.1002/biot.20$32#1700161

Arnold FH (2017) Directed evolution: bringing new chemistry to life. Angew Chem Int Ed Engl 57:4143

Cases I, de Lorenzo V (2005) Genetically modified organisms for the environment: stories of success and failure and what we have learned from them. Int Microbiol 8:213–222

Celiker H, Gore J (2014) Clustering in community structure across replicate ecosystems following a long-term bacterial evolution experiment. Nat Commun 5:4643

Ceroni F, Algar R, Stan GB, Ellis T (2015) Quantifying cellular capacity identifies gene expression designs with reduced burden. Nat Methods 12:415–418

Chae TU, Choi SY, Kim JW, Ko YS, Lee SY (2017) Recent advances in systems metabolic engineering tools and strategies. Curr Opin Biotechnol 47:67–82

Champer J, Buchman A, Akbari OS (2016) Cheating evolution: engineering gene drives to manipulate the fate of wild populations. Nat Rev Genet 17:146

Choi KR, Lee SY (2016) CRISPR technologies for bacterial systems: current achievements and future directions. Biotechnol Adv 34:1180–1209

Conrad HE, Hedegaard J, Gunsalus IC (1965) Lactone intermediates in the microbial oxidation of (+)-camphor. Tetrahedron Lett 10:561–565

de las Heras A, de Lorenzo V (2011) In situ detection of aromatic compounds with biosensor *Pseudomonas putida* cells preserved and delivered to soil in water-soluble gelatin capsules. Anal Bioanal Chem 400:1093–1104

de Lorenzo V (2017) Seven microbial bio-processes to help the planet. Microb Biotechnol 10:995–998

de Lorenzo V, Schmidt M (2018) Biological standards for the Knowledge-Based BioEconomy: what is at stake. N Biotechnol 40:170–180

de Lorenzo V, Fraile S, Jiménez J (2010) Emerging systems and synthetic biology approaches to hydrocarbon biotechnology. In: Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 1411–1435

de Lorenzo V, Prather KLJ, Chen GQ, O’Day E, von Kameke C, Oyarzún DA et al (2018) The power of synthetic biology for bioproduction, remediation and pollution control. EMBO Rep 19 (4): e45658

Dejonghe W, Goris J, El Fantroussi S, Hofte M, De Vos P, Verstraete W, Top EM (2000) Effect of dissemination of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation plasmids on 2,4-D degradation and on bacterial community structure in two different soil horizons. Appl Environ Microbiol 66:3297–3304

Donnelly AE, Murphy GS, Digianantonio KM, Hecht MH (2018) A de novo enzyme catalyzes a life-sustaining reaction in *Escherichia coli*. Nat Chem Biol. https://doi.org/10.1038/nchembi$32#o.2550
Dvorak P, Nikel PI, Damborsky J, de Lorenzo V (2017) Bioremediation 3.0: engineering pollutant-removing bacteria in the times of systemic biology. Biotechnol Adv 35:845–866
Ellis LB, Roe D, Wackett LP (2006) The University of Minnesota biocatalysis/biodegradation database: the first decade. Nucleic Acids Res 34:D517–D521
Eriksen M, Lebreton LC, Carson HS, Thiel M, Moore CJ, Borerro JC et al (2014) Plastic pollution in the world's oceans: more than 5 trillion plastic pieces weighing over 250,000 tons afloat at sea. PLoS One 9:e111913
French HK, Kastner M, van der Zee SE (2014) New approaches for low-invasive contaminated site characterization, monitoring and modelling. Environ Sci Pollut Res Int 21:8893–8896
Gomez MJ, Pazos F, Guijarro FJ, de Lorenzo V, Valencia A (2007) The environmental fate of organic pollutants through the global microbial metabolism. Mol Syst Biol 3:114
Gunsalus IC, Gunsalus CF, Stanier RY (1953) The enzymatic conversion of mandelic acid to benzoic acid. I. Gross fractionation of the system into soluble and particulate components. J Bacteriol 66:538–542
Gyorgy A, Del Vecchio D (2014) Modular composition of gene transcription networks. PLoS Comput Biol 10:e1003486
Hadadi N, Hafner J, Shajkofci A, Zisaki A, Hatzimanikatis V (2016) ATLAS of biochemistry: a repository of all possible biochemical reactions for synthetic biology and metabolic engineering studies. ACS Synth Biol 5:1155–1166
Harms H, Wells MC, van der Meer JR (2006) Whole-cell living biosensors-are they ready for environmental application? Appl Microbiol Biotechnol 70:273–280
Jordan A, Gathergood N (2015) Biodegradation of ionic liquids–a critical review. Chem Soc Rev 44:8200–8237
Kan SB, Lewis RD, Chen K, Arnold FH (2016) Directed evolution of cytochrome c for carbon-silicon bond formation: bringing silicon to life. Science 354:1048–1051
Karig DK (2017) Cell-free synthetic biology for environmental sensing and remediation. Curr Opin Biotechnol 45:69–75
Kellogg ST, Chatterjee DK, Chakrabarty AM (1981) Plasmid-assisted molecular breeding: new technique for enhanced biodegradation of persistent toxic chemicals. Science 214:1133–1135
Latino DA, Wicker J, Gütlein M, Schmid E, Kramer S, Fenner K (2017) Eawag-Soil in enviPath: a new resource for exploring regulatory pesticide soil biodegradation pathways and half-life data. Environ Sci Process Impacts 19:449–464
Lindow SE, Panopoulos NJ, McFarland BL (1989) Genetic engineering of bacteria from managed and natural habitats. Science 244:1300–1307
Loftie-Eaton W, Yano H, Burleigh S, Simmons RS, Hughes JM, Rogers LM et al (2015) Evolutionary paths that expand plasmid host-range: implications for spread of antibiotic resistance. Mol Biol Evol 33:885–897
Mayr A, Klambauer G, Unterthiner T, Hochreiter S (2016) DeepTox: toxicity prediction using deep learning. Front Environ Sci 3:80
Mena E, Villaseñor J, Cañizares P, Rodrigo M (2016) Effect of electric field on the performance of soil electro-bioremediation with a periodic polarity reversal strategy. Chemosphere 146:300–307
Mertens B, Boon N, Verstraete W (2006) Slow-release inoculation allows sustained biodegradation of gamma-hexachlorocyclohexane. Appl Environ Microbiol 72:622–627
Nikel PI, Martinez-Garcia E, de Lorenzo V (2014) Biotechnological domestication of pseudomonads using synthetic biology. Nat Rev Microbiol 12:368–379
Nikel PI, Chavarria M, Danchin A, de Lorenzo V (2016) From dirt to industrial applications: *Pseudomonas putida* as a Synthetic Biology chassis for hosting harsh biochemical reactions. Curr Opin Chem Biol 34:20–29
Oye KA, Esvelt K, Appleton E, Catteruccia F, Church G, Kuiken T et al (2014) Regulating gene drives. Science 345:626–628

Pazos F, Guijas D, Valencia A, de Lorenzo V (2005) MetaRouter: bioinformatics for bioremediation. Nucleic Acids Res 33:D588–D592
Perez-Pantoja D, Nikel PI, Chavarria M, de Lorenzo V (2013) Endogenous stress caused by faulty oxidation reactions fosters evolution of 2,4-dinitrotoluene-degrading bacteria. PLoS Genet 9: e1003764
Pérez-Pantoja D, Donoso R, Junca H, Gonzalez B, Pieper D (2016) Phylogenomics of aerobic bacterial degradation of aromatics. In: Aerobic utilization of hydrocarbons, oils and lipids. Springer International Publishing AG. pp 1–48
Ponomarova O, Gabrielli N, Sevin DC, Mulleder M, Zirngibl K, Bulyha K et al (2017) Yeast creates a niche for symbiotic lactic acid bacteria through nitrogen overflow. Cell Syst 5:345–357.e346
Raina V, Suar M, Singh A, Prakash O, Dadhwal M, Gupta SK et al (2008) Enhanced biodegradation of hexachlorocyclohexane (HCH) in contaminated soils via inoculation with *Sphingobium indicum* B90A. Biodegradation 19:27–40
Ramos JL, Andersson P, Jensen LB, Ramos C, Ronchel MC, Diaz E et al (1995) Suicide microbes on the loose. Biotechnology (NY) 13:35–37
Rampley CPN, Davison PA, Qian P, Preston GM, Hunter CN, Thompson IP et al (2017) Development of SimCells as a novel chassis for functional biosensors. Sci Rep 7:7261
Ricaurte DE, Martinez-Garcia E, Nyerges A, Pal C, de Lorenzo V, Aparicio T (2018) A standardized workflow for surveying recombinases expands bacterial genome-editing capabilities. Microb Biotechnol 11:176–188
Rovner AJ, Haimovich AD, Katz SR, Li Z, Grome MW, Gassaway BM et al (2015) Recoded organisms engineered to depend on synthetic amino acids. Nature 518:89–93
Schmidt M, de Lorenzo V (2012) Synthetic constructs in/for the environment: managing the interplay between natural and engineered Biology. FEBS Lett 586:2199–2206
Schmidt M, de Lorenzo V (2016) Synthetic bugs on the loose: containment options for deeply engineered (micro)organisms. Curr Opin Biotechnol 38:90–96
Schwarzenbach RP, Escher BI, Fenner K, Hofstetter TB, Johnson CA, Von Gunten U, Wehrli B (2006) The challenge of micropollutants in aquatic systems. Science 313:1072–1077
Taleb NN (2007) The black swan: the impact of the highly improbable. Random House, New York
Teuben A, Verhoef H (1992) Relevance of micro-and mesocosm experiments for studying soil ecosystem processes. Soil Biol Biochem 24:1179–1183
Top EM, Van Daele P, De Saeyer N, Forney LJ (1998) Enhancement of 2,4-dichlorophenoxyacetic acid (2,4-D) degradation in soil by dissemination of catabolic plasmids. Antonie Van Leeuwenhoek 73:87–94
van der Meer JR, Belkin S (2010) Where microbiology meets microengineering: design and applications of reporter bacteria. Nat Rev Microbiol 8:511–522
von Wintersdorff CJH, Penders J, van Niekerk JM, Mills ND, Majumder S, van Alphen LB et al (2016) Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front Microbiol 7:173
Waltz E (2016) Gene-edited CRISPR mushroom escapes US regulation. Nature 532:293
Wicker J, Lorsbach T, Gutlein M, Schmid E, Latino D, Kramer S, Fenner K (2016) enviPath – the environmental contaminant biotransformation pathway resource. Nucleic Acids Res 44: D502–D508
Zhou K, Qiao K, Edgar S, Stephanopoulos G (2015) Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nat Biotechnol 33:377–383

Developing Bioremediation Technologies for Commercial Application: An Insider's View

2

Robert J. Steffan

Contents

1 Introduction 22
2 Lesson 1: Money Rules 23
3 Lesson 2: Know the Market 24
4 Lesson 3: Learn New Languages 27
5 Lesson 4: Be Flexible 28
6 Lesson 5: Be Decisive 28
7 Lesson 6: It Is All About Marketing 29
8 Lesson 7: Learn to Predict the Future 30
9 Research Needs 31
References 32

Abstract

For several decades, there has been a conflict between those who release pollutants into the environment and/or own polluted sites and those who wish those releases to be curtailed and want contaminated sites to be remediated. The polluters have generally been businesses that are most interested in controlling costs and maximizing profits or government agencies who often had other priorities of national interest (e.g., war efforts). Those opposed to pollution have sought to reduce the exposure risks of toxic chemicals and to ensure the sustainability of the natural environment. Some of these conflicts came to a peak in the 1970s in the United States, and they ultimately led to the creation of strong environmental laws that forced polluters to control emissions and to reduce the risk associated with contaminants on their sites. As a result of these new rules, a profit-driven environmental remediation and pollution control industry also

R. J. Steffan (✉)
Blue Crab Lure Company, Cape Coral, FL, USA
e-mail: Drrjs@comcast.net

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_26

emerged. The goal of this industry is primarily to help the polluters meet their regulatory requirements by developing and applying remedial technologies, helping the companies or agencies evaluate and implement technologies as they emerge, and/or ensuring regulatory compliance. During my 25-year career in this environmental remediation industry, I observed how technologies were selected for application at given sites, and I learned about the many subtle factors and conflicting interests that affected such decisions. I also observed the role that scientists can play in this high-stakes game that pits the profit motives of two parties and the legal requirements of a third party against one another to, hopefully, meet the needs of all parties. Herein I describe some of the lessons I learned with the hope of helping current and future scientists and entrepreneurs to develop and market successful technologies to improve waste treatment and pollution remediation.

1 Introduction

The implementation of strong environmental laws since the 1970s led to the creation of an industry focused on cleaning up contaminated sites and treating waste streams. The industry is composed of many profit-driven firms that vary in size from sole proprietorships to multi-national corporations. These remediation firms typically are dominated by engineers, and they focus on choosing and implementing technologies for clients who are forced to comply with the environmental regulations. Technology developers attempt to find solutions for addressing these remediation and waste treatment challenges and then either implement them themselves or sell them to the end users. In most cases the developed technologies are based on solid science and engineering principles. During my 25-year career as a technology developer and scientist in the environmental remediation industry I gained a unique perspective on how the business of remediation operates, and its relationship to scientific research. During this time I observed how particular technologies were chosen for application at a given site, and more importantly, why there was often resistance to the application of novel techniques or technologies. Herein, I attempt to provide some insight into how I saw the industry operate over the period from 1990 to 2015, with the goal of helping others prepare to work in this industry, but also to help researchers better understand forces that drive the selection and application of remedial technologies. The views I express here are mine alone.

After completing my graduate and postdoctoral studies, I began my career in a start-up environmental bioremediation company known as Envirogen, Inc (Envirogen). Envirogen was a venture capital-funded company that planned to follow the biotechnology industry model of performing cutting edge bioremediation research and then commercializing the research product at high profit margins. The founders' vision was to find biological solutions to remediate the most challenging contaminants (i.e., those that were not easily treated by using available conventional technologies), and also to develop improved treatment systems (i.e., bioreactors) that could be deployed to apply the new technologies. During this time, genetic engineering technologies were revolutionizing the biotechnology industry, and a few

releases of genetically engineered microorganisms (GEMs) into the environment had been performed (Wilson and Lindow 1993). Consequently, the development and application of GEMs for site remediation was a priority. The initial target contaminants chosen by the company were polychlorinated biphenyls (PCBs) which had been widely used as transformer oils and chlorinated solvents (e.g., perchloroethylene [PCE] and trichloroethylene [TCE]) that were used in a wide range of cleaning applications. The company had raised sufficient funds to support both the initial research efforts and company administration. Within a short time, Envirogen built a commendable reputation by recruiting well-known and well-connected scientists and officers, hiring enthusiastic laboratory researchers, and by presenting its work in traditional scientific ways (i.e., publications and presentations at scientific conferences, etc.). Below are some of the lessons that I learned during this time. I believe many of these lessons are still true today and will be valuable to future entrepreneurs and scientists trying to navigate the business of environmental remediation and waste treatment.

2 Lesson 1: Money Rules

In the early days of my career, I often struggled with understanding how remedial technologies were selected (or not selected), and I saw that certain technologies often were chosen and applied at sites even if they were unlikely to result in complete or rapid remediation of the contaminants of concern. For example, pump-and-treat technologies that relied on pumping groundwater to the surface and treating it by air stripping or carbon adsorption were often applied to chlorinated solvent-contaminated aquifers, even though years of experience had demonstrated that these systems must typically be operated for decades to effectively remove contaminant sources. In most cases, technology selection appeared to be based primarily on cost. Furthermore, I learned that cost has many faces.

It is important to realize that the goal of many companies with polluted sites is not to clean up a particular site, but rather to meet their regulatory requirements while minimizing expenditures. Any money spent to remediate a site is money that cannot be used for producing products or rewarding shareholders. Environmental issues are “liabilities” not “assets.” Furthermore, many of these liabilities are the result of past activities yet they negatively impact current business success. Thus, this concept of spending as little as possible on site remediation makes perfect sense from a business perspective. Likewise, the timing of expenditures can be equally as important to a company as the overall cost. That is, expenses spread over a long period may have far less impact on a current balance sheet than expenses expended over a short period, even if the overall cost for long-term expenditure is much higher. Therefore, some understanding of the time value of money is necessary.

Evaluating the true cost of a technology is a daunting task. Costs that must be considered include equipment costs, installation costs, power consumption, waste disposal, and sampling and analysis costs. An important cost that is often overlooked by technology developers is regulatory compliance. Environmental remediation

is almost always driven by environmental regulations rather than by company altruism. Consequently, there is a cost associated with meeting these regulatory requirements. These costs include those associated with collecting the required amount of monitoring data, preparing reports from those data, and evaluating the technology's performance over months or years to prove effectiveness. In the case of innovative technologies, there is often the added cost of educating and convincing state and federal regulators of a technology's efficacy. These costs can be very high, especially in jurisdictions where the regulators are either extremely skeptical of anything new, distrusting of companies and their consultants, generally unsophisticated and/or uneducated regarding emerging technologies, or some combination of these factors. Thus, some technologies are not selected simply because it is not worth the effort (i.e., cost) of trying to convince a responsible regulator to accept something new. Often, this somewhat hidden cost is known by a target customer who has a historic relationship with the site regulators, but not by the technology vendor/developer who is trying to advance a new technology.

Another often overlooked cost is the cost of research and development. I am a strong believer that fundamental research provides the building blocks that ultimately can be cemented together to create solutions to remediation and waste treatment challenges. The cost of research, however, must be factored into the cost of developing and applying any innovative technology. Historically, many large companies in the United States and elsewhere maintained large and active research staffs focused on addressing environmental issues. Oil companies, for examples, studied the fate of spilled oil in the environment, while producers and users of PCBs evaluated remedial alternatives for these chemicals. Today, many of these company-funded research efforts have been reduced or completely eliminated because the cost of such work (reducing liabilities) outweighed the overall financial benefit. Some of this research is now contracted to university laboratories to reduce overall costs and/or is funded at a fundamental level by government entities such as the National Science Foundation or research programs and laboratories of the US Department of Defense, as many environmental issues cross government/industry boundaries. I was once told by a business development leader in our company to remove all "researchy" words, including test, evaluate, research, etc., from a proposal I was preparing for his client. He said, "Nobody wants to pay for research"!

Having rudimentarily addressed cost issues, this retrospective could really end here. Researchers who have a goal of developing and applying innovative solutions to better protect and/or clean the environment simply have to face the reality that cost drives every decision, from pollution control to site remediation. Like it or not, cost is king.

3 Lesson 2: Know the Market

As a start-up biotechnology company in 1990, Envirogen selected chlorinated solvents and PCBs as its primary target markets. Research and market analysis showed that these contaminants were widespread and there were no readily

available, low-cost, and effective technologies to treat them. Thus, they appeared to be perfect target markets that were (1) large enough to justify investing in research, the cost of which could be recovered over time, and (2) challenging enough that an innovative solution could justify large profit margins and rapid return on investments. Even the development and application of GEMs appeared worth the investment and regulatory costs.

Enthusiastic business people and scientists have a tendency to overestimate market size. For example, nearly all scientific papers and research proposals related to a particular treatment approach for an environmental contaminant (e.g., bioremediation) begin with an introduction that describes its enormous distribution and a "dooms day" scenario concerning its potential impact on man and nature. They also typically insist that no other technology can effectively treat or otherwise manage the described contaminant. Similar assumptions led to Envirogen's hopes of huge "value added" profit margins from successful technologies. What became apparent during my early days in the remediation business was that there are always alternatives for site remediation and pollution control, and there are always cost ceilings that limit the potential for large profit margins.

PCBs, for example, certainly were, and still are, challenging contaminants to remediate, and they constituted a potentially large market. PCBs were nonflammable oils composed of biphenyl molecules that had been chlorinated to improve their stability and utility in a wide range of electrical applications. Consequently, they were mixtures of chemicals containing many chlorinated congeners with different degrees of chlorination. While some congeners could be biodegraded either anaerobically or aerobically by some bacteria, complete remediation of PCB contamination was extremely challenging. Thus, they appeared to be a prime target for which high profit margins could be generated if someone could develop an effective low-cost remediation technology. What was overlooked, however, was that even in the early 1990s, effective PCB remediation technologies did exist for many contaminated media. Namely, "dig and haul" where the contaminated media were excavated and emplaced in special landfills or incinerated at high temperature to destroy the PCBs. While these technologies are and were unpopular and unacceptable to some, from a company's or agency's perspective, they could remove an environmental liability. Furthermore, even though these technologies were expensive to implement, they did create a cost ceiling. Any new, innovative technology had to fall well below this ceiling to have a chance at widespread implementation. Thus, the expected value added profit margin of any new technology had an upper limit. Speculation in the 1990s even suggested that some companies did cost analyses that showed that long-term legal litigation was more cost effective, by about half, than dig and haul remediation (i.e., lawyers were half as expensive as digging and landfilling!). Thus, cost considerations might have dictated that companies engage in long-term litigation to delay the costs of actually remediating a PCB-contaminated site.

In the final analysis, the market for PCB bioremediation appears to have been far smaller than originally estimated, and the potential for large profits had a ceiling. Many smaller sites were remediated by removing contaminated soils to landfills or immobilizing the contaminants in place. Regulatory limitations on PCB uses

reduced the creation of new polluted sites, thereby further limiting the attractiveness of PCBs as a high profit margin target. Likewise, research advancements have demonstrated that some natural attenuation of PCBs does occur in the environment, and natural attenuation strategies now appear to be the primary remedies sought for PCB-contaminated sites. Clearly, however, some contaminated environments, including harbor and river sediments, still have some luster for entrepreneurial scientists and business people.

Another market that appears to have been greatly overstated is the remediation of hydrocarbon contaminated soils and groundwater at gasoline stations. There are many thousands of gasoline stations in the United States and elsewhere. Even with the implementation of new rules that limit subsurface contamination via corroded underground storage tanks, releases of hydrocarbons at these sites are common. This market appeared especially fruitful with the discovery of methyl-*tert*-butyl ether (MTBE) from gasoline in groundwaters in the mid-1990s (Squillace et al. 1996) because MTBE was recalcitrant and not easily removed by conventional technologies (i.e., natural attenuation or air stripping and adsorption onto granular activated carbon). For Envirogen, despite the successful discovery of microbes that could degrade MTBE by co-metabolism (Steffan et al. 1997) or that could grow on MTBE as a sole carbon source (Hatzinger et al. 2001), and the demonstration of potential remedial technologies (Steffan et al. 2000; Vainberg et al. 2002), the structure of the gasoline station market proved limiting as a source of value-added profit margins. Specifically, although some large oil companies or their subsidiaries own significant numbers of gasoline stations in the USA, most stations are franchisees or independently owned either individually or as a small regional group of stations. Environmental management of the oil company-owned stations historically has been performed regionally through contracting, on a low cost/margin basis, to smaller local consulting companies that could manage a few or several stations. These smaller firms typically applied traditional, low lost, yet relatively reliable, technologies including air sparging and pump-and-treat approaches regardless of the presence of MTBE. Likewise, the smaller companies and independent operators typically relied upon application of the lowest cost remedial alternatives, often with the goal of low-cost, long-term operation that did not interfere with the ongoing business at the station. Thus, the lack of consolidation of station ownership and site management within the oil industry limited the ability of the gas station remediation market, even with MTBE, to support the implementation of high profit margin technologies that could justify research and other costs associated with innovative technologies.

A final example of how the market can impact the success of an environmental remediation business can be found in the remediation of chlorinated solvents (primarily PCE and TCE). Chlorinated solvent contamination is clearly widespread around the globe as a result of the use of these chemicals as degreasing and dry cleaning agents. In the early 1990s, there were few remedial alternatives for sites with chlorinated solvent contamination in groundwater. Although some research-developed bioremediation alternatives were demonstrated (McCarty et al. 1998; Steffan et al. 1999), it was the discovery of complete anaerobic dechlorination of

these compounds (Freedman and Gossett 1989) and the subsequent identification of a *Dehalococcoides* sp. (DHC) microbial catalyst (Maymó-Gatell et al. 1997, 1999) that led to the rapid expansion of bioremediation as a preferred method for chlorinated solvent remediation (Steffan and Schaefer 2016).

One company that capitalized early-on on the discovery of DHC as a biocatalyst for chlorinated solvent degradation was Geosyntec, Inc (Geosyntec). Through research performed at the University of Toronto (Duhamel et al. 2004), the company acquired a DHC-containing culture known as KB-1, and they demonstrated its efficacy during a US Department of Defense-funded field project (Major et al. 2002). The company then rapidly commercialized the culture and began selling it at seemingly very high profit margins. Their ability to demand these high prices, however, was short lived because our company, using existing fermentation technology and expertise, was able to produce very large, high DHC-density cultures (Vainberg et al. 2009) and deliver them at approximately one-fourth the price that Geosyntec was able to demand. This forced Geosyntec to reduce their prices to be competitive in the market. Thus, this example demonstrates that regardless of what one thinks the market might be, and the technology prices that it may support, if the market is fruitful (i.e., large and profitable) it will attract competitors.

4 Lesson 3: Learn New Languages

One of the hallmarks of Envirogen's early success in the environmental biotechnology industry was the founders' foresight to incorporate a holistic, systems-focused approach to the business. This approach combined scientists who could understand biocatalysts with engineers who could develop reactor systems to apply the biotechnology and/or hydrogeologists who could design, construct, deploy, and evaluate in situ systems in aquifers. They also employed technical sales people who could market the technologies, and business people who could manage and run the company. Success depended on strong leadership and consistent cooperation. Research scientists (primarily molecular biologists and microbiologists) working for the firm were soon forced to learn the meaning of many new terms such as hydraulic residence time, mass transfer, pressure drop, porosity, hydraulic conductivity, groundwater velocity, as well as budget, gross margin, net margin, profit margin, direct costs, indirect costs, and more. Some also found themselves speaking to outsiders who had their own languages. These included patent attorneys who use terms like specifications, claims, prior art, novelty, and dependent claims to name a few, or regulators who spoke of EPA regulations, QA/QC, sentinel wells, and the like. Similarly, scientists in the environmental industry often are called upon to interpret and translate these foreign languages for someone with limited or no technical expertise, be it an investor, regulator, salesperson, or business owner. Thus, anyone seeking to pursue a career in the environmental industry, or even seeking to do applied research in an academic setting, would be well advised to cross-train to the extent possible in these many fields. Ultimately, these fields will all play a role

in the successful (or unsuccessful) development and application of any new environmental technology.

5 Lesson 4: Be Flexible

Becoming a scientist typically involves gaining specific expertise in a given area. This might be, for example, biodegradation of PCBs or TCE, or maybe biochemistry of DHC. My experience in the environmental industry, however, has taught me that success and sustainability often requires individuals to become generalists or "Jacks of all trades." Rarely can one sustain themselves by performing the same type of work that they performed during their graduate or postgraduate studies. At the very least, individuals must acquire the ability to assimilate information quickly and then be able to apply that knowledge to adapt to rapidly changing circumstances. One hallmark of site remediation and pollution is that no two sites/waste streams are the same. Within any given day, a scientist in an environmental firm might be asked to answer questions about chlorinated solvents in groundwater, hydrocarbons in soils, the use of bioreactors to treat ammonium perchlorate or MTBE, or any other number of issues or topics related to remediation or waste treatment.

6 Lesson 5: Be Decisive

Scientists struggle with decisiveness. We use phrases like "the data suggest...," "it appears that...," and "more research is needed...." People who manage environmental liabilities for companies or regulate sites for the government have to make decisions. The decisions they make are typically going to cost money, and wrong decisions could affect their careers. Often, site managers or regulators are not experts in a technology under evaluation, and they are hesitant to select technologies that are unfamiliar to them. Scientists and engineers working for technology developers, therefore, are often called upon to help these people make decisions, or even to be the person who will ultimately take the blame if something fails. It is not uncommon for a technology vendor to guarantee performance and absorb costs if the technology fails. Typically, as scientists in this industry, we are asked to make these decisions with less data than we desire. One of the greatest challenges scientists face in this situation is to have enough confidence to be decisive in the face of what always appears to be insufficient information.

In the early days of my career, I was on a conference call with a potential client discussing the option of adding a carbon substrate to stimulate in situ biodegradation of TCE in an aquifer on his site, a technology that is now widely accepted (Steffan and Schaefer 2016). We had not yet applied this approach in the field, but we had done significant laboratory testing with positive results. Moreover, the approach was becoming better understood overall and had been performed successfully in the field by others. When the client asked if we had done this at full-scale before, I answered truthfully, but told him I "thought" it would work at his site based on site data and

what we then knew about the technology. He did not select our approach. In the eyes of the client, I apparently was not decisive enough to convince him of the technology's potential and to invest his company's hard-earned money.

As a leader of a technology development group, it was a great challenge to develop the necessary level of decisiveness in our scientists. It also appeared that the better the scientific training the scientist had, the greater was the challenge to get them to commit to a decisive answer. Ultimately, those scientists that developed the ability to walk the fine line between decisiveness and innate scientific resistance to commitment were most successful in the long run, whether they were writing research proposals or papers, or meeting with clients.

7 Lesson 6: It Is All About Marketing

An often overlooked component of a successful technology business is marketing. During my graduate studies, I developed a method for using a novel technology, polymerase chain reaction (PCR), for monitoring bacteria in the environment (Steffan and Atlas 1988). At the time, PCR was a largely unknown technique, PCR kits were not yet commercially available, and PCR machines were nonexistent. After our paper was published, my advisor was contacted by Roche Pharmaceuticals who owned the rights to develop PCR machines and PCR technologies for environmental applications. With their support, and one of the first ever PCR machines, we set out to develop a PCR technology for detecting coliform bacteria, including *E. coli,* in water samples (Bej et al. 1990). The project was successful and led to a patent (United States Patent 1994). I naïvely expected that the world would beat a path to our door, but I was quickly informed by our Roche sponsor that "marketing is king." If someone did not actively market and sell the technology, it would have no commercial value. This turned out to be true and was a valuable lesson on the importance of marketing irrespective of whether or not one holds patents in the environmental field.

As Envirogen began its journey to develop and commercialize remediation and waste treatment technologies, marketing was always critical. Few environmental managers are willing to be the first to apply a new technology because of the risks described above. Consequently, the sales cycle for a new technology (i.e., from first engaging a client to making a sale) can be very long, and extensive marketing and strong salesmanship are required to fully commercialize a new remediation technology. This is certainly not unique to the remediation industry, but because of the nature of the business (with liability-driven and risk-averse clients), it is almost always true.

In my experience, most scientists are not natural sales people, especially when it comes to closing a deal. As a result, a close working relationship between sales people and technology developers is essential. Scientists and engineers must teach the sales people about the technology and also help them to develop materials that can be used to promote its most important aspects. Likewise, scientists and engineers are often critical in discussions with decision makers (e.g., buyers and regulators) to

help promote acceptance of technologies. Good sales people have the ability to keep discussions moving forward and getting buyers to commit resources. The ability of a scientist to be confident in sales situations and to help make sales is an obvious asset, so any training that can be done to enhance these capabilities is beneficial to any entrepreneurial scientist. Unbeknownst to many of us, however, scientists are always selling themselves; whether it is writing a research proposal, publishing a paper, or presenting our work at scientific conferences. Like it or not, marketing rules the day.

8 Lesson 7: Learn to Predict the Future

Any successful business starts with a vision of future sales and business success. As the business progresses and marketplaces change, the business must adapt to take advantage of new opportunities. The most successful businesses are those that foresee events that change the markets. These events could include the emergence of a new contaminant or market niche (e.g., sediments, groundwater, etc.), changes in regulations, or a new targeted effort by a funding agency. As a contract research laboratory, for example, we often had a competitive advantage when seeking research funding if we had sufficient preliminary data to support suppositions we made in our research proposals. Likewise, to capture market share in developing markets, we had an advantage if we had foreseen the market developing and prepared ourselves by collecting necessary data before the market emerged. In our case, decisions to attack a new market were rarely made by management and passed down to the research and development personnel (i.e. top-down). Rather, the scientists who had the training to understand the challenges of treating new contaminants or solving new problems were responsible for communicating important information to the business management (i.e., bottom-up).

The ability to predict the future comes with experience. For those of us at Envirogen it was essential to maintain contacts in the industry, attend conferences, and pay attention to subtle changes in communications about particular contaminants or markets. Often there are small amounts of smoke before there is a fire. By maintaining open relationships with our funding agencies, clients, regulators, and our friends and colleagues, we often identified subtle clues about pending actions and work opportunities that triggered us to expend resources to be prepared for the coming changes. When attempting to predict the future, it is rarely an option to sit and wait for the phone to ring.

One area where we did not predict the future well was in our decision to invest in the development of GEMs for site remediation. In the mid 1980s through the early 1990s, several field-scale applications of GEMS were planned or performed (e.g., Wilson and Lindow 1993). This led us to believe that the market for GEMs for otherwise recalcitrant contaminants like chlorinated solvents and PCBs was emerging and suitable for sizable research and development investment. The market for GEMs for bioremediation, however, never materialized.

I believe the reasons that the bioremediation market for GEMs never developed are many. First, initial proposals to release GEMs into the environment were met

with extreme reactions from the anti-genetic engineering community. This led to vicious protests and demonstrations and fear mongering that predicted catastrophic outcomes suitable for horror films. Importantly, these protests led to hesitation by potential users of the technology who did not want to add the publicity of the anti-GEM movement to the already negative stigma of their polluted sites.

Secondly, the regulatory framework for applying GEMS in the field created great uncertainty. Technology developers could not be sure about what data would be necessary to obtain a permit to release a particular GEM. This uncertainty meant that the cost of applying GEMs could not be readily calculated. As an example, I was once asked by an environmental manager at an east coast military base where we proposed to inject a mutant (non-GEM) Pseudomonad into groundwater for TCE remediation if I could insure him that the added culture would not kill salmon on the west coast of the US. Generating this additional data would be costly, indeed!

A third, yet often overlooked, limitation to the use of GEMs is that it is likely that naturally occurring microorganisms able to do what we are trying to with GEMs will eventually be discovered. It has long been proposed that the diversity and adaptability of natural microbial communities are so great that they may be infallible in their ability to respond to new contaminants (Gale 1952). Many compounds initially thought to be recalcitrant and good targets for GEMs are now known to be treatable by using naturally occurring microorganisms. Furthermore, with the possible exception of DHC for chlorinated solvent remediation and a few other similar cultures, exogenous cultures added to the environment to degrade contaminants rarely survive and perform well, even if short-term laboratory studies might suggest otherwise (DeFlaun and Steffan 2002). All of the above factors continue to affect current and future applications of GEMs for site remediation, and it is unlikely that they will be widely used in the foreseeable future.

9 Research Needs

Technology development for pollution remediation can be an exciting and fruitful career for the well-suited scientist or entrepreneur. It is very rewarding to experience one's technology selected for implementation at a site and then meet the goals of site remediation or successful waste treatment. Scientists entering this field, however, must be prepared for the factors that actually drive technology selection and implementation, and they also must be aware that there are real limits on the profits that can be generated by a given application. Most importantly, one must never forget that, in the business world, cost drives every decision, and altruism is rare. Furthermore, stakeholders are many, and often careers or businesses are dependent on the success of treatment applications, so technology selections are taken very seriously. I have attempted to provide some insight into how I saw the industry function during my career. Cross-disciplinary research focused on understanding how and why remediation and technology decisions are made in actual remediation or pollution control situations, and how to better focus academic research to meet these real demands will better prepare scientists and engineers who seek careers involving pollution control and remediation and also ensure that limited research funding is used efficiently.

References

Bej AK, Steffan RJ, DiCesare J, Haff L, Atlas RM (1990) Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl Environ Microbiol 56:307–314

DeFlaun MF, Steffan RJ (2002) Bioaugmentation. In: Bitton G (ed) Encyclopedia of environmental microbiology. Wiley, New York, pp 434–442

Duhamel M, Mo K, Edwards EA (2004) Characterization of a highly enriched *Dehalococcoides*-containing culture that grows on vinyl chloride and trichloroethene. Appl Environ Microbiol 70:5538–5545

Freedman DL, Gossett JM (1989) Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl Environ Microbiol 55:2144–2151

Gale EF (1952) The chemical activities of bacteria. Academic, New York, p 5

Hatzinger PB, McClay K, Vainberg S, Tugusheva M, Condee CW, Steffan RJ (2001) Biodegradation of methyl *tert*-butyl ether (MTBE) by a pure bacterial culture. Appl Environ Microbiol 67:5601–5607

Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW (2002) Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environ Sci Technol 36:5106–5116

Maymó-Gatell X, Chien YT, Gossett JM, Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276:1568–1571

Maymó-Gatell X, Anguish T, Zinder SH (1999) Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by *"Dehalococcoides ethenogenes"* 195. Appl Environ Microbiol 65:3108–3113

McCarty PL, Goltz MN, Hopkins GD, Dolan ME, Allan JP, Kawakami BT, Carrothers TJ (1998) Full-scale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection. Environ Sci Technol 32:88–100

Squillace PJ, Zogorski JS, Wilber WG, Price CV (1996) Preliminary assessment of the occurrence and possible sources of MTBE in groundwater in the United States, 1993–1994. Environ Sci Technol 30:1721–1730

Steffan RJ, Atlas RM (1988) DNA amplification to enhance the detection of genetically engineered microorganisms in environmental samples. Appl Environ Microbiol 54:2185–2191

Steffan RJ, Schaefer CE (2016) Current and future bioremediation applications: bioremediation from a practical and regulatory perspective. In: Adrian L, Loffler FE (eds) Organohalide-respiring bacteria. Springer, Berlin, pp 517–540

Steffan RJ, McClay K, Vainberg S, Condee CW, Zhang D (1997) Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME) by propane oxidizing bacteria. Appl Environ Microbiol 63:4216–4222

Steffan RJ, Sperry KL, Walsh MT, Vainberg S, Condee CW (1999) Field-scale evaluation of in situ bioaugmentation for remediation of chlorinated solvents in groundwater. Environ Sci Technol 33:2771–2781

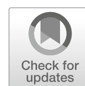
Steffan RJ, Condee C, Quinnan J, Walsh M, Abrams SH, Flanders J (2000) In situ application of propane sparging for MTBE bioremediation. In: Wickramanayake GB, Gavaskar AR, Alleman BC, Magar VS (eds) Bioremediation and phytoremediation of chlorinated and recalcitrant compounds. Battelle Press, Columbus, pp 157–165

United States Patent (1994) Process for detection of water-born microbial pathogens and indicators of human fecal contamination in water samples and kits therefor. U. S. Patent #5,298,392, 29 Mar 1994

Vainberg S, Togna AP, Sutton PM, Steffan RJ (2002) Treatment of MTBE-contaminated water in a fluid bed bioreactor (FBR). J Environ Eng 128:842–851

Vainberg S, Condee CW, Steffan RJ (2009) Large-scale production of bacterial consortia for remediation of chlorinated solvent-contaminated groundwater. J Ind Microbiol Biotechnol 36:1189–1197

Wilson M, Lindow SE (1993) Release of recombinant microorganisms. Annu Rev Microbiol 47:913–944



Utility of Industrial Experimental Sites for Developing Analytical, Monitoring, and Remediation Technologies

3

D. L. Freedman and R. Yu

Contents

1 Introduction 34
2 Chlorinated Ethenes 37
3 Chlorinated Ethanes 38
4 Halogenated Methanes 38
5 Chlorinated Aromatics 39
6 Research Needs 40
References 40

Abstract

The use of industrial sites for developing analytical, monitoring, and bioremediation technologies has been central to advances in the cleanup of hazardous waste sites. While that may seem obvious, since so many contaminated sites are industrial, site owners are sometimes reluctant to allow the results of experimental work on their property to reach publication, out of concern that it might reveal unfavorable information. Those barriers have gradually come down to the benefit of the entire bioremediation field and site owners. Increasingly, results are being reported based on research and fieldwork on industrial sites, often without revealing the identity of the site or the site owners. An overview of critical advances in bioremediation by major category of contaminants shows that, while laboratory studies regularly provide insight and theoretical background,

D. L. Freedman (✉) · R. Yu
Department of Environmental Engineering and Earth Sciences, Clemson University, Clemson, SC, USA
e-mail: dfreedm@clemson.edu; rongy@clemson.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_17

in situ applications at contaminated industrial sites have driven the pattern of development of bioremediation technology. Examples are offered for developments in remediation of chlorinated ethenes, chlorinated ethanes, halogenated methanes, and chlorinated aromatics. These reinforce the importance of promoting application of laboratory and field studies at industrial sites. Industrial sites are likely to play a major role in advancing remediation of emerging contaminants such as 1,4-dioxane and perfluoroalkyl compounds.

1 Introduction

Over the past four decades, the practice of bioremediation has made great strides. Most practitioners would agree that when bioremediation can be used to address contamination problems at a hazardous waste site, it is likely to be the most economical remediation option. This is a somewhat remarkable development when considering that the vast majority of contaminants that are the subject of remediation were once thought to be biologically inert or at least highly refractory. Our understanding of the science of biodegradation has advanced rapidly. The pace of these advances has been driven by the interplay between fundamental discoveries in the laboratory and applications in the field. The latter has required access to contaminated sites. These can roughly be divided into industrial facilities and those operated by government agencies, especially facilities operated by the Department of Defense. The involvement of industrial sites as a proving ground for advances in bioremediation is the focus of this chapter.

Having access to samples from contaminated sites, let alone access to the sites themselves for pilot-scale evaluations, has not always been the norm. One metric for this may be gleaned by the frequency with which industrial and DOD sites are featured in presentations made at the semiannual Battelle symposium on bioremediation. This symposium has been held every other year since 1991; its name has changed over the years, yet bioremediation has remained the core theme. As shown in Fig. 1, the number of presentations increased substantially over the years, reflecting the increased importance of bioremediation to the remediation field. The percentage of presentations that featured work at industrial and DOD sites has grown considerably, to the point that by 2015 nearly one-half featured some element of work performed at an industrial hazardous waste site. On the one hand, that may seem obvious, since so many of the contaminated sites are industrial and site owners stand to gain from advances in the technology available for remediation. Nevertheless, site owners are sometimes reluctant to allow the results of experimental work on their property to reach publication, out of concern that it might reveal unfavorable information. Those barriers have gradually come down to the benefit of the entire bioremediation field. Increasingly, results are being reported based on research and fieldwork on industrial sites without revealing the identity of the site or the site owners. A number of larger companies actively support research on remediation;

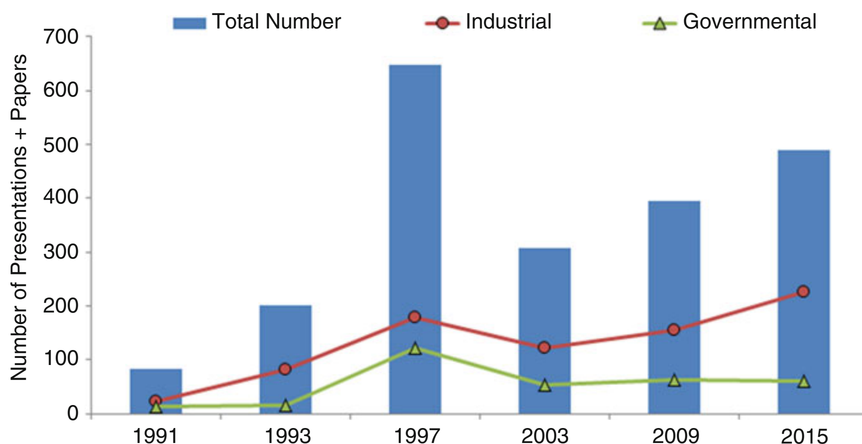


Fig. 1 Total number of presentations and/or papers presented at several of the Battelle conferences that focus on bioremediation and the percentages that mention industrial and/or governmental sites

DuPont (Fung et al. 2009; Hendrickson et al. 2002; Justicia-Leon et al. 2014; Passeport et al. 2016) and General Electric (Bedard et al. 2007) are good examples.

Through the establishment of the Remediation Technologies Development Forum (RTDF; https://rtdf.clu-in.org/), the US Environmental Protection Agency (EPA) sought to foster collaboration between the public and private sectors in developing innovative solutions to mutual hazardous waste problems. Since its establishment in 1992, the RTDF has become a clearinghouse for information on bioremediation and other technologies. The Bioremediation Consortium was established in 1993 and has played an important role in the development of cometabolic bioventing, intrinsic bioremediation, and accelerated anaerobic biodegradation.

An early example of the importance of relating laboratory studies to “real-world” conditions at hazardous waste sites comes from a pivotal advance in understanding the pathway for reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE). These are two of the most frequently encountered contaminants at hazardous waste sites. During the 1980s, the first reports emerged that chlorinated organic compounds are subject to microbial alteration via reductive dechlorination. Suflita et al. (1982) used inocula from lake sediment and sewage sludge to develop enrichments capable of removing chlorines from chlorobenzoates. Microbial dechlorination of PCE and TCE was demonstrated by a number or researchers with a variety of inocula, e.g., muck, surface water, and marls from the South Florida Everglades (Parsons et al. 1984).

The exciting news was that chlorinated organic compounds such as PCE and TCE underwent reductive dechlorination to dichloroethene (DCE; predominantly the *cis* isomer, although *trans* was occasionally predominant) and vinyl chloride (VC) (Vogel et al. 1987). As exciting as these observations were, the realization soon set

in that VC is hardly a desirable endpoint, since it poses an even greater toxicological risk than the "parent" compounds. At around the same time, Wilson and Wilson (1985) made their seminal discovery that TCE underwent complete oxidation under aerobic conditions when microcosms were amended with natural gas. This was critical because a pathway for microbial transformation was revealed that did not involve VC as an intermediate. Interest in anaerobic transformation waned. However, Freedman and Gossett (1989) subsequently demonstrated that complete dechlorination of PCE and TCE to ethene is possible under anaerobic conditions. This was a critical discovery because ethene is a nonhazardous endpoint for groundwater. The inoculum used in their study was sludge from a municipal anaerobic digester in Ithaca, NY. It is possible that the source of inoculum was not the determining factor in this discovery, but rather the measurement technique used, i.e., a gas chromatograph equipped with a flame ionization detector (which "sees" non-chlorinated volatile organics such as ethene) instead of the more commonly used electron capture detector (which is more sensitive to halogenated compounds but is "blind" to nonhalogenated compounds such as ethene). The ubiquity of dechlorinating microbes in environments not previously exposed to halogenated organics has since been demonstrated (Krzmarzick et al. 2012). On the other hand, the practice for disposal of laboratory waste in those days included dumping them down the drain, such that wastewater from Cornell University and nearby industries may have contained these solvents, thereby selecting for dechlorinating microbes in the anaerobic digester (since the hydrophobic solvents tend to adsorb to organic particles that ended up in the digester).

The report by Freedman and Gossett (1989) led to renewed interest in anaerobic bioremediation. Anaerobic processes for groundwater remediation have a considerable advantage in that most aquifers have little or no oxygen present and delivery of oxygen is problematic due to its low solubility; anaerobic processes do not face this hurdle. A critical question remained, however: Did the process observed in the laboratory, using inoculum from a municipal digester operating at 35 °C, have any relevance to in situ conditions? That question was quickly answered by Major and colleagues in 1990. At an industrial site in Toronto, a vinyl chloride plume was observed to be the same size as a *cis*-DCE plume, even though vinyl chloride is more mobile in groundwater. Complete dechlorination of PCE to ethene was confirmed by measuring ethene both in groundwater samples and in microcosms prepared with soil and groundwater from the site (Major et al. 1991). This is a perfect example of how results from a laboratory study provided the insight for a more thorough investigation at an industrial site. The door was now open for confirmation of complete dechlorination at numerous other sites.

In the sections that follow, similar examples are provided for critical advances in bioremediation that resulted in part because samples were available from industrial sites, or the sites themselves were used for pilot studies. The examples are organized according to major categories of contaminants, i.e., chlorinated ethenes, chlorinated ethanes, halogenated methanes, and chlorinated aromatics. Although advances in bioremediation of fuel hydrocarbons is also linked to research involving industrial sites, fuel hydrocarbons are not featured in this review.

2 Chlorinated Ethenes

The enrichment culture developed by Freedman and Gossett (1989) that completely dechlorinated chlorinated ethenes to ethene eventually led to the discovery of the key microbe involved, i.e., *Dehalococcoides* (Maymó-Gatell et al. 1997). Because this genus is phylogenetically unique, it was possible to develop a qPCR technique based on its 16S rRNA gene. That all-important tool led to a field study of sites throughout North America and Europe to test for the occurrence of *Dehalococcoides*. Of the 24 sites evaluated, 15 were industrial; this demonstrated the widespread occurrence of *Dehalococcoides* at sites where chlorinated ethenes were completely dechlorinated to ethene (Hendrickson et al. 2002). The use of qPCR to quantify *Dehalococcoides* is now a commonly used tool in bioremediation.

Arguably the most critical step in the dechlorination process is reduction of VC to ethene, since VC is relatively more toxic than the other chlorinated ethenes. Not all strains of *Dehalococcoides* are capable of mediating this reaction at a high rate. Two key enzymes, *vcrA* and *bvcA*, are associated with a rapid rate of VC dechlorination. *vcrA* was identified in *Dehalococcoides* strain VS, which originated from an industrial site in Victoria, Texas (Müller et al. 2004). *bvcA* was identified in *Dehalococcoides* strain BAV1, which originated from the Bachman Road dry-cleaning site in Oscoda, Michigan (He et al. 2003). Along with qPCR for *Dehalococcoides*, quantification of the *vcrA* and *bvcA* genes is now a standard tool used to assess the occurrence of complete dechlorination in the field. The interplay between laboratory studies and fieldwork made this development possible.

Bioaugmentation now plays a major role in bioremediation strategies because some sites lack the microbes needed to achieve treatment in a reasonable timeframe. The first and one of the most widely used cultures for this purpose is KB-1, marketed by SiREM. KB-1 was developed using soil and groundwater from a contaminated industrial site in southern Ontario (Wehr 2001). KB-1 was used in one of the first bioaugmentation trials (Major et al. 2002) and has since been characterized in detail (Duhamel et al. 2002; Liang et al. 2015).

Aerobic and sequential anaerobic-aerobic biodegradation of chlorinated ethenes are also important processes, especially as it applies to monitored natural attenuation. Original observations of these processes were made at industrial sites in California and New Hampshire (Cox et al. 1995; Edwards and Cox 1997). The use of VC as a growth substrate has been demonstrated with *Mycobacterium*, *Nocardioides*, *Ochrobactrum*, and *Pseudomonas*. Coleman et al. (2002) sampled 38 sites for the presence of microbes capable of metabolizing VC, five of which were industrial; VC-degrading isolates were obtained from four of these. The *Pseudomonas* isolate capable of growth on VC was obtained from a former industrial lagoon (Danko et al. 2004).

Recognition of the role of abiotic degradation of chlorinated ethenes has grown and industrial sites have played a role. Most of the early work was done in laboratories with iron containing compounds (e.g., zero valent iron, iron minerals, and iron sulfide) (Arnold and Roberts 2000; Butler and Hayes 2001; Lee and Batchelor 2002a, b). Darlington et al. (2008) demonstrated the occurrence of abiotic transformations using ^{14}C-TCE and ^{14}C-cDCE with samples of fractured sandstone

and groundwater from an industrial site in southern California. A key development in characterization of fractured rock sites such as these is the continuous packer for temporary borehole seals (Flexible Liner Underground Technologies Ltd. [FLUTe] blank liner) and a depth-discrete multilevel monitoring system (MLS) (the Water FLUTe) for temporary or permanent monitoring (Cherry et al. 2007).

Compound specific isotope analysis (CSIA) is a tool of increasing importance in documenting degradation of chlorinated ethenes, as well as numerous other contaminants. Not that long ago, CSIA was strictly a research tool. As the cost for sample analysis has decreased, commercial laboratories now offer the service and it is routinely applied to samples from industrial sites (Hunkeler et al. 2008). One of the applications includes contaminant forensics, with the intent of identifying the parties responsible for multiple plumes at complex sites (Blessing et al. 2009).

3 Chlorinated Ethanes

Industrial sites have been front and center in advancing the practice of bioremediation for chlorinated ethanes. Using groundwater and solids from a multilayered aquifer at a former chlorinated solvent disposal facility in West Louisiana, Grostern and Edwards (2009) developed an enrichment culture that transforms 1,2-dichloroethane (DCA) and 1,1,2-trichloroethane (TCA) to ethene. Both *Dehalobacter* and *Dehalococcoides* were involved in dichloroelimination of 1,2-DCA to ethene and 1,1,2-TCA to VC, while only *Dehalococcoides* were responsible for VC reduction to ethene. Samples from the same site and another industrial site in Louisiana were used to demonstrate carbon isotopic fractionation during aerobic biodegradation of 1,2-DCA (Hirschorn et al. 2004) and the potential for anoxic biodegradation of 1,2-DCA with nitrate as the electron acceptor (Dinglasan-Panlilio et al. 2006). Grostern and Edwards also developed an enrichment culture from a northeastern United States industrial area contaminated with high concentrations of 1,1,1-TCA (38 μM) and TCE (8 μM). *Dehalobacter* were responsible for halorespiration of 1,1,1-TCA to chloroethane; removal of 1,1,1-TCA was essential for TCE dechlorination by the KB-1 culture, which is inhibited by 1,1,1-TCA. This led to the development of a coculture that can be used at sites with both chlorinated ethenes and 1,1,1-TCA.

Industrial sites have also served as the test bed for natural attenuation of 1,2-DCA in the Netherlands (Bosma et al. 1997) and the United States (Cox et al. 1998), and for bioaugmentation using *Desulfitobacterium dichloroeliminans* strain DCA1 in Belgium (Maes et al. 2006). Strain DCA1 also respires vicinal dichloropropanes and dichlorobutanes (De Wildeman et al. 2003).

4 Halogenated Methanes

Samples of soil and groundwater from an industrial site in southern California were used in a microcosm study to assess biodegradation of carbon tetrachloride (CT), chloroform (CF), and trichlorofluoromethane (CFC-11). Biostimulation was

effective, although bioaugmentation with an acclimated enrichment culture was faster (Shan et al. 2010). Evaluation of the bioaugmentation culture confirmed the potential for complete defluorination of CFC-11 (Shan et al. 2014).

The 1,1,1-TCA enrichment culture developed by Grostern and Edwards (2006) with groundwater and soil from a northeastern United States industrial area was subsequently shown to use CF as an electron acceptor, with accumulation of dichloromethane (DCM) (Grostern et al. 2010). The *Dehalobacter* present in the enrichment appear to be specialized for substrates with three halogen substituents on the same carbon atom. Although biodegradation of CF had been demonstrated in numerous previous studies, this was the first to demonstrate that CF can undergo halorespiration; this is accompanied by a significant level of carbon isotope fractionation (Chan et al. 2012).

Unlike CF, DCM does not undergo halorespiration. It can be degraded aerobically (Leisinger et al. 1994), while anaerobic biodegradation occurs via organohalide fermentation. The first isolate obtained that carries out this process, *Dehalobacterium formicoaceticum*, emanated from industrially polluted, anaerobic groundwater (Mägli et al. 1998; Stromeyer et al. 1991). *Dehalobacter* were subsequently identified to possess this same capability, although consortium RM was developed from pristine river sediment rather than from an industrial site (Justicia-Leon et al. 2012). A mixed *Dehalobacter* consortium capable of respiring CF to DCM and fermenting DCM was used in a bioaugmentation microcosms study using samples from an industrial site in California (Justicia-Leon et al. 2014). Complete dechlorination was demonstrated, although amendment with 10 mM of bicarbonate was required. Complete anaerobic dechlorination of CF by *Dehalobacter* has also been described by Lee et al. (2012), although it is unclear if the inoculum derived from an industrial site.

5 Chlorinated Aromatics

Chlorinated benzenes are among the most prevalent contaminants at industrial sites, so it stands to reason that many of these sites have provided opportunities to advance bioremediation. Evidence in support of natural attenuation of chlorobenzenes, BTEX, and chlorinated aliphatic compounds was documented at an industrial area of Bitterfeld/Wolfen, about 130 km south of Berlin in Germany (Heidrich et al. 2004). Elango et al. (2010) used samples of soil, crushed dolomite and groundwater from an industrial site to evaluate strategies for remediation of chlorinated benzenes, chlorinated ethenes, and lindane.

The favorability of aromatic dechlorination decreases as the number of chlorines decreases, such that relatively few observations of chlorobenzene reduction to benzene have been made. Using samples from the DuPont Chambers Works site adjacent to the Delaware River in Salem County, New Jersey, Fung et al. (2009) developed an enrichment culture capable of consistent reduction of chlorobenzene to benzene. Dehalobacter were subsequently identified as the dechlorinating microbes (Nelson et al. 2011). The *Dehalobacter* enrichment was combined with a benzene-fermenting culture to achieve complete aromatic degradation (Liang et al. 2013).

The benzene fermenting culture was developed with sediment and groundwater collected from an oil refinery in Oklahoma.

The range of chlorinated aromatics that are respired by *Dehalococcoides* continues to grow. For example, Fennell et al. (2004) demonstrated dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin, 2,3,4,5,6-pentachlorobiphenyl, 1,2,3,4-tetrachloronaphthalene, and hexachlorobenzene by strain 195. Bedard (2008) reviewed the history of anaerobic microbial polychlorinated biphenyl (PCB) dechlorination using the Housatonic River (Massachusetts) as an example. Most the literature on this topic relates to remediation of contaminated waterways rather than industrial sites, although the industrial sites are the origin of the environmental releases.

6 Research Needs

Industrial sites will continue to be a fertile source of microbes for use in bioremediation, as well as the proving ground for advances in analysis, monitoring, and remediation. Progress will likely continue with bioremediation of halogenated organic compounds. At the same time, there is a growing need for advances in bioremediation of "emerging" contaminants such as 1,4-dioxane and perfluoroalkyl compounds (PFCs). 1,4-Dioxane has been identified at numerous sites, yet the options for in situ remediation are still limited (Adamson et al. 2014, 2015). Aerobic biodegradation, either via microbes that metabolize the compound or degrade it via cometabolism, is well-established in the laboratory (Sales et al. 2011), while field trials are more limited (Li et al. 2010; Lippincott et al. 2015). Definitive evidence for anaerobic biodegradation is still lacking. There is a critical need to evaluate samples from sites with anaerobic conditions at which 1,4-dioxane appears to be attenuating. As was the initial case with PCE and TCE, it may be that the best source of microbes is an anaerobic digester that receives a continuous input of 1,4-dioxane.

PFCs are industrial chemicals that have become disseminated globally in soils, sediments, waters, and the atmosphere over the previous 50 years. Perfluorooctanoic acid (PFOA) is the PFC that receives the most attention. Despite their ubiquity, relatively little is known about the biodegradability of PFCs in general and PFOA in particular. The few studies that have been done this far indicate that PFOA is microbially recalcitrant (Liou et al. 2010). Industrial sites with a long history of PFC contamination may be the ideal locations to find the microbes capable of degrading these compounds, thereby opening the door to developing new bioremediation technologies.

References

Adamson DT, Mahendra S, Walker KL, Rauch SR, Sengupta S, Newell CJ (2014) A multisite survey to identify the scale of the 1,4-dioxane problem at contaminated groundwater sites. Environ Sci Technol Lett 1(5):254–258

Adamson DT, Anderson RH, Mahendra S, Newell CJ (2015) Evidence of 1,4-dioxane attenuation at groundwater sites contaminated with chlorinated solvents and 1,4-dioxane. Environ Sci Technol 49(11):6510–6518

Arnold WA, Roberts AL (2000) Pathways and kinetics of chlorinated ethylene and chlorinated acetylene reaction with Fe(0) particles. Environ Sci Technol 34(9):1794–1805

Bedard DL (2008) A case study for microbial biodegradation: anaerobic bacterial reductive dechlorination of polychlorinated biphenyl-from sediment to defined medium. Annu Rev Microbiol 62(1):253–270

Bedard DL, Ritalahti KM, Loffler FE (2007) The *Dehalococcoides* population in sediment-free mixed cultures metabolically dechlorinates the commercial polychlorinated biphenyl mixture Aroclor 1260. Appl Environ Microbiol 73(8):2513–2521

Blessing M, Schmidt TC, Dinkel R, Haderlein SB (2009) Delineation of multiple chlorinated ethene sources in an industrialized area – a forensic field study using compound-specific isotope analysis. Environ Sci Technol Lett 43(8):2701–2707

Bosma TNP, van Aalst MA, Rijnaarts HHM (1997) Intrinsic dechlorination of 1,2-dichloroethane at an industrial site. In: Alleman BC, Leeson A (eds) In Situ and On-Site Bioremediation: volume 3. Battelle Press, Columbus, pp 155–159

Butler EC, Hayes KF (2001) Factors influencing rates and products in the transformation of trichloroethylene by iron sulfide and iron metal. Environ Sci Technol 35(19):3884–3891

Chan CCH, Mundle SOC, Eckert T, Liang X, Tang S, Lacrampe-Couloume G, Edwards EA, Sherwood Lollar B (2012) Large carbon isotope fractionation during biodegradation of chloroform by *Dehalobacter* cultures. Environ Sci Technol 46(18):10154–10160

Cherry JA, Parker BL, Keller C (2007) A new depth-discrete multilevel monitoring approach for fractured rock. Ground Water Monit R 27(2):57–70

Coleman NV, Mattes TE, Gossett JM, Spain JC (2002) Phylogenetic and kinetic diversity of aerobic vinyl chloride-assimilating bacteria from contaminated sites. Appl Environ Microbiol 68 (12):6162–6171

Cox E, Edwards E, Lehmicke L, Major D (1995) Intrinsic biodegradation of trichloroethene and trichloroethane in a sequential anaerobic-aerobic aquifer. In: Hinchee RE, Wilson JT, Downey DC (eds) Intrinsic Bioremediation. Battelle Press, Columbus, pp 223–232

Cox EE, McMaster M, Major DW, Lehmicke L, Neville S (1998) Natural attenuation of 1,2-dichloroethane and chloroform in groundwater at a Superfund site. Proceedings of the First International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Battelle Press, Columbus, OH

Danko AS, Luo M, Bagwell CE, Brigmon RL, Freedman DL (2004) Involvement of linear plasmids in aerobic biodegradation of vinyl chloride. Appl Environ Microbiol 70(10):6092–6097

Darlington R, Lehmicke L, Andrachek RG, Freedman DL (2008) Biotic and abiotic anaerobic transformations of trichloroethene and *cis*-1,2-dichloroethene in fractured sandstone. Environ Sci Technol 42(12):4323–4330

De Wildeman S, Diekert G, Van Langenhove H, Verstraete W (2003) Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. Appl Environ Microbiol 69(9):5643–5647

Dinglasan-Panlilio MJ, Dworatzek S, Mabury S, Edwards EA (2006) Microbial oxidation of 1,2-dichloroethane under anoxic conditions with nitrate as electron acceptor in mixed and pure cultures. FEMS Microbiol Ecol 56:355–364

Duhamel M, Wehra SD, Yua L, Rizvia H, Seepersada D, Dworatzeka S, Cox EE, Edwards EA (2002) Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, *cis*-dichloroethene and vinyl chloride. Water Res 36 (17):4193–4202

Edwards EA, Cox EE (1997) Field and laboratory studies of sequential anaerobic-aerobic chlorinated solvent biodegradation. In: Alleman BC, Leeson A (eds) In Situ and On-Site Bioremediation. Battelle Press, New Orleans, pp 261–265

Elango V, Cashwell JM, Bellotti MJ, Marotte R, Freedman DL (2010) Bioremediation of hexachlorocyclohexane isomers, chlorinated benzenes, and chlorinated ethenes in soil and fractured dolomite. Bioremediation J 14(1):10–27

Fennell DE, Nijenhuis I, Wilson SF, Zinder SH, Haggblom MM (2004) *Dehalococcoides ethenogenes* strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. Environ Sci Technol 38(7):2075–2081

Freedman DL, Gossett JM (1989) Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. Appl Environ Microbiol 55 (9):2144–2151

Fung JM, Weisenstein BP, Mack EE, Vidumsky JE, Ei TA, Zinder SH (2009) Reductive dehalogenation of dichlorobenzenes and monochlorobenzene to benzene in microcosms. Environ Sci Technol 43(7):2302–2307

Grostern A, Edwards EA (2006) A 1,1,1-trichloroethane-degrading anaerobic mixed microbial culture enhances biotransformation of mixtures of chlorinated ethenes and ethanes. Appl Environ Microbiol 72(12):7849–7856

Grostern A, Edwards EA (2009) Characterization of a *Dehalobacter* coculture that dechlorinates 1,2-dichloroethane to ethene and identification of the putative reductive dehalogenase gene. Appl Environ Microbiol 75(9):2684–2693

Grostern A, Duhamel M, Dworatzek S, Edwards EA (2010) Chloroform respiration to dichloromethane by a *Dehalobacter* population. Environ Microbiol 12(4):1053–1060

He J, Ritalahti KM, Yang K-L, Koeningsberg SS, Löffler FE (2003) Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424:62–65

Heidrich S, Weiß H, Kaschl A (2004) Attenuation reactions in a multiple contaminated aquifer in Bitterfeld (Germany). Environ Pollut 129(2):277–288

Hendrickson ER, Payne JA, Young RM, Starr MG, Perry MP, Fahnestock S, Ellis DE, Ebersole RC (2002) Molecular analysis of *Dehalococcoides* 16S ribosomal DNA from chloroethene-contaminated sites throughout North America and Europe. Appl Environ Microbiol 68(2):485–495

Hirschorn SK, Dinglasan MJ, Elsner M, Mancini SA, Lacrampe-Couloume G, Edwards EA, Sherwood Lollar B (2004) Pathway dependent isotopic fractionation during aerobic biodegradation of 1,2-dichloroethane. Environ Sci Technol 38(18):4775–4781

Hunkeler D, Meckenstock RU, Sherwood Lollar B, Schmidt TC, Wilson JT (2008) A Guide for Assessing Biodegradation and Source Identification of Organic Ground Water Contaminants using Compound Specific Isotope Analysis (CSIA). EPA 600/R-08/148. https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=202171

Justicia-Leon SD, Ritalahti KM, Mack EE, Löffler FE (2012) Dichloromethane fermentation by a *Dehalobacter* sp. in an enrichment culture derived from pristine river sediment. Appl Environ Microbiol 78(4):1288–1291

Justicia-Leon SD, Higgins S, Mack EE, Griffiths DR, Tang S, Edwards EA, Löffler FE (2014) Bioaugmentation with distinct *Dehalobacter* strains achieves chloroform detoxification in microcosms. Environ Sci Technol 48:1851–1858

Krzmarzick MJ, Crary BB, Harding JJ, Oyerinde OO, Leri AC, Myneni SCB, Novak PJ (2012) Natural niche for organohalide-respiring chloroflexi. Appl Environ Microbiol 78(2):393–401

Lee W, Batchelor B (2002a) Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing soil minerals. 1. Pyrite and magnetite. Environ Sci Technol 36(23):5147–5154

Lee W, Batchelor B (2002b) Abiotic reductive dechlorination of chlorinated ethylenes by iron-bearing soil minerals. 2. Green rust. Environ Sci Technol 36(24):5348–5354

Lee M, Low A, Zemb O, Koenig J, Michaelsen A, Manefield M (2012) Complete chloroform dechlorination by organochlorine respiration and fermentation. Environ Microbiol 14 (4):883–894

Leisinger T, Bader R, Hermann R, Schmid-Appert M, Vuilleumier S (1994) Microbes, enzymes and genes involved in dichloromethane utilization. Biodegradation 5:237–284

Li M, Fiorenza S, Chatham JR, Mahendra S, Alvarez PJJ (2010) 1,4-Dioxane biodegradation at low temperatures in Arctic groundwater samples. Water Res 44(9):2894–2900

Liang X, Devine CE, Nelson J, Sherwood Lollar B, Zinder S, Edwards EA (2013) Anaerobic conversion of chlorobenzene and benzene to CH_4 and CO_2 in bioaugmented microcosms. Environ Sci Technol 47(5):2378–2385

Liang X, Molenda O, Tang S, Edwards EA (2015) Identity and substrate specificity of reductive dehalogenases expressed in *Dehalococcoides*-containing enrichment cultures maintained on different chlorinated ethenes. Appl Environ Microbiol 81(14):4626–4633

Liou JSC, Szostek B, DeRito CM, Madsen EL (2010) Investigating the biodegradability of perfluorooctanoic acid. Chemosphere 80(2):176–183

Lippincott D, Streger SH, Schaefer CE, Hinkle J, Stormo J, Steffan RJ (2015) Bioaugmentation and propane biosparging for in situ biodegradation of 1,4-dioxane. Groundw Monit Remediat 35 (2):81–92

Maes A, VanRaemdonck H, Smith K, Ossieur W, Lebbe L, Verstraete W (2006) Transport and activity of *Desulfitobacterium dichloroeliminans* strain DCA1 during bioaugmentation of 1,2-DCA-contaminated groundwater. Environ Sci Technol 40(17):5544–5552

Mägli A, Messmer M, Leisinger T (1998) Metabolism of dichloromethane by the strict anaerobe *Dehalobacterium formicoaceticum*. Appl Environ Microbiol 64(2):646–650

Major DW, Hodgins EW, Butler BJ (1991) Field and laboratory evidence of in situ biotransformation of tetrachloroethene to ethene and ethane at a chemical transfer facility in North Toronto. In Hinchee RE, Olfenbuttel RF (ed), On-Site Bioreclamation. Butterworth-Heinemann, Stoneham, MA 147–171

Major DW, McMaster ML, Cox EE, Edwards EA, Dworatzek SM, Hendrickson ER, Starr MG, Payne JA, Buonamici LW (2002) Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. Environ Sci Technol 36(23):5106–5116

Maymó-Gatell X, Chien Y-T, Gossett JM, Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276(June 6):1568–1571

Müller JA, Rosner BM, von Abendroth G, Meshulam-Simon G, McCarty PL, Spormann AM (2004) Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. Appl Environ Microbiol 70(8):4880–4888

Nelson JL, Fung JM, Cadillo-Quiroz H, Cheng X, Zinder SH (2011) A role for *Dehalobacter* spp. in the reductive dehalogenation of dichlorobenzenes and monochlorobenzene. Environ Sci Technol 45(16):6806–6813

Parsons F, Wood PR, DeMarco J (1984) Transformations of tetrachloroethene and trichloroethene in microcosms and groundwater. J Am Water Works Assoc 76(2):56–59

Passeport E, Landis R, Lacrampe-Couloume G, Lutz EJ, Mack EE, West K, Morgan S, Sherwood Lollar B (2016) Sediment monitored natural recovery evidenced by compound specific isotope analysis and high-resolution pore water sampling. Environ Sci Technol 50(22):12197–12204

Sales CM, Mahendra S, Grostern A, Parales RE, Goodwin LA, Woyke T, Nolan M, Lapidus A, Chertkov O, Ovchinnikova G, Sczyrba A, Alvarez-Cohen L (2011) Genome sequence of the 1,4-dioxane-degrading *Pseudonocardia dioxanivorans* strain CB1190. J Bacteriol 193 (17):4549–4550

Shan H, Kurtz HD Jr, Freedman DL (2010) Evaluation of strategies for anaerobic bioremediation of high concentrations of halomethanes. Water Res 44(5):1317–1328

Shan H, Wang H, Yu R, Jacob P, Freedman D (2014) Biodegradation of high concentrations of halomethanes by a fermentative enrichment culture. AMB Express 4(1):48

Stromeyer SA, Winkelbauer W, Kohler H, Cook AM, Leisinger T (1991) Dichloromethane utilized by an anaerobic mixed culture: acetogenesis and methanogenesis. Biodegradation 2:129–137

Suflita JM, Horowitz A, Shelton DR, Tiedje JM (1982) Dehalogenation: a novel pathway for the anaerobic biodegradation of haloaromatic compounds. Science 218:1115–1117

Vogel TM, Criddle CS, McCarty PL (1987) Transformations of halogenated aliphatic compounds. Environ Sci Technol 21(8):722–736

Wehr S (2001) Characterization of Anaerobic Dechlorinating Enrichment Cultures Maintained on Different Chlorinated Ethenes. Master's of Applied Science, University of Toronto, National Library of Canada. http://www.collectionscanada.gc.ca/obj/s4/f2/dsk3/ftp04/MQ58751.pdf

Wilson JT, Wilson BH (1985) Biotransformation of trichloroethylene in soil. Appl Environ Microbiol 49(1):242–243

Bioremediation of Marine Oil Spills

4

Roger C. Prince and Ronald M. Atlas

Contents

1 Introduction 46
2 Fate of Oil in the Sea 46
3 Eventual Fate of Spilled Oil 48
4 Bioremediation by Alleviating Limiting Factors – Biostimulation 49
4.1 Dispersants 49
4.2 Fertilizers 53
5 Bioremediation by Adding Oil-Degrading Microbes – Bioaugmentation 57
6 Conclusions 58
7 Research Needs 60
References 60

Abstract

Spills of crude and fuel oils from tankers, freighters, pipelines, wells, and storage facilities into the marine environment capture the public's attention and demand prompt and environmentally sensitive response technologies. Sometimes it is possible to contain the oil with booms and collect it with skimmers or burn it, but in many cases this is impractical, and aiding natural attenuation (largely by microbial biodegradation) is all that can be done without causing further environmental damage. One approach, biostimulation, is to at least partially alleviate those factors slowing the growth of indigenous oil-degrading microbes. While an oil slick is floating or emerging from a

R. C. Prince (✉)
ExxonMobil Biomedical Sciences, Inc., Annandale, NJ, USA
e-mail: rogercprince@gmail.com

R. M. Atlas
Department of Biology, University of Louisville, Louisville, KY, USA
e-mail: r.atlas@louisville.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_13

wellhead, this can be done by spraying dispersants to encourage the oil to disperse as tiny droplets in the water column, dramatically enhancing the surface area for microbial colonization. If oil reaches a shoreline, biodegradation can be stimulated by carefully delivering biologically available nitrogen and phosphorus to at least partially reduce their limitation on microbial growth. These approaches of biostimulation have been successfully employed many times. The other tactic, bioaugmentation, aiming to add exogenous microbes in the hope that they will “jump-start” biodegradation has yet to be shown to be effective in real oil-spill situations and likely is unnecessary given the ubiquitous distribution of oil-degrading bacteria in the sea.

1 Introduction

Much of the world’s oil production of almost 93 million barrels per day in 2014 (Energy Information Agency 2016a; 1 barrel = 42 gallons (US), 159 l) is transported by sea. The International Tanker Owners Pollution Federation (ITOPF 2016) reported that seaborne oil trade has averaged 100 trillion barrel-miles per year since 2000. Despite the best efforts of the industry, some does get into the marine environment. Fortunately the amount of oil released from oil transportation is very small as a percentage of the volume transported and is getting smaller (Etkin 2001; ITOPF 2016). Indeed, releases of petroleum from oil tankers in the 1990s amounted to only 2% of the total oil released to North American waters and 8% worldwide (National Research Council 2002) and is less today (ITOPF 2016). In contrast, natural oil seeps, the largest contributors, accounted for 47% of the oil entering the world’s oceans (National Research Council 2002). These seeps have likely been active for millions of years and provided the carbon and energy source and selection pressure for the numerous oil-degrading microbial genera (Prince 2010, Prince et al. 2010, and this series), and it is these organisms that are the foundation of bioremediation strategies – and perhaps even of some fisheries (Levy and Lee 1988)!

2 Fate of Oil in the Sea

Crude oils and refined fuels are very complex mixtures that vary widely depending on their source (Tissot and Welte 1984; Robbins and Hsu 1996; USDOE 2008; Speight 2014). They contain thousands of molecular species, which are often grouped into four categories: saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes. The first two are amenable to gas chromatography, and have been intensely studied. The last two contain heteroatoms such as oxygen, sulfur, and nitrogen and are not amenable to gas chromatography. Until recently their identity was unknown, but recent developments in high resolution mass spectrometry (e.g., Kim et al. 2005; Hughey et al. 2007; Cho et al. 2015) are beginning to reveal

their composition. Commercially, crude oils are classified by their density in units of API (American Petroleum Institute) gravity, which is defined as [141.5/(specific gravity)] – 131.5 and expressed as degrees (°). Thus distilled water has an API gravity of 10°, and denser fluids will have lower API gravities. The median API gravity of crude oil processed in the USA in 2015 was 31.68 (Energy Information Agency 2016b), and this is probably typical worldwide. Almost all oils in commerce float on seawater, although some very heavy bitumen and bunker fuels may "float" beneath the surface of freshwater, as happened with the 1989 *Presidente Rivera* spill in the Delaware River (Wiltshire and Corcoran 1991).

When oils enter the oceans from seeps, urban runoff, or a spill, they become subject to a variety of processes collectively termed "weathering" (Prince et al. 2003b). The actual weathering processes are highly dependent on environmental factors (Lee et al. 2015). Almost all oils float, allowing the smallest molecules to evaporate (Betancourt et al. 2005; Fingas 2013). These molecules are either photochemically degraded (e.g., Hamilton et al. 2003; Wu et al. 2014) or washed from the atmosphere in rain and then biodegraded (e.g., Arzayus et al. 2001; Castro-Jiménez et al. 2012; Chen et al. 2016). Evaporation is usually limited to molecules with less than about 15 carbon atoms, so evaporation is the likely fate of most of a gasoline spill at sea, three-quarters or more of a diesel spill, and perhaps 20–40% of a typical crude oil. Heavy fuels such as marine Bunker fuels do not contain a significant volatile fraction.

Aliphatic hydrocarbons are almost insoluble in water, but small aromatics, particularly the notorious BTEX (benzene, toluene, ethylbenzene, and the xylenes) and small polar molecules such as naphthenic acids dissolve and leave the bulk oil (Lafargue and Le Thiez 1996; Wang et al. 2016b) – their eventual fate is biodegradation (Prince et al. 2007).

Two disparate processes compete when water and oil mix; water may become entrained in the oil to form an emulsion or mousse, as eventually happens at most spills (Fingas and Fieldhouse 2012), or oil can disperse into the water column as a suspension of small droplets, as happened during the 1993 *Braer* spill off the Shetland Islands that occurred in a major storm (Thomas and Lunel 1993). Mousses are thought to be the precursors of tarballs, which can last for decades (Goodman 2003; Warnock et al. 2015). As we shall see below, chemical dispersants that break emulsions and stimulate the natural dispersion process are effective tools in the oil spill response "toolkit."

Oil also interacts with small mineral particles in a process termed "Oil-Mineral Fines Interactions" (Owens and Lee 2003; Wang et al. 2013). Like dispersion, this dramatically increases the surface area of the oil and stimulates biodegradation (Weise et al. 1999).

Aromatic hydrocarbons, particularly the larger and more alkylated ones, can be photochemically oxidized (Garrett et al. 1998; Aeppli et al. 2012), converting them to polar and probably polymerized species. Since light cannot penetrate very far into a dark oil slick, photooxidation has little effect on the bulk properties of spilled oil, but it may be important in generating a polymerized "skin" that stabilizes tarballs and "pavements" on beaches. Layers of immobile, hardened oil and sediment,

termed pavements, form when oil reaches a shoreline as a heavy, thick slick. Oil becomes trapped in the sediment, and the oil and the sediment become saturated with each other (Owens et al. 2008). Oil incorporated into such pavements is effectively preserved from weathering processes until this heavy, solidified material is physically disrupted, so a major goal of spill cleanup operations is to prevent the formation of pavements.

3 Eventual Fate of Spilled Oil

The weathering processes described above distribute and change the oil in various ways, but they do not actually remove oil from the environment. Only two processes, combustion and biodegradation, actually eliminate oil by converting it to carbon dioxide and water. Some spills do accidentally ignite, as happened in the 1991 *Haven* spill in the Mediterranean (Martinelli et al. 1995) where some 70% of the cargo was destroyed in the fire. Deliberate ignition is an accepted response option in some situations, such as that of the wood chip carrier *New Carissa* off the coast of Oregon in 1999 (Gallagher et al. 2001). Oil on water needs to be several mm thick to sustain combustion and in general that requires corralling in a boom, preferably a fire-proof one. Under optimal conditions, burning may consume >90% of oil so contained, but there is usually only a small window of opportunity for success (Buist 2003). Burning boomed oil was a significant response to the 2010 *Deepwater Horizon* blowout (Allen et al. 2011).

Far more generally, it is biodegradation that removes oil from the environment. As mentioned above, a diverse group of microorganisms (Prince 2010; Prince et al. 2010) has evolved to degrade hydrocarbons, and many are able to grow on hydrocarbons as their sole source of carbon. They are ubiquitous and obviously very effective, since they have been consuming the vast majority of the oil entering the world's oceans from natural seeps for millions of years (600,000 t, 700 million liters per year; National Research Council 2002). Nevertheless, the biodegradability depends on the oil; McMillen et al. (1995) examined the short-term biodegradability of 17 crude oils in soil microcosms and found that >61% of the most degradable oil (46° API) was lost in 4 weeks, while only 10% of the least degradable oil (15° API) was consumed under the same conditions. Nevertheless, further degradation occurred on a longer timescale and the literature reports biodegradation potentials as high as 97% for particularly light oils (Prince 1993). The rate of biodegradation depends on the oil composition, with the saturate fraction typically being more rapidly biodegradable than the aromatic fraction (e.g., Sugiura et al. 1997). In a very neat experiment, Uraizee et al. (1998) showed that the amount of asphaltenes (e.g., Sirota 2005; McKenna et al. 2013) in the oil affected the biodegradation of the hydrocarbons, progressively inhibiting biodegradation as their weight fraction increased. Thus the biodegradation of the complex mixtures that compose crude oils and refined product is complicated and shows clear preferences for some molecules before others (Prince and Walters 2016). Nevertheless it is a robust process that has been active for millions of years. The focus of this chapter is the

different approaches that have been taken to stimulate this biodegradation to clean up oils spilled in the marine environment.

4 Bioremediation by Alleviating Limiting Factors – Biostimulation

As we have seen above, crude oils and refined fuels are very biodegradable, but they are unusual carbon sources for microorganisms for at least two reasons. For one thing, they are essentially insoluble, so the surface area for microbial colonization and consumption is likely to limit biodegradation. For another, they provide energy without providing the nitrogen and phosphorus required for life, so availability of these, and perhaps other nutrients, is likely to limit degradation. Biostimulation protocols have focused on alleviating these two distinct limitations; the former while the spill is floating as a slick and the latter if oil reaches a shoreline.

4.1 Dispersants

Dispersants are mixtures of surfactants in a hydrocarbon solvent (e.g., Nalco 2014) that together aid the dispersion of oil into water as <100 μm neutrally buoyant droplets. They can do this even if the oil has begun to absorb water en route to becoming a mousse (Lessard and DeMarco 2000; National Research Council 2005). Once applied to the oil, the mixing action of waves and currents both generates the droplets and distributes them into the water column. This usually reduces oil concentrations so that droplets do not coalesce to floating sheens, and allows dilution to the sub ppm level within a day (Cormack and Nichols 1977; McAuliffe et al. 1980; Lichtenthaler and Daling 1983; Prince 2015). Such levels are below levels of acute concern for marine life (Gardiner et al. 2013; Incardona et al. 2013; Adams et al. 2014) and provide a very large surface area for microbial colonization and biodegradation (Prince and Butler 2014; Prince 2015). The rate and effectiveness of the dispersion process depends on the nature of the spilled oil (its API gravity and viscosity, degree of evaporation, extent of emulsification, pour point, etc.), the ability of the dispersant formulation to mix with the oil, and the sea conditions (Lessard and DeMarco 2000), but most oils can be dispersed with enough dispersant and enough energy (typical application rates are five gallons per acre – about 50 l per hectare). It is important to recognize that the effectiveness tests for dispersants mandated by the USEPA for listing on the *National Oil and Hazardous Substances Pollution Contingency Plan Product Schedule* (USEPA 2014), while suitable for purpose in discriminating between good and poor dispersants, substantially underestimate efficacy in the field and in wave tanks. To pass the test, dispersants must disperse at least 45% of Prudhoe Bay or South Louisiana crude oil in a standard “swirling flask” test (USEPA 2006). But the swirling energy used in the test is insignificant compared to even mild turbulence at sea. Tests in the OHMSETT facility, a 200 m wave tank in New Jersey that is 20 m wide and 2.5 m deep, routinely measure

dispersant efficiencies >95%, even at low temperatures with ice in the water (Belore et al. 2009), and the wave energy in that facility pales before the energy in even mild sea states.

An important environmental consideration is assessing the trade-off between intentionally exposing water column organisms to dispersed oil and the often significant effects of unrecovered oil left to drift to potentially strand on a shoreline. In most cases, these considerations suggest a net environmental benefit to the use of dispersants because the short-term, transient exposure of water column communities has much less ecological effect than the prolonged, and often widespread, contamination of oil reaching shorelines (McCay and Graham 2014; Prince 2015; Bejarano and Mearns 2015).

Dispersed oil droplets have only a transient lifetime because their large surface to volume ratio provides a much increased colonizing substrate for oil-degrading bacteria. Furthermore, their dispersion in the water column allows the low levels of biologically available nitrogen and phosphorus in the ocean to support bacterial degradation and maintain a viable degrader community (Swannell and Daniel 1999; Venosa and Holder 2007; Prince et al. 2013; McFarlin et al. 2014). Laboratory studies have shown that oil-degrading microbes colonize the droplets within 2–4 days, and a full heterotrophic community of oil, bacteria, protozoa, and even nematodes soon follows (MacNaughton et al. 2003). Furthermore, the chemical composition of some dispersants may enhance the initial rate of oil degradation because they serve as initial substrates for nascent bacterial growth (Varadaraj et al. 1995; Swannell and Daniel 1999). Dispersant formulations have been proposed that combine dispersion with the delivery of nutrients for microbial growth (Gatellier et al. 1983; Lepain and Bronchart 1986), but these are not currently commercially available and are probably unnecessary given the enormous dilution that occurs with effective dispersion. A persistent myth is that dispersants are unusually toxic, and that nontoxic formulations could be developed. In fact, dispersants such as Corexit 9500 are made from materials "generally regarded as safe for food contact" (Nalco 2014), and their inherent toxicity to marine organisms is lower than that of common dishwashing products (Word et al. 2013): the toxicity of dispersant-enhanced oil dispersions is due to the dispersed oil, not the dispersant (Hemmer et al. 2011).

Dispersants are stockpiled around the world (e.g., Marine Spill Response Corporation 2016), and many regulatory agencies recognize their use as an effective tool (Chapman et al. 2007). The largest use of dispersants to date occurred following the BP *Deepwater Horizon* accident. On April 20, 2010, high-pressure oil and gas escaped from BP's *Deepwater Horizon* exploratory well in Mississippi Canyon Block 252 located 77 km offshore. In the subsequent fire and explosions, 11 men tragically lost their lives. The *Deepwater Horizon* drilling rig burned and 2 days later sank in 1500 m. The blowout prevention device (BOP) at the wellhead and all the emergency shut-off equipment failed.

Upon sinking, the 21 in. (53 cm) riser pipe from the wellhead to the drilling platform collapsed onto the sea floor. Oil leaked from multiple locations along the riser pipe and the top of the BOP. In all, it took 84 days to stop the flow of oil. The oil

from this well is typical of light Louisiana crude from petroleum reservoirs more than 5000 m deep; it has an API gravity of 35.2 (The Federal Interagency Solutions Group: Oil Budget Calculator Science and Engineering Team 2010).

The BP *Deepwater Horizon* oil release was much larger and vastly different from the *Exxon Valdez* spill (Atlas and Hazen 2011). The actual volume of oil and gas released from the Deepwater Horizon well is difficult to determine, but a US Federal Judge (Barbier 2015) adjudicated that 3.19 million barrels of oil entered the water (134 million US gallons, 507 million liters). This is slightly smaller than (admittedly uncertain) assessments of the 1979 spill from the IXTOC-I well blowout in the Bay of Campeche (Jernelöv and Lindén 1981), estimated at 147 million gallons (556 million liters). Both are dwarfed by the deliberate release of oil into the Arabian Gulf in 1991 (250–450 million gallons, Gupta et al. 1993), which in turn was only a small part of the estimated >50 billion gallons of oil that spewed out of Kuwaiti oil wells destroyed by Iraqi troops as they withdrew (Husain 1995).

One of the strategies employed to defray the environmental and safety impact of the oil from the *Deepwater Horizon* was to inject the dispersant COREXIT 9500 directly at the wellhead (and end of the riser pipe before that was disconnected) at a water depth of 1500 m. The goal was to disperse the oil at depth, thereby preventing large slicks from forming at the surface directly above the wellhead where many ships were gathered to stop the leak, and to prevent the oil from impacting the shoreline (Rorick et al. 2012). The EPA established a rigorous, daily water sampling program, once it was demonstrated in early May that less oil was coming to the surface immediately above the wellhead within 4 h of injecting COREXIT 9500 at the wellhead, making it safer for leak operations.

As a result of dispersant addition and the forceful physical injection of the oil in the deepwater of the Gulf of Mexico, fine droplets of dispersed oil formed and moved away from the wellhead in the water column. Droplets between 10 and 60 μm are neutrally buoyant and were picked up by the current between 900 and 1300 m. Fluorescence measurements indicated that these finely dispersed oil droplets moved predominantly to the Southwest at a depth of approximately 1100 m (Camilli et al. 2010). While this was often referred to as a "plume," its concentration of <1 ppm oil (Wade et al. 2016) was invisible to the human eye, although readily detected by sensitive fluorometers. Larger droplets moved to the surface and formed surface slicks, some of which moved toward the shorelines. Surface applications of dispersant were used to break up these slicks to protect ecologically and commercially sensitive shorelines.

Almost immediately, Hazen et al. (2010) reported that there was rapid biodegradation of saturated hydrocarbons in the finely dispersed oil within the deep water even though temperatures were about 5 °C. The average half-life of C_{13}–C_{26} alkanes from four different field samples and two different lab microcosm assays was 3 days (Hazen et al. 2010). During the release (April–July), concentrations of polynuclear aromatic hydrocarbons also decreased rapidly with distance from the release point (the wellhead) and were seen to reach <1.0 ppb within 15–20 mi (24–32 km) in all directions other than to the southwest, where a small number of samples exceeded 1 ppb out to 40 mi (64 km) (Boehm et al. 2016).

The <1 ppm "deep sea plume of oil" was associated with an oxygen dip indicative of ongoing hydrocarbon biodegradation, and substantial enrichment of indigenous oil degrading microorganisms (*Colwelliaceae, Pseudomonas, Cycloclasticus, Oceanospirillales,* and *Pseudoalteromonas*) was detected in association with the deep plume of oil (Hazen et al. 2010; Baelum et al. 2012; Dubinsky et al. 2013). Measured half-lives for nC_{13}–nC_{26} alkanes ranged from 1.6–9.5 days (Hazen et al. 2010). The available data also shows that bacterial communities evolved sequential capabilities to degrade *n*-alkanes and aromatic hydrocarbons (Dubinsky et al. 2013; Valentine et al. 2012). Valentine et al. (2012) postulated that autoinoculation of oil degrading bacteria occurred as oil circulated around the wellhead before moving to the Southwest.

In a laboratory simulation using dispersed Macondo oil and Norwegian seawater collected below the thermocline in Trondheim fjord, Brakstad et al. (2015b) studied the biodegradation of dispersed oil at two different droplet sizes (10 µm or 30 µm median oil droplets) at a nominal concentration of 2 ppm oil. There was a clear correlation between oil biodegradation and bacterial succession, the latter dominated by *Gammaproteobacteria*. While *Oceanospirillales* and *Colwellia* were associated with *n*-alkane biodegradation, the later abundances of *Cycloclasticus, Marinobacter, Pseudomonas,* and *Pelagibacter* corresponded with degradation of aromatic HCs, and possibly complex alkanes. The enrichment of *Pelagibacter* at the end of the experiment hinted at a return to baseline conditions. The dispersions with the larger oil droplet distribution (30 µm) showed subtly slower biodegradation of some of the oil hydrocarbons than in the smaller droplet dispersion (10 µm), and this was reflected in delayed appearances of some bacterial groups, mainly associated with biodegradation of aromatic HCs. Several of the bacterial groups enriched during biodegradation of the Macondo oil in Norwegian seawater were also associated with oil biodegradation in the deepwater plume from the *Deepwater Horizon* accident and in laboratory studies with Gulf of Mexico deepwater. However, there were subtly different patterns of bacterial successions during biodegradation when compared to Gulf of Mexico water, suggesting differences between different geographical localities, depths, and environmental conditions.

A rather similar study with suspended droplets (Wang et al. 2016a) used water collected at depth near the wellhead and showed that most hydrocarbons were effectively degraded within 64 days. Some compounds exhibited significant lag times, but once this was completed, the half-lives of biodegradation showed good agreement with the alkane (nC_{13}–C_{26}) half-lives of 0.6–9.5 days reported for in situ biodegradation from the *Deepwater Horizon* plume samples and laboratory efforts to recreate in situ conditions (Hazen et al. 2010). The results also matched other chemically dispersed oil studies conducted at low temperatures (−1–8 °C), in which *n*-alkane half-lives ranged from 2 to 10 days while median PAHs half-lives ranged from 2 to 37 days (Siron et al. 1995; Venosa and Holder 2007; McFarlin et al. 2014; Prince et al. 2013).

As the dilute and finely dispersed oil and gas moved away from the *Deepwater Horizon* wellhead, the microbial community exhibited a dynamic successional response (Kimes et al. 2013; King et al. 2015). *Gammaproteobacteria* appear to

have been especially important hydrocarbon degraders that became enriched in the path of the oil and gas. Metagenome analyses showed that bacteria of the order *Oceanospirillales*, which are capable of alkane degradation, were dominant early (Mason et al. 2012). PhyloChip and 16S sequence analysis showed that the *Oceanospirillales* was subsequently replaced by *Cycloclasticus* and *Colwellia* that are capable of degrading aromatic and gaseous saturated hydrocarbons (Hazen et al. 2010; Valentine et al. 2010; Baelum et al. 2012; Redmond and Valentine 2012; Dubinsky et al. 2013), followed by methylotrophic bacteria (Kessler et al. 2011).

Studies that evaluated metabolic potential using protein-coding sequence microarrays (GeoChip) indicated the enrichment of genes involved in both aerobic and anaerobic hydrocarbon degradation in the plume (Lu et al. 2012). Metagenome and metatranscriptome sequence data showed that *Oceanospirillales* were actively involved in aerobic hydrocarbon degradation in May 2010 and that genes for enzymes involved in aerobic alkane degradation were expressed at relatively high levels in the path of the plume (Mason et al. 2012).

4.2 Fertilizers

The necessity of biologically available nitrogen and phosphorus for culturing hydrocarbon degrading microbes has been known for more than 65 years (e.g., Bushnell and Haas 1941), and exploiting the phenomenon to stimulate oil biodegradation dates back more than 40 years (e.g., Davis and Raymond 1964). The use of fertilizers for remediating oil spills at sea was first suggested by Atlas and Bartha (1972), who recognized that the fertilizers would have to stay with the oil for maximal effectiveness. They suggested using paraffinized urea and dioctylpyrophosphate as oleophilic sources of nitrogen and phosphorus, respectively (Bartha and Atlas 1976). Since then there has been a lot of research to test and improve this hypothesis, much of it reviewed in Prince (1993), Swannell et al. (1996), Lee and Merlin (1999), Prince and Atlas (2005), and Head et al. (2006).

Although Atlas and Bartha's initial experiments were aimed at floating oil slicks, the major use of oleophilic and slow release fertilizers has been in stimulating the biodegradation of beached oil. By far the largest use was in the response to the 1989 spill from the *Exxon Valdez*, where about 15% of the 12,600 km shoreline of Prince William Sound and the Gulf of Alaska became oiled to some degree (Galt et al. 1991; Harrison 1991). Most of the oiled beaches consisted of coarse gravel, sometimes protected by boulder armor. The primary treatment of heavily oiled shorelines was washing with cold or warm seawater, without surfactants, and collection of the liberated oil with skimmers (Nauman 1991). Then these shorelines, together with those that were lightly oiled and needed no prior treatment, were treated with fertilizers: an oleophilic liquid product designed to adhere to oil, known as Inipol EAP22 (Ladousse and Tramier 1991) for surface oil, and a slow-release encapsulated agricultural product known as Customblen to deliver nutrients to subsurface oil (Pritchard et al. 1992). Inipol EAP22 was a microemulsion with an internal aqueous solution of urea in an external oil phase of oleic acid and trilaureth-4-phosphate,

co-solubilized by butoxy-ethanol (Gautier et al. 1984). It contained 7.4% nitrogen and 0.7% phosphorus by weight and was applied with airless paint sprayers transported on small pontoon catamarans. Customblen was a high-quality agricultural fertilizer designed to release its nutrients into water percolating the shorelines over several weeks. It consisted primarily of ammonium nitrate, calcium phosphate, and ammonium phosphate, encased in polymerized linseed oil. Customblen contained 28% nitrogen and 3.5% phosphorus by weight and was applied with broadcast spreaders by workers walking the beaches.

More than 250,000 l of Inipol EAP22 was applied in 1989 and about half that in 1990 when there was much less surface oil and bioremediation was the principal cleanup technique. In addition, more than 16 t of Customblen were applied in 1989, and >50 t were applied in 1990 when the residual oil was principally beneath the very surface of the beaches. Small amounts of both fertilizers were applied in 1991 to the patches of residual oil, and the cleanup was declared complete by the Alaska and Federal governments in 1991. Almost 50 t of biologically available nitrogen had been delivered to the oiled shorelines in the 3 years (Prince and Bragg 1997), with no detectable adverse environmental impact.

The bioremediation operations were studied in detail by a team from Exxon, USEPA, and the Alaska Department of Environmental Conservation (ADEC). Careful monitoring of three representative shorelines demonstrated that fertilizer applications were generally successful at delivering nitrogen nutrients throughout the oiled sediment (Prince et al. 1994b). Within days, the indigenous microbial populations responded by increasing their oxygen consumption and their ability to mineralize radiolabeled hydrocarbons in laboratory assays (Lindstrom et al. 1991). And changes in the oil composition on the beaches showed that the rate of biodegradation had been stimulated up to fivefold (Bragg et al. 1994; Prince et al. 1994b). Environmental monitoring and toxicity testing showed that this stimulation was achieved with no detectable adverse environmental impact (Prince et al. 1994b).

The rates of oil biodegradation (determined by loss of hydrocarbons with respect to hopane used as an internal marker within the oil; Prince et al. 1994a), based on field measurements from April 1989–May 1990 at three sites on Knight Island, ranged from ~0.6 to 3.4 g oil/kg sediment per year for surface oil and ~0.3–3.6 g oil/kg sediment per year for subsurface oil (Bragg et al. 1994). This equated to a mean loss in the mass of residual oil of about 31% per year for surface oil and 12% per year for subsurface oil. Oil removal by biodegradation contributed to the total rate of oil removed from shorelines by all factors (physical cleanup, waves, storms, etc.), which amounted to 75–90% of the oil from 1989 through the winter of 1990 (Koons and Jahns 1992; Wolfe et al. 1994).

While much of the oil was biodegraded, some did become sequestered in subsurface sediments where there was little or no water flow (Short et al. 2007). Boufadel and colleagues proposed trying to bioremediate the residual subsurface oil by using high pressure injection of nutrient enriched water (Boufadel and Bobo 2011), and Venosa et al. (2010) performed microcosm studies that showed that additional biodegradation would occur if enriched water did reach the sequestered

oil. However, Pope et al. (2013) argued that water would more likely flow around the sequestered oil and would not deliver the necessary oxygen and nutrients. Therefore, Atlas and Bragg (2009, 2013) argued that this was a case where bioremediation would be ineffective and unnecessary since the oil was sequestered and not in contact with living biota.

The next use of bioremediation on a real spill seems to have been the work of Rosenberg and colleagues on a 1994 spill of heavy crude oil on a sandy beach north of Haifa, Israel (Rosenberg et al. 1996). They were concerned that approaches such as those used in Alaska did not deliver the nitrogen fertilizer specifically to the oil-degrading microbes, so they developed an enrichment culture of oil-degrading microbes able to get their nitrogen from polymerized formaldehyde-urea resin (Rosenberg et al. 1996; Rosenberg and Ron 1998). The spill of several hundred tons of crude oil contaminated a large amount of sand, and 30,000 m^2 were treated with a mixed bacterial culture plus their polymerized fertilizer and tilled twice a week. Overall, the treatment apparently resulted in the degradation of 88% of the oil after 4 months, during which time there was virtually no change in the oil concentration of a control plot that was tilled in the same way. Unfortunately no chemical analyses were done on the residual oil to confirm that the loss was due to biodegradation rather than physical loss, perhaps due to surfactants produced by the organisms, but the results appeared very promising. Later this group favored the use of uric acid as an insoluble lipophilic fertilizer (Koren et al. 2003), perhaps even in the form of guano (Knezevic et al. 2006), and showed that one of the more abundant hydrocarbon degraders, *Alcanivorax* (e.g., Cappello et al. 2007b; Harayama et al. 2004), is able to use uric acid as a nitrogen source.

Although bioremediation by adding fertilizers played an important role in the *Exxon Valdez* response, this success has not been followed by widespread use. This can be attributed to the fact that many spills get little if any cleanup, while those that do are treated with dispersants that prevent serious shoreline oiling. Furthermore, many high-visibility spills are near beaches that are amenable to rapid physical cleanup. For example, the vast majority of the oil from the 1996 *Sea Empress* spill was dispersed at sea or collected when it landed on sandy beaches (Colcomb et al. 1997; Lunel et al. 1997; Law and Kelly 2004). Swannell et al. (1999) did a small field trial with soluble fertilizer applied once a week, and a slow-release fertilizer held in bags, on a small cobble beach, and showed that both stimulated biodegradation without any detectable adverse environmental impact. Nevertheless, the technique was not used on a larger scale.

Bioremediation was also used on small experimental scales following the 2002 spill of very heavy fuel oil (API gravity of 11°) from the *Prestige*. Jiménez et al. (2006) showed that an oleophilic fertilizer rather similar to Inipol EAP22 significantly enhanced the biodegradation rate of high molecular weight *n*-alkanes, alkylcyclohexanes, and benzenes when applied to oil that had been on a cobble beach for 10 months, even though the experiment was done over the winter of 2003–2004. In contrast, Fernández-Álvarez et al. (2006) found no beneficial effects

of adding soluble fertilizer or bacterial cultures, although their site seems to have contained more weathered oil than that of Jiménez et al. (2006). But in any case, cleanup from this spill relied principally on physical removal because the heavy oil mousse did not penetrate into most beaches (Fernández-Álvarez et al. 2006).

There have, however, been many field trials on experimental oil spills, and the experiments of Lee and colleagues in Canada, Sveum and colleagues in Norway, and Swannell and colleagues in the UK have been reviewed in detail elsewhere (Prince 1993; Lee and Merlin 1999; Swannell et al. 1996; Prince and Atlas 2005; Head et al. 2006). More recent trials include the work of Venosa et al. (1996) on a beach in the Delaware estuary, Prince et al. (2003a) on a shoreline in Spitsbergen, Maki et al. (2003) on a beach in the Sea of Japan, Xu et al. (2005) on a beach in Singapore, and Mills et al. (2004) in a wetland in Texas. All demonstrated that nutrient addition stimulated the rate of biodegradation of at least some components of their oils by severalfold – quite in line with the field data from the *Exxon Valdez* spill (Bragg et al. 1994).

In contrast, Oudot et al. (1998) found no stimulation when they did ostensibly similar experiments in the Bay of Brest, which they attributed to high background levels of nutrients throughout their test site. Indeed, background levels were also rather high in the Delaware Bay (Venosa et al. 1996), and the stimulation of biodegradation by fertilizer was correspondingly small. This demonstrates that there is no point in undertaking a nutrient bioaugmentation program if lack of nutrients is not significantly limiting biodegradation. On the other hand, both these experiments involved small amounts of oil, so the requirement for nutrients at the sites was only a small fraction of that naturally available. It is not unreasonable to imagine that a large spill at those sites would deplete the ecosystem of nutrients, and that nutrient addition might then have a markedly positive effect.

Similarly, Venosa et al. (2002) saw very little stimulation of biodegradation in a nutrient amended freshwater wetland on the St. Lawrence River, Canada, and Tate et al. (2012) saw little stimulation of biodegradation by nutrients in a *Spartina alterniflora* dominated Louisiana salt marsh. Here it seems likely that oxygen rather than nutrients was the major intrinsic limitation on biodegradation.

When fertilizers are to be applied, the question arises “How much?” This is not a trivial issue, since excess nutrients washing off a shoreline might stimulate algal growth and even be toxic to invertebrates and fish. Several groups have suggested aiming for a nominal fertilizer nitrogen to oil carbon ratio of about 1:10, but this is a fruitless pursuit since oil concentrations typically follow log-normal distribution on a shoreline (see Limpert et al. 2001). To avoid potentially deleterious effects, it is appropriate to aim for nutrient levels near 100 μM biologically available nitrogen in the interstitial water of the oiled sediment (Prince et al. 1994b; Boufadel et al. 1999; Prince and Atlas 2005), which can be measured with simple hand-held colorimetric tests on site as appropriate (Prince et al. 2003a). No adverse effects have been seen at such levels (see Prince and Atlas 2005). The precise form of nitrogen does not seem to be very important – as discussed above, ammonium, nitrate, urea, uric acid, and polymerized forms have all been used with success.

5 Bioremediation by Adding Oil-Degrading Microbes – Bioaugmentation

The publicity around the first patented genetically engineered organism (Chakrabarty 1981), which could indeed degrade hydrocarbons, has led many to imagine that bioremediation means adding genetically enhanced bacteria to stimulate biodegradation. But in fact there have been very few cases where bacteria have been added in order to stimulate oil spill cleanup, and those few cases have used naturally occurring strains.

The first well-publicized use of added bacteria was on the 1990 spill from the *Mega Borg* in the Gulf of Mexico (Holden 1990, and see Leveille 1991), but nothing beyond anecdotal results were ever reported. It is only fair to note, however, that the logistical problems involved in monitoring the treatment were not settled before the spill dispersed.

A more recent use of added bacteria has been on the 1997 spill of Medium Fuel Oil from the *Nakhodka* in the Sea of Japan (Tsutsumi et al. 2000), but the only analyses reported in the paper are image analyses showing that the very heavy oil left the treated rocks – to what extent this was due to biodegradation is unknown.

Laboratory experiments have not been more optimistic. Indeed the most insightful demonstration was performed by the USEPA when testing potential inoculants for stimulating oil biodegradation in Alaska following the *Exxon Valdez* oil spill (Venosa et al. 1992). Eight microbial inocula were tested in small laboratory reactors that allowed substantial degradation of oil by the indigenous organisms of Prince William Sound; all eight potential inoculants had a greater stimulatory effect on alkane degradation if they were autoclaved prior to addition. This suggests that the indigenous organisms readily out-competed the added products but that autoclaving the products released some trace nutrient that was able to stimulate the growth of the endogenous organisms. Nevertheless, tests continue (e.g., Neralla and Weaver 1997; Tam and Wong 2008; Bao et al. 2012).

Putting aside the huge technological challenge of promptly adding a substantial number of viable and thriving organisms to a significant spill, the fundamental basis of bioaugmentation may rest on erroneous preconceptions. It makes the assumption that hydrocarbon-degrading microbes are relatively rare in the environment and so will be slow to colonize a spill. But there is no evidence this is true. Rather it seems that hydrocarbon-degraders are ubiquitous but typically limited by the availability of substrate. Thus when a spill occurs, they rapidly increase in numbers, indeed they “bloom.” Actual quantitation is fraught with experimental biases, not least because it is widely believed that only 1% or less of bacteria seen in environmental samples with a microscope can be cultured. Nevertheless, experiments that use consistent methods can give comparative results even if absolute numbers may be underestimates. Perhaps the most thorough sampling to date was done by Braddock et al. (1995) following the *Exxon Valdez* oil spill in Prince William Sound Alaska. Total bacteria were counted using epifluorescence microscopy (i.e., without culturing) while oil-degrading organisms were counted with a dilution method that detects organisms

by their ability to grow and disrupt a floating sheen of oil (Brown and Braddock 1990). Oil-degrading microorganisms defined in this way made up only about 0.002% of the total bacterial population, detected microscopically, on beaches outside the path of the spill, but 2% of the population on oiled shorelines. Thus the presence of oil was correlated with a thousand-fold increase in the population of culturable oil-degrading microbes. These changes can occur within a few days – Kasai et al. (2002) and Cappello et al. (2007a) showed that well-known hydrocarbon degraders, such as *Cycloclasticus* and *Alcanivorax,* were detected within days in oiled mesocosms flushed with seawater and that these numbers increased, as did their activity, when fertilizers were present. And these organisms do appear to be ubiquitous (Yakimov et al. 2007). Interestingly, blooms of oil-degrading organisms occur even following spills of very heavy oils not usually thought to be prime targets for bioremediation, such as the spill from the *Nakhodka* (Maruyama et al. 2003), indicating that even these heavy oils can lift the carbon limitation of oil-degrading microbes in the sea.

Data from the *Deepwater Horizon* blowout add further support to the notion that oil-degrading microbes are ubiquitous and ready to respond rapidly if oil enters the environment. Molecular data indicated that offshore sediments close to the wellhead became enriched in hydrocarbon degrading bacteria (Kimes et al. 2013). Single-cell sequencing revealed that surficial deep-sea sediments (0–1 cm) had elevated hydrocarbon concentrations and high populations of *Gammaproteobacteria*, including a strain of *Colwellia* sp. that had metabolic potential to degrade a wide range of hydrocarbons (Mason et al. 2014). Metagenomic analysis and qPCR-targeted functional gene assays of subsurface (1.5–3 cm) deep-sea sediment cores from September to October 2010 revealed increased levels of Deltaproteobacteria (which include anaerobic hydrocarbon-degrading sulfate reducers) and genes associated with the anaerobic degradation of aliphatic and aromatic hydrocarbons (e.g., benzoyl-CoA reductase genes and *bssA* and *assA* which encode benzyl- and alkyl succinate synthase, respectively) in the sediments located within 3 km of the wellhead (Kimes et al. 2013). Fully concordant with these microbial populations, the residual oil in these deep-sea sediments was biodegraded (Stout and Payne 2016).

Just as in the gravel shores of Prince William Sound (Lindstrom et al. 1991; Braddock et al. 1995), the arrival of oil from the *Deepwater Horizon* on sandy shorelines resulted in significant increases in oil-degrading microbes, especially *Gammaproteobacteria* (Kostka et al. 2011; Newton et al. 2013; Lamendella et al. 2014; Kappell et al. 2014; Rodriguez-R et al. 2015) and fungi (Bik et al. 2012). The hydrocarbon-utilizing bacterium *Desulfococcus oleovorans* comprised over 50% of the microbial community in heavily oil-contaminated anaerobic marsh sediments (Atlas et al. 2015).

6 Conclusions

Biodegradation is the ultimate fate of the majority of the hydrocarbon that enters the marine environment, so stimulating this biodegradation is an environmentally responsible approach to minimizing the environmental impact of oil spills if it can

be done with net environmental benefit (API 2013). But it is essential that the intervention address the factors actually limiting biodegradation (Atlas and Bragg 2009).

Dispersants are the optimal tool for this approach, for dispersed oil has a "half-life" by biodegradation of a couple of weeks or so (Hazen et al. 2010; Baelum et al. 2012; Prince et al. 2013, 2016; Prince and Butler 2014; Brakstad et al. 2015a; Wang et al. 2016a). This can be compared to a lifetime of months to years if oil reaches a shoreline and is not completely collected (Bragg et al. 1994; Aeppli et al. 2014). Dispersants must be used promptly, however, for their efficacy decreases as oil evaporates and incorporates water (Lessard and DeMarco 2000). This is principally a function of dispersant incorporation into the oil slick, since penetration becomes more difficult as oil viscosity increases (Canevari 1985). Once dispersants are incorporated, even in calm conditions, they will aid dispersion when rougher weather arrives (Lewis et al. 2010). Insistence that dispersants be seen to be effective on a trial basis before large-scale application can begin may thus hinder effective response.

Timely and effective oil dispersion should minimize, or even eliminate oil stranding. But some accidents occur so close to shore that oil will reach the shoreline and most likely require some physical cleanup, perhaps involving bioremediation. Again it is essential that bioremediation protocols address the factors actually limiting biodegradation (Atlas and Bragg 2009). For example, there is little point in adding nitrogenous nutrients to a site where such nutrients are not the fundamental limitation, either because there is already a sufficient supply (e.g., Oudot et al. 1998; Venosa et al. 1996) or because something else, such as oxygen, is the likely limiting factor (e.g., Venosa et al. 2002; Tate et al. 2012). To date there have been no successful demonstrations of the stimulation of biodegradation of oil under anaerobic conditions (e.g., Mills et al. 2004). Microbial degradation clearly occurs in such environments (Mahmoudi et al. 2013; Looper et al. 2013; Atlas et al. 2015), but no environmentally benign approach to stimulating the process has yet been achieved.

Beyond that, the composition of the oil must be borne in mind. For example, it is generally accepted that bioremediation is not a very useful technology for very heavy oils, such as those from the *Nakhodka* (Tsutsumi et al. 2000), *Erika* (Oudot 2000), or the *Prestige* (Díez et al. 2005), since only a small fraction of such oils will be biodegraded in a reasonably short timeframe. In the *Erika* case, only 11% of the oil was degraded in 80 days under what were thought to be optimal conditions. As discussed above, this is enough to cause a microbial bloom (Maruyama et al. 2003) but not enough to warrant consideration as a remediation approach unless the environmental impact of the most degradable fraction is a particular local concern. Similar considerations apply to the weathered residua of oils that were initially more degradable. For example, Díez et al. (2005) demonstrated that the rate of biodegradation of the three and four ring aromatic compounds in *Prestige* cargo oil decreased substantially after all the *n*-alkanes had been degraded, even in the presence of nutrients, and Venosa et al. (1996) saw a similar effect in their field trial on the Delaware Bay, as did Wrenn et al. (2006) in continuous flow microcosms. This may be a reflection of co-metabolism (Kanaly and Harayama 2000), or perhaps the fact that alkane degraders such as *Alcanivorax* grow much faster than aromatic degraders

such as *Cycloclasticus* (Kasai et al. 2002; Yakimov et al. 2007). In either case, while it does not indicate that biodegradation has ceased, it does suggest that the system is unlikely to be fertilizer limited once the alkanes are degraded (Atlas and Bragg 2009).

The biodegradation of oil pavements (Owens et al. 2008) is also unlikely to be nutrient limited. As discussed above, a photochemically polymerized surface layer likely protects encased hydrocarbons from microbial attack. But a thin coating of oil on sediment in a nutrient poor area, for example, one not subject to anthropogenic run-off, would be a prime target for nutrient assisted bioremediation.

In conclusion, bioremediation by the use of dispersants or fertilizers epitomizes modern environmental thinking, working with natural processes to remedy an accident in an environmentally responsible way. By its very nature, bioremediation addresses the most bioavailable hydrocarbons first, thereby minimizing exposure to higher trophic levels. Neither dispersants nor fertilizers are the panaceas that have sometimes been portrayed, but they are important tools in reducing the ecological impact of many oil spills (Canadian Coast Guard 1995; Owens 1996; NOAA 2016). In some cases, they are essentially the only approach for treating marine oil spills effectively.

7 Research Needs

There are currently no reliable bioremediation treatments for anaerobic environments, such as marsh sediments.

References

Adams J, Sweezey M, Hodson PV (2014) Oil and oil dispersant do not cause synergistic toxicity to fish embryos. Environ Toxicol Chem 33:107–114

Aeppli C, Carmichael CA, Nelson RK, Lemkau KL, Graham WM, Redmond MC, Valentine DL, Reddy CM (2012) Oil weathering after the *Deepwater Horizon* disaster led to the formation of oxygenated residues. Environ Sci Technol 46:8799–8807

Aeppli C, Nelson RK, Radović JR, Carmichael CA, Valentine DL, Reddy CM (2014) Recalcitrance and degradation of petroleum biomarkers upon abiotic and biotic natural weathering of *Deepwater Horizon* oil. Environ Sci Technol 48:6726–6734

Allen AA, Jaeger D, Mabile NJ, Costanzo D (2011) The use of controlled burning during the Gulf of Mexico *Deepwater Horizon* MC-252 oil spill response. In: Proceedings of the International oil spill conference paper 194. American Petroleum Institute

API (2013) Net environmental benefit analysis for effective oil spill preparedness and response. www.api.org/~/media/Files/EHS/Clean_Water/Oil_Spill_Prevention/NEBA/NEBA-Net-Environmental-Benefit-Analysis-July-2013.pdf. Accessed 1 Aug 2016

Arzayus KM, Dickhut RM, Canuel EA (2001) Fate of atmospherically deposited polycyclic aromatic hydrocarbons (PAHs) in Chesapeake Bay. Environ Sci Technol 35:2178–2183

Atlas RM, Bartha R (1972) Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorus. Biotechnol Bioeng 14:309–318

Atlas RM, Bragg JR (2009) Bioremediation of marine oil spills: when and when not – the *Exxon Valdez* experience. J Microbial Biotechnol 2:213–221

Atlas RM, Bragg JR (2013) Removal of oil from Shorelines: biodegradation and bioremediation. In: Wiens JA (ed) Oil in the environment: legacies and lessons of the *Exxon Valdez* oil spill. Cambridge University Press, Cambridge, pp 176–200

Atlas RM, Hazen TC (2011) Oil biodegradation and bioremediation: a tale of the two worst spills in US history. Environ Sci Technol 45:6709–6715

Atlas RM, Stoeckel DM, Faith SA, Minard-Smith A, Thorn JR, Benotti MJ (2015) Oil biodegradation and oil-degrading microbial populations in marsh sediments impacted by oil from the *Deepwater Horizon* well blowout. Environ Sci Technol 49:8356–8366

Baelum J, Borglin S, Chakraborty R, Fortney JL, Lamendella R, Mason OU, Auer M, Zemla M, Bill M, Conrad ME, Malfatti SA, Tringe SG, Holman HY, Hazen TC, Jansson JK (2012) Deep-sea bacteria enriched by oil and dispersant from the *Deepwater Horizon* spill. Environ Microbiol 14:2405–2416

Bao MT, Wang LN, Sun PY, Cao LX, Zou J, Li YM (2012) Biodegradation of crude oil using an efficient microbial consortium in a simulated marine environment. Mar Pollut Bull 64:1177–1785

Barbier CJ (2015) MDL – 2179 oil spill by the oil rig "Deepwater horizon." http://www.laed.uscourts.gov/sites/default/files/OilSpill/Orders/1152015FindingsPhaseTwo.pdf. Accessed 1 Aug 2016

Bartha R, Atlas RM (1976) Biodegradation of oil on water surfaces. US Patent 3,959,127

Bejarano AC, Mearns AJ (2015) Improving environmental assessments by integrating Species Sensitivity Distributions into environmental modeling: examples with two hypothetical oil spills. Mar Pollut Bull 93:172–182

Belore RC, Trudel K, Mullin JV, Guarino A (2009) Large-scale cold water dispersant effectiveness experiments with Alaskan crude oils and Corexit 9500 and 9527 dispersants. Mar Pollut Bull 58:118–128

Betancourt F, Palacio A, Rodriguez A (2005) Effects of the mass transfer process in oil spill. Am J Appl Sci 2:939–946

Bik HM, Halanych KM, Sharma J, Thomas WK (2012) Dramatic shifts in benthic microbial eukaryote communities following the *Deepwater Horizon* oil spill. PLoS One 7(6):e38550

Boehm PD, Murray KJ, Cook LL (2016) Distribution and attenuation of polycyclic aromatic hydrocarbons in Gulf of Mexico seawater from the *Deepwater Horizon* oil accident. Environ Sci Technol 50:584–592

Boufadel MC, Bobo AM (2011) Feasibility of high pressure injection of chemicals into the subsurface for the bioremediation of the *Exxon Valdez* oil. Ground Water Monit Remidiat 31 (1):59–67

Boufadel MC, Reeser P, Suidan MT, Wrenn BA, Cheng J, Du X, Huang TL, Venosa AD (1999) Optimal nitrate concentration for the biodegradation of *n*-heptadecane in a variably-saturated sand column. Environ Technol 20:191–199

Braddock JF, Lindstrom JE, Brown EJ (1995) Distribution of hydrocarbon-degrading microorganisms in sediments from Prince William Sound, Alaska, following the *Exxon Valdez* spill. Mar Pollut Bull 30:125–132

Bragg JR, Prince RC, Harner EJ, Atlas RM (1994) Effectiveness of bioremediation for the *Exxon Valdez* oil spill. Nature 368:413–418

Brakstad OG, Nordtug T, Throne-Holst M (2015a) Biodegradation of dispersed *Macondo* oil in seawater at low temperature and different oil droplet sizes. Mar Pollut Bull 93:144–152

Brakstad OG, Throne-Holst M, Netzer R, Stoeckel DM, Atlas RM (2015b) Microbial communities related to biodegradation of dispersed *Macondo* oil at low seawater temperature with Norwegian coastal seawater. J Microbial Biotechnol 8:989–998

Brown EJ, Braddock JE (1990) Sheen Screen, a miniaturized most probable number method for enumeration of oil-degrading microorganisms. Appl Environ Microbiol 56:3895–3896

Buist I (2003) Window-of-opportunity for in situ burning. Spill Sci Technol Bull 8:341–346

Bushnell LD, Haas HF (1941) The utilization of certain hydrocarbons by microorganisms. J Bacteriol 41:653–673

Camilli R, Reddy CM, Yoerger DR, Van Mooy BA, Jakuba MV, Kinsey JC, McIntyre CP, Sylva SP, Maloney JV (2010) Tracking hydrocarbon plume transport and biodegradation at *Deepwater Horizon*. Science 330:201–204

Canadian Coast Guard (1995) Oil spill response field guide. Canadian Coast Guard, Ottawa. ISBN 0–660–16112–5.
Canevari GP (1985) The effect of crude oil composition on dispersant performance. In: Proceedings of the international oil spill conference. American Petroleum Institute, pp 441–444
Cappello S, Caruso G, Zampino D, Monticelli LS, Maimone G, Denaro R, Tripodo B, Troussellier M, Yakimov M, Giuliano L (2007a) Microbial community dynamics during assays of harbour oil spill bioremediation: a microscale simulation study. J Appl Microbiol 102:184–194
Cappello S, Denaro R, Genovese M, Giuliano L, Yakimov MM (2007b) Predominant growth of *Alcanivorax* during experiments on "oil spill bioremediation" in mesocosms. Microbiol Res 162:185–190
Castro-Jiménez J, Berrojalbiz N, Wollgast J, Dachs J (2012) Polycyclic aromatic hydrocarbons (PAHs) in the Mediterranean Sea: atmospheric occurrence, deposition and decoupling with settling fluxes in the water column. Environ Pollut 166:40–47
Chakrabarty AN (1981) Microorganisms having multiple compatible degradative energy-generating plasmids and preparation thereof. US Patent 4,259,444
Chapman H, Purnell K, Law RJ, Kirby MF (2007) The use of chemical dispersants to combat oil spills at sea: a review of practice and research needs in Europe. Mar Pollut Bull 54:827–838
Chen Y, Lin T, Tang J, Xie Z, Tian C, Li J, Zhang G (2016) Exchange of polycyclic aromatic hydrocarbons across the air-water interface in the Bohai and Yellow Seas. Atmos Environ 141:153–160
Cho Y, Ahmed A, Islam A, Kim S (2015) Developments in FT-ICR MS instrumentation, ionization techniques, and data interpretation methods for petroleomics. Mass Spectrom Rev 34:248–263
Colcomb K, Bedborough D, Lunel T, Swannell R, Wood P, Rusin J, Bailey N, Halliwell C, Davies L, Sommerville M, Dobie A, Mitchell D, McDonagh M, Lee K, Shimwell S, Davies B, Harries D (1997) Shoreline cleanup and waste disposal issues during the *Sea Empress* incident. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 195–203
Cormack D, Nichols JA (1977) The concentrations of oil in sea water resulting from natural and chemically induced dispersion of oil slicks. In: Proceedings of the international oil spill conference. American Petroleum Institute, pp 381–385
Davis JB, Raymond RL (1964) Microbial disposal of oily wastes. US Patent 3,152,983
Díez S, Sabaté J, Viñas M, Bayona JM, Solanas AM, Albaigés J (2005) The *Prestige* oil spill. I. Biodegradation of a heavy fuel oil under simulated conditions. Environ Toxicol Chem 24:2203–2317
Dubinsky EA, Conrad ME, Chakraborty R, Bill M, Borglin SE, Hollibaugh JT, Mason OU, Piceno YM, Reid FC, Stringfellow WT, Tom LM, Hazen TC, Andersen GL (2013) Succession of hydrocarbon-degrading bacteria in the aftermath of the *Deepwater Horizon* oil spill in the Gulf of Mexico. Environ Sci Technol 47:10860–10867
Energy Information Agency (2016a) International energy statistics. https://www.eia.gov/cfapps/ipdbproject/iedindex3.cfm?tid=5&pid=53&aid=1&cid=regions&syid=2010&eyid=2015&unit=TBPD. Accessed 1 Aug 2016
Energy Information Agency (2016b) Crude oil input qualities. https://www.eia.gov/dnav/pet/pet_pnp_crq_a_EPC0_YCG_d_a.htm. Accessed 1 Aug 2016
Etkin DS (2001) Analysis of oil spill trends in the US and worldwide. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 1291–1300
Fernández-Álvarez P, Vila J, Garrido-Fernández JM, Grifoll M, Lema JM (2006) Trials of bioremediation on a beach affected by the heavy oil spill of the *Prestige*. J Hazard Mater 137:1523–1531
Fingas MF (2013) Modeling oil and petroleum evaporation. J Petrol Sci Res 2:104–115
Fingas M, Fieldhouse B (2012) Studies on water-in-oil products from crude oils and petroleum products. Mar Pollut Bull 64:272–283

Gallagher JJ, Hile HB, Miller JA (2001) The old *New Carissa*; a study in patience. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 85–90

Galt JA, Lehr WL, Payton DL (1991) Fate and transport of the *Exxon Valdez* oil spill. Environ Sci Technol 25:202–209

Gardiner WW, Word JQ, Word JD, Perkins RA, McFarlin KM, Hester BW, Word LS, Ray CM (2013) The acute toxicity of chemically and physically dispersed crude oil to key arctic species under arctic conditions during the open water season. Environ Toxicol Chem 32:2284–2300

Garrett RM, Pickering IJ, Haith CE, Prince RC (1998) Photooxidation of crude oils. Environ Sci Technol 32:3719–3723

Gatellier C, Plessis A, Gateau P, Durand JP (1983) Dispersant and biodegradant formulations for treating hydrocarbon-polluted waters. US Patent 4,382,873

Gautier JC, Sirvins A, Tellier J, Tramier B (1984) Micro-emulsion of nutrient substances. US Patent 4,460,692

Goodman R (2003) Tar balls: the end state. Spill Sci Technol Bull 8:117–121

Gupta RS, Fondekar SP, Alagarsamy R (1993) State of oil pollution in the northern Arabian Sea after the 1991 Gulf oil spill. Mar Pollut Bull 27:85–91

Hamilton JF, Lewis AC, Bloss C, Wagner V, Henderson AP, Golding BT, Wirtz K, Martin-Reviejo M, Pilling MJ (2003) Measurements of photo-oxidation products from the reaction of a series of alkyl-benzenes with hydroxyl radicals during EXACT using comprehensive gas chromatography. Atmos Chem Phys 3:1999–2014

Harayama S, Kasai Y, Hara A (2004) Microbial communities in oil-contaminated seawater. Curr Opin Biotechnol 15:205–214

Harrison OR (1991) An overview of the *Exxon Valdez* oil spill. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 313–320

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Borglin SE, Fortney JL, Stringfellow WT, Bill M, Conrad ME, Tom LM, Chavarria KL, Alusi TR, Lamendella R, Joyner DC, Spier C, Baelum J, Auer M, Zemla ML, Chakraborty R, Sonnenthal EL, D'haeseleer P, Holman H-YN, Osman S, Lu Z, Van Nostrand JD, Deng Y, Zhou J, Mason OU (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 330:204–208

Head IM, Jones DM, Röling WFM (2006) Marine micro-organisms make a meal of oil. Nat Rev Microbiol 4:173–182

Hemmer MJ, Barron MG, Greene RM (2011) Comparative toxicity of eight oil dispersants, Louisiana sweet crude oil (LSC), and chemically dispersed LSC to two aquatic test species. Environ Toxicol Chem 30:2244–2252

Holden C (1990) Gulf slick a free lunch for bacteria. Science 249:120

Hughey CA, Galasso SA, Zumberge JE (2007) Detailed compositional comparison of acidic NSO compounds in biodegraded reservoir and surface crude oils by negative ion electrospray Fourier transform ion cyclotron resonance mass spectrometry. Fuel 86:758–768

Husain T (1995) Kuwaiti oil fires; regional environmental perspective. Pergamon Press, UK.

Incardona JP, Swarts TL, Edmunds RC, Linbo TL, Aquilina-Beck A, Sloan CA, Gardner LD, Block BA, Scholz NL (2013) *Exxon Valdez* to *Deepwater Horizon*: comparable toxicity of both crude oils to fish early life stages. Aquat Toxicol 142–143:303–316

ITOPF (2016) Oil tanker spill statistics. www.itopf.com/fileadmin/data/Documents/Company_Lit/Oil_Spill_Stats_2016.pdf. Accessed 1 Aug 2016

Jernelöv A, Lindén O (1981) *Ixtoc I*: a case study of the world's largest oil spill. Ambio 1981:299–306

Jiménez N, Viñas M, Sabaté J, Díez S, Bayona JM, Solanas AM, Albaiges J (2006) The *Prestige* oil spill. 2. Enhanced biodegradation of a heavy fuel oil under field conditions by the use of an oleophilic fertilizer. Environ Sci Technol 40:2578–2585

Kanaly RA, Harayama S (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067

Kappell AD, Wei Y, Newton RJ, Van Nostrand JD, Zhou J, McLellan SL, Hristova KR (2014) The polycyclic aromatic hydrocarbon degradation potential of Gulf of Mexico native coastal microbial communities after the *Deepwater Horizon* oil spill. Front Microbiol 5:285

Kasai Y, Kishira H, Harayama S (2002) Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. Appl Environ Microbiol 68:5625–5633

Kessler JD, Valentine DL, Redmond MC, Du M, Chan EW, Mendes SD, Quiroz EW, Villanueva CJ, Shusta SS, Werra LM, Yvon-Lewis SA (2011) A persistent oxygen anomaly reveals the fate of spilled methane in the deep Gulf of Mexico. Science 331:312–315

Kim S, Stanford LA, Rodgers RP, Marshall AG, Walters CC, Qian K, Wenger LM, Mankiewicz P (2005) Microbial alteration of the acidic and neutral polar NSO compounds revealed by Fourier transform ion cyclotron resonance mass spectrometry. Org Geochem 36:1117–1134

Kimes NE, Callaghan AV, Aktas DF, Smith WL, Sunner J, Golding BT, Drozdowska M, Hazen TC, Suflita JM, Morris PJ (2013) Metagenomic analysis and metabolite profiling of deep–sea sediments from the Gulf of Mexico following the *Deepwater Horizon* oil spill. Front Microbiol 4:50

King GM, Kostka JE, Hazen TC, Sobecky PA (2015) Microbial responses to the *Deepwater Horizon* oil spill: from coastal wetlands to the deep sea. Ann Rev Mar Sci 7:377–401

Knezevic V, Koren O, Ron EZ, Rosenberg E (2006) Petroleum bioremediation in seawater using guano as the fertilizer. Biorem J 10:83–91

Koons CB, Jahns HO (1992) The fate of oil from the *Exxon Valdez*: a perspective. Mar Technol Soc J 26:61–69

Koren O, Knezevic V, Ron EZ, Rosenberg E (2003) Petroleum pollution bioremediation using water-insoluble uric acid as the nitrogen source. Appl Environ Microbiol 69:6337–6339

Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, Delgardio J, Norton N, Hazen TC, Huettel M (2011) Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the *Deepwater Horizon* oil spill. Appl Environ Microbiol 77:7962–7974

Ladousse A, Tramier B (1991) Results of 12 years of research in spilled oil bioremediation: inipol EAP22. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 577–582

Lafargue E, Le Thiez P (1996) Effect of water washing on light ends compositional heterogeneity. Org Geochem 24:1141–1150

Lamendella R, Strutt S, Borglin S, Chakraborty R, Tas N, Mason OU, Hultman J, Prestat E, Hazen TC, Jansson JK (2014) Assessments of the *Deepwater Horizon* oil spill impact on Gulf coast microbial communities. Front Microbiol 5:13

Law RJ, Kelly C (2004) The impact of the *Sea Empress* oil spill. Aquat Living Resour 17:389–394

Lee K, Merlin FX (1999) Bioremediation of oil on shore-line environments: development of techniques and guidelines. Pure Appl Chem 71:161–171

Lee K, Boufadel M, Chen B, Foght J, Hodson P, Swanson S, Venosa A (2015) Expert panel report on the behavior and environmental impacts of crude oil released into aqueous environments. Royal Society of Canada, Ottawa. www.rsc.ca/sites/default/files/pdf/OIW%20Report_1.pdf. Accessed 1 Aug 2016

Lepain A, Bronchart RDEM (1986) Method for treating oil slicks. US Patent 4,623,468

Lessard RR, DeMarco G (2000) The significance of oil spill dispersants. Spill Sci Technol Bull 6:59–68

Leveille TP (1991) The *Mega Borg* fire and oil spill: a case study. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 273–278

Levy EM, Lee K (1988) Potential contribution of natural hydrocarbon seepage to benthic productivity and the fisheries of Atlantic Canada. Can J Fish Aquat Sci 45:349–352

Lewis A, Trudel BK, Belore RC, Mullin JV (2010) Large-scale dispersant leaching and effectiveness experiments with oils on calm water. Mar Pollut Bull 60:244–254

Lichtenthaler RG, Daling PS (1983) Dispersion of chemically treated crude oil in Norwegian offshore waters. In: Proceedings of the international oil spill conference. American Petroleum Institute, pp 7–14

Limpert E, Stahel WA, Abbt M (2001) Log-normal distributions across the sciences: keys and clues. Bioscience 51:341–352

Lindstrom JE, Prince RC, Clark JR, Grossman MJ, Yeager TR, Braddock JF, Brown EJ (1991) Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V *Exxon Valdez* oil spill. Appl Environ Microbiol 57:2514–2522

Looper JK, Cotto A, Kim BY, Lee MK, Liles MR, Chadhain SM, Son A (2013) Microbial community analysis of *Deepwater Horizon* oil-spill impacted sites along the Gulf coast using functional and phylogenetic markers. Evnviron Sci Process Impacts 15:2068–2079

Lu Z, Deng Y, Van Nostrand JD, He Z, Voordeckers J, Zhou A, Lee YJ, Mason OU, Dubinsky EA, Chavarria KL, Tom LM (2012) Microbial gene functions enriched in the *Deepwater Horizon* deep-sea oil plume. ISME J 6:451–460

Lunel T, Rusin J, Halliwell C, Davies L (1997) The net environmental benefit of a successful dispersant operation at the *Sea Empress* incident. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 185–194

MacNaughton SJ, Swannell R, Daniel F, Bristow L (2003) Biodegradation of dispersed Forties crude and Alaskan North Slope oils in microcosms under simulated marine conditions. Spill Sci Technol Bull 8:179–186

Mahmoudi N, Porter TM, Zimmerman AR, Fulthorpe RR, Kasozi GN, Silliman BR, Slater GF (2013) Rapid degradation of *Deepwater Horizon* spilled oil by indigenous microbial communities in Louisiana saltmarsh sediments. Environ Sci Technol 47:13303–13312

Maki H, Hirayama N, Hiwatari T, Kohata K, Uchiyama H, Watanabe M, Yamasaki F, Furuki M (2003) Crude oil bioremediation field experiment in the Sea of Japan. Mar Pollut Bull 47:74–77

Marine Spill Response Corporation (2016) https://www.oilspillresponse.com/globalassets/technical-library/factsheets/gds_tis_jan-2016.pdf. Accessed 1 Aug 2016

Martinelli M, Luise A, Tromellini E, Sauer TC, Neff JM, Douglas GS (1995) The M/C Haven oil spill: environmental assessment of exposure pathways and resource injury. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 679–685

Maruyama A, Ishiwata H, Kitamura K, Sunamura M, Fujita T, Matsuo M, Higashihara T (2003) Dynamics of microbial populations and strong selection for *Cycloclasticus pugetii* following the *Nakhodka* oil spill. Microb Ecol 46:442–453

Mason OU, Hazen TC, Borglin S, Chain PS, Dubinsky EA, Fortney JL, Han J, Holman HY, Hultman J, Lamendella R, Mackelprang R (2012) Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to *Deepwater Horizon* oil spill. ISME J 6:1715–1727

Mason OU, Han J, Woyke T, Jansson JK (2014) Single-cell genomics reveals features of a *Colwellia* species that was dominant during the *Deepwater Horizon* oil spill. Front Microbiol 5:332

McAuliffe CD, Johnson JC, Greene SH, Canevari GP, Searl TD (1980) Dispersion and weathering of chemically treated crude oils on the ocean. Environ Sci Technol 14:1509–1518

McCay DF, Graham E (2014) Quantifying tradeoffs-net environmental benefits of dispersant use. In: Proceedings of the international oil spill conference. American Petroleum Institute, pp 762–775

McFarlin KM, Prince RC, Perkins R, Leigh MB (2014) Biodegradation of dispersed oil in arctic seawater at-1 C. PLoS One 9:e84297

McKenna AM, Marshall AG, Rodgers RP (2013) Heavy petroleum composition. 4. Asphaltene compositional space. Energy Fuel 27:1257–1267

McMillen SJ, Requejo AG, Young GN, Davis PS, Cook PD, Kerr JM, Gray NR (1995) Bioremediation potential of crude oil spilled on soil. In: Hinchee RE, Vogel CM, Brockman FJ (eds) Microbial processes for bioremediation. Battelle Press, Columbus, pp 91–99

Mills MA, Bonner JS, Page CA, Autenrieth RL (2004) Evaluation of bioremediation strategies of a con-trolled oil release in a wetland. Mar Pollut Bull 49:425–435
Nalco (2014) COREXIT® Ingredients. http://www.nalcoesllc.com/nes/1602.htm. Accessed 1 Aug 2016
National Research Council (2002) Oil in the Sea III: inputs, fates and effects. National Academy Press, Washington, DC
National Research Council (2005) Oil spill dispersants: efficacy and effects. National Academy Press, Washington, DC
Nauman SA (1991) Shoreline clean-up: equipment and operations. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 141–148
Neralla S, Weaver RW (1997) Inoculants and biodegradation of crude oil floating on marsh sediments. Biorem J 1:89–96
Newton RJ, Huse SM, Morrison HG, Peake CS, Sogin ML, McLellan SL (2013) Shifts in the microbial community composition of Gulf Coast beaches following beach oiling. PLoS One 8: e74265
NOAA (2016) Characteristics of response strategies: a guide for spill response planning in marine environments. http://response.restoration.noaa.gov/oil-and-chemical-spills/oil-spills/resources/characteristics-response-strategies.html
Oudot J (2000) Biodégradabilité du fuel de *l'Erika*/Bio-degradability of the *Erika* fuel oil. C R Acad Sci III 323:945–950
Oudot J, Merlin FX, Pinvidic P (1998) Weathering rates of oil components in a bioremediation experiment in estuarine sediments. Mar Environ Res 45:113–125
Owens EH (1996) Field guide for the protection and cleanup of arctic oiled shorelines. Environment Canada, Prairie and Northern region, Yellowknife NWT, p 213pp
Owens EH, Lee K (2003) Interaction of oil and mineral fines on shorelines: review and assessment. Mar Pollut Bull 47:397–405
Owens EH, Taylor E, Humphrey B (2008) The persistence and character of stranded oil on coarse-sediment beaches. Mar Pollut Bull 56:14–26
Pope G, Gordon KD, Bragg JR (2013) Understanding subsurface contamination using conceptual mathematical models. In: Wiens JA (ed) Oil in the Environment: legacies and Lessons of the *Exxon Valdez* oil spill. Cambridge University Press, Cambridge, pp 144–175
Prince RC (1993) Petroleum spill bioremediation in marine environments. Crit Rev Microbiol 19:217–242
Prince RC (2010) Eukaryotic hydrocarbon degraders. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin/Heidelberg, pp 2066–2078
Prince RC (2015) Oil spill dispersants: boon or bane? Environ Sci Technol 49:6376–6384
Prince RC, Atlas RM (2005) Bioremediation of marine oil spills. In: Atlas RM, Philp JC (eds) Bioremediation: applied microbial solutions for real-world environmental cleanup. ASM Press, Washington, DC, pp 269–292
Prince RC, Bragg JM (1997) Shoreline bioremediation following the *Exxon Valdez* oil spill in Alaska. Biorem J 1:97–104
Prince RC, Butler JD (2014) A protocol for assessing the effectiveness of oil spill dispersants in stimulating the biodegradation of oil. Environ Sci Pollut Res 21:9506–9510
Prince RC, Walters CC (2016) Biodegradation of oil and its implications for source identification. In: Stout SA, Wang Z (eds) Standard handbook oil spill environmental forensics, 2nd edn. Academic Press, Burlington, pp 869–916
Prince RC, Elmendorf DL, Lute JR, Hsu CS, Haith CE, Senius JD, Dechert GJ, Douglas GS, Butler EL (1994a) 17α(H)-21β(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ Sci Technol 28:142–145
Prince RC, Clark JR, Lindstrom JE, Butler EL, Brown EJ, Winter G, Grossman MJ, Parrish RR, Bare RE, Braddock JF, Steinhauer WG, Douglas GS, Kennedy JM, Barter PJ, Bragg JR, Harner EJ, Atlas RM (1994b) Bioremediation of the *Exxon Valdez* oil spill: monitoring safety and

efficacy. In: Hinchee RE, Alleman BC, Hoeppel RE, Miller RN (eds) Hydrocarbon remediation. Lewis Publishers, Boca Raton, pp 107–124

Prince RC, Bare RE, Garrett RM, Grossman MJ, Haith CE, Keim LG, Lee K, Holtom GJ, Lambert P, Sergy GA, Owens EH, Guénette CC (2003a) Bioremediation of stranded oil on an Arctic shoreline. Spill Sci Technol Bull 8:303–312

Prince RC, Garrett RM, Bare RE, Grossman MJ, Townsend T, Suflita JM, Lee K, Owens EH, Sergy GA, Braddock JF, Lindstrom JE, Lessard RR (2003b) The roles of photooxidation and biodegradation in long-term weathering of crude and heavy fuel oils. Spill Sci Technol Bull 8:145–156

Prince RC, Parkerton TF, Lee C (2007) The primary aerobic biodegradation of gasoline hydrocarbons. Environ Sci Technol 41:3316–3321

Prince RC, Gramain A, McGenity TJ (2010) Prokaryotic hydrocarbon degraders. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin/Heidelberg, pp 1672–1692

Prince RC, McFarlin KM, Butler JD, Febbo EJ, Wang FC, Nedwed TJ (2013) The primary biodegradation of dispersed crude oil in the sea. Chemosphere 90:521–526

Prince RC, Kelley BA, Butler JD (2016) Three widely-available dispersants substantially increase the biodegradation of otherwise undispersed oil. J Mar Sci 6:2

Pritchard PH, Mueller JG, Rogers JC, Kremer FV, Glaser JA (1992) Oil spill bioremediation; experiences, lessons and results from the *Exxon Valdez* oil spill in Alaska. Biodegradation 3:315–335

Redmond MC, Valentine DL (2012) Natural gas and temperature structured a microbial community response to the *Deepwater Horizon* oil spill. Proc Natl Acad Sci U S A 109:20292–20297

Robbins WK, Hsu CS (1996) Petroleum Composition. In: Kroschwitz JI (ed) Kirk-othmer encyclopedia of chemical technology, 4th edn. Wiley, New York, pp 352–370

Rodriguez-R LM, Overholt WA, Hagan C, Huettel M, Kostka JE, Konstantinidis KT (2015) Microbial community successional patterns in beach sands impacted by the Deepwater Horizon oil spill. ISME J 9:1928–1940

Rorick R, Nedwed T, DeMarco G, Cooper C (2012) Comment on "A tale of two spills: novel science and policy implications of an emerging new oil spill model". Bioscience 62:1009–1010

Rosenberg E, Ron EZ (1998) Non-polluting compositions to degrade hydrocarbons and microorganisms. US Patent 5,780,290

Rosenberg E, Legman R, Kushmaro A, Adler E, Abir H, Ron EZ (1996) Oil bioremediation using insoluble nitrogen source. J Biotechnol 51:273–278

Short JW, Irvine GV, Mann DH, Maselko JM, Pella JJ, Lindeberg MR, Payne JR, Driskell WB, Rice SD (2007) Slightly weathered *Exxon Valdez* oil persists in Gulf of Alaska beach sediments after 16 years. Environ Sci Technol 41:1245–1250

Siron R, Pelletier É, Brochu C (1995) Environmental factors influencing the biodegradation of petroleum hydrocarbons in cold seawater. Arch Environ Contam Toxicol 28:406–416

Sirota EB (2005) Physical structure of asphaltenes. Energy Fuel 19:1290–1296

Speight JG (2014) The chemistry and technology of petroleum, 5th edn. CRC press, Boca Raton

Stout SA, Payne JR (2016) Macondo oil in deep-sea sediments: Part 1–sub-sea weathering of oil deposited on the seafloor. Mar Pollut Bull 111: 365. in press.

Sugiura K, Ishihara M, Shimauchi T, Harayama S (1997) Physicochemical properties and biodegradability of crude oil. Environ Sci Technol 31:45–51

Swannell RPJ, Daniel F (1999) Effect of dispersants on oil biodegradation under simulated marine conditions. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 169–176

Swannell RPJ, Lee K, McDonagh M (1996) Field evaluations of marine spill bioremediation. Microbiol Rev 60:342–365

Swannell RPJ, Mitchell D, Lethbridge G, Jones D, Heath D, Hagley M, Jones M, Petch S, Milne R, Croxford R, Lee K (1999) A field demonstration of the efficacy of bioremediation to treat oiled shore-lines following the Sea Empress incident. Environ Technol 20:863–873

Tam NFY, Wong YS (2008) Effectiveness of bacterial inoculum and mangrove plants on remediation of sediment contaminated with polycyclic aromatic hydrocarbons. Mar Pollut Bull 57:716–726

Tate PT, Shin WS, Pardue JH, Jackson WA (2012) Bioremediation of an experimental oil spill in a coastal Louisiana salt marsh. Water Air Soil Pollut 223:1115–1123

The Federal Interagency Solutions Group: Oil Budget Calculator Science and Engineering Team (2010) Oil budget calculator technical documentation. http://www.restorethegulf.gov/sites/default/files/documents/pdf/OilBudgetCalc_Full_HQ-Print_111110.pdf. Accessed 1 Aug 2016

Thomas R, Lunel T (1993) The Braer incident; dispersion in action. In: Proceedings of the 16th Arctic and Marine Oil Spill Programme (AMOP) technical seminar, alberta, environment Canada, Edmonton, pp 843–859

Tissot BP, Welte DH (1984) Petroleum formation and occurrence. Springer, Berlin

Tsutsumi H, Kono M, Takai K, Manabe T (2000) Bioremediation on the shore after an oil spill from the *Nakhodka* in the Sea of Japan. III. Field test of a bioremediation agent with microbiological cultures for the treatment of an oil spill. Mar Pollut Bull 40:320–324

Uraizee FA, Venosa AD, Suidan MT (1998) A Model for diffusion controlled bioavailability of crude oil components. Biodegradation 8:287–296

USDOE (2008) Strategic petroleum reserve crude oil assay manual. www.spr.doe.gov/reports/docs/CrudeOilAssayManual.pdf. Accessed 1 Aug 2016

USEPA (2006) Swirling flask dispersant effectiveness test, revised standard dispersant toxicity test, and bioremediation agent effectiveness test. http://www.gpo.gov/fdsys/pkg/CFR-2006-title40-vol27/xml/CFR-2006-title40-vol27-part300-appC.xml. Accessed 1 Aug 2016

USEPA (2014) National contingency plan Subpart J. http://www2.epa.gov/emergency-response/national-contingency-plan-subpart-j#testing. Accessed 1 Aug 2016

Valentine DL, Kessler JD, Redmond MC, Mendes SD, Heintz MB, Farwell C, Hu L, Kinnaman FS, Yvon-Lewis S, Du M, Chan EW (2010) Propane respiration jump-starts microbial response to a deep oil spill. Science 330:208–211

Valentine DL, Mezić I, Maćešić S, Črnjarić-Žic N, Ivić S, Hogan PJ, Fonoberov VA, Loire S (2012) Dynamic autoinoculation and the microbial ecology of a deep water hydrocarbon irruption. Proc Natl Acad Sci U S A 109:20286–20291

Varadaraj R, Robbins ML, Bock J, Pace S, MacDonald D (1995) Dispersion and biodegradation of oil spills on Water. In: Proceedings of the international oil spill conference. American Petroleum Institute, Washington, DC, pp 101–106

Venosa AD, Holder EL (2007) Biodegradability of dispersed crude oil at two different temperatures. Mar Pollut Bull 54:545–553

Venosa AD, Haines JR, Nisamaneepong W, Govind R, Pradhan S, Siddique B (1992) Efficacy of commercial products in enhancing biodegradation in closed laboratory reactors. J Ind Microbiol 10:13–23

Venosa AD, Suidan MT, Wrenn BA, Strohmeier KL, Haines JR, Eberhart BL, King D, Holder E (1996) Bioremediation of an experimental oil spill on the shoreline of Delaware Bay. Environ Sci Technol 30:1764–1775

Venosa AD, Lee K, Suidan MT, Garcia-Blanco S, Cobanli S, Moteleb M, Haines JR, Tremblay G, Hazelwood M (2002) Bioremediation and biorestoration of a crude oil-contaminated freshwater wetland on the St. Lawrence River. Biorem J 6:261–281

Venosa AD, Campo P, Suidan MT (2010) Biodegradability of lingering crude oil 19 years after the *Exxon Valdez* oil spill. Environ Sci Technol 44:7613–7621

Wade TL, Sericano JL, Sweet ST, Knap AH, Guinasso NL (2016) Spatial and temporal distribution of water column total polycyclic aromatic hydrocarbons (PAH) and total petroleum hydrocarbons (TPH) from the *Deepwater Horizon* (Macondo) incident. Mar Pollut Bull 103:286–293

Wang W, Zheng Y, Lee K (2013) Chemical dispersion of oil with mineral fines in a low temperature environment. Mar Pollut Bull 72:205–212

Wang J, Sandoval K, Ding Y, Stoeckel D, Minard-Smith A, Andersen G, Dubinsky EA, Atlas R, Gardinali P (2016a) Biodegradation of dispersed *Macondo* crude oil by indigenous Gulf of Mexico microbial communities. Sci Total Environ 557–558:453–468

Wang G, Xue Y, Wang D, Shi S, Grice K, Greenwood PF (2016b) Biodegradation and water washing within a series of petroleum reservoirs of the Panyu Oil Field. Org Geochem 96:65–76

Warnock AM, Hagen SC, Passeri DL (2015) Marine tar residues: a review. Water Air Soil Pollut 226:1–24

Weise AM, Nalewajko C, Lee K (1999) Oil-mineral fine interactions facilitate oil biodegradation in seawater. Environ Technol 20:811–824

Wiltshire GA, Corcoran L (1991) Response to the *Presidente Rivera* major oil spill, Delaware River. In: Proceedings of the international oil spill conference. American Petroleum Institute, pp 253–258

Wolfe DA, Hameedi MJ, Galt JA, Watabayashi G, Short J, O'CLAIRE C, Rice S, Michel J, Payne JR, Braddock J, Hanna S (1994) The fate of the oil spilled from the *Exxon Valdez*. Environ Sci Technol 28:560A–568A

Word JQ, Clark JR, Word LS (2013) Comparison of the acute toxicity of Corexit 9500 and household cleaning products. Hum Ecol Risk Assess 21:707–725

Wrenn BA, Sarnecki KL, Kohar ES, Lee K, Venosa AD (2006) Effects of nutrient source and supply on crude oil biodegradation in continuous-flow beach microcosms. J Environ Eng 132:75–84

Wu R, Pan S, Li Y, Wang L (2014) Atmospheric oxidation mechanism of toluene. J Phys Chem A 118:4533–4547

Xu R, Yong LC, Lim YG, Obbard JP (2005) Use of slow-release fertilizer and biopolymers for stimulating hydrocarbon biodegradation in oil-contaminated beach sediments. Mar Pollut Bull 51:1101–1110

Yakimov MM, Timmis KN, Golyshin PN (2007) Obligate oil-degrading marine bacteria. Curr Opin Biotechnol 18:257–266

Oil Biodegradation in Deep Marine Basins

5

Terry C. Hazen and Stephen M. Techtmann

Contents

1 Introduction 72
2 Deep Marine Bacterial Oil Degraders 74
3 *Colwellia psychrerythraea* in Deep Marine Basins 80
4 *Thaumarchaeota* in Deep Marine Basins (Canary in the Coal Mine) 81
5 Cometabolic Biodegradation of Oil 81
6 Biogeochemistry and Oil Biodegradation 82
7 Environmental Systems and Synergistic Effects in Deep Marine Oil Biodegradation 83
8 Research Needs 84
References 84

Abstract

Nine important hydrocarbon (oil) basins where offshore petroleum leases have been licensed are compared. These nine basins (Gulf of Mexico, Eastern Mediterranean's Nile Deep-Sea Fan, Central Mediterranean and the Sirte Basin, North Sea, Caspian Sea, Angola, Trinidad and Tobago, Great Australian Bight, and Brazil's Amazonian Deep-Sea Basin) are geographically separated and are impacted by very different water masses. The geochemical parameters of these basins are quite distinct, for example, salinities ranging from 39 psu in

T. C. Hazen (✉)
Department of Civil and Environmental Engineering, Department of Earth and Planetary Sciences, Department of Microbiology, Institute for Secure and Sustainable Environment, University of Tennessee, Knoxville, TN, USA

Oak Ridge National Laboratory, Oak Ridge, TN, USA
e-mail: tchazen@utk.edu

S. M. Techtmann
Department of Biological Sciences, Michigan Technological University, Houghton, MI, USA
e-mail: smtechtm@mtu.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_22

the Eastern Mediterranean to 12 psu in the Caspian. Additionally, parameters like temperatures of the bottom water are also very distinct, with the temperature in the deep water of the Eastern Mediterranean being between 12 °C and 14 °C and the temperature of the deep water in the North Sea being −2 °C. Each basin represents a unique ecosystem in which distinct microbes may thrive. These distinct environmental parameters may act to constrain the extent of hydrocarbon degradation in these basins. Another potential constraint on hydrocarbon degradation is the extent of natural hydrocarbon seeps in the area. Though many basins have similar if not 16S rRNA identical strains of oil-degrading bacteria, *Colwellia psychrerythraea* from different basins showed that a mixture of natural selection and neutral evolution has contributed to the divergence of these. Most if not all deep ocean basin microbial communities are dominated by *Thaumarchaeota* below 200 m. These microaerophilic, ammonium oxidizer, psychrophiles are very adapted to an oligotrophic lifestyle, and though many in this group will degrade oil, they are rapidly outcompeted by other bacteria in oil or high hydrocarbon intrusions, thus the virtual “canary in the coal mine.” Cometabolic biodegradation of oil is well documented but could be an important natural attenuation mechanism for oil in deep marine basins with episodic methane seeps. Microbial community structure can also predict concentrations of oil in deep basins. Many other synergistic effects require more research in environmental systems biology in deep marine basins.

1 Introduction

Deep marine basins have been exposed to oil and oil analogs for millions of years. Indeed, as hydrocarbons fall through the water column to the ocean floor, the easiest to degrade components are biodegraded, leaving the more recalcitrant components like polyaromatic hydrocarbons and asphaltenes. Since much of this hydrocarbon originates as phytoplankton from surface waters, it is not surprising that in deep marine basins that microorganisms have adapted to degrading these recalcitrant hydrocarbons as carbon and energy sources in these oligotrophic environments. Indeed, many deep basins also have natural oil seeps from oil reservoirs that are more than 6000 m in the subsurface. At these depths, the marine phytoplankton that originally made up the oil strata underwent extreme pressures and temperature for the diagenesis of the oils in these reservoirs. Thus, oil like other hydrocarbons reaching deep marine basins is natural and is expected to have microorganisms capable of degrading it, over the millennia of adaptation that would have occurred (Hazen et al. 2016). Oil in subsurface reservoirs is 100–400 million years old (Tissot and Welte 1984). Oil-degrading taxa are identified in all three domains of the tree of life (Fig. 1).

In deep marine basins, oil is dominated by natural seeps, 600,000 tonnes/year of petroleum worldwide from 1990 to 1999 averages (NAS 2003). Anthropogenic sources during this same period accounted for 678,000 tonnes/year or 53% (Fig. 2) (NAS 2003). Oil can come into the deep basin from the surface (spills), natural seeps, and leaks from production wells in the basin (Fig. 3). Recent studies on the *Deepwater Horizon* (DWH) oil spill have resulted in many new studies in deep water which previously had received little attention (Hazen et al. 2016).

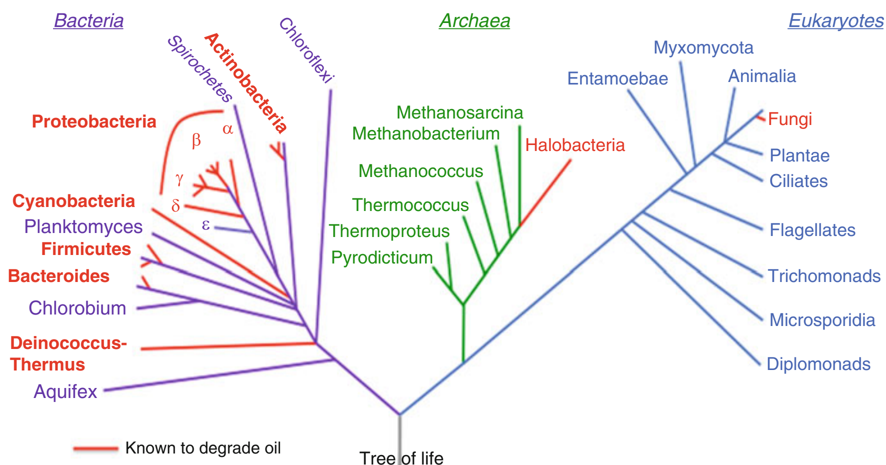


Fig. 1 Oil-degrading microbial taxa. Oil-degrading microbial phyla, highlighted in red, have been identified from all three domains of life. (After Hazen et al. 2016)

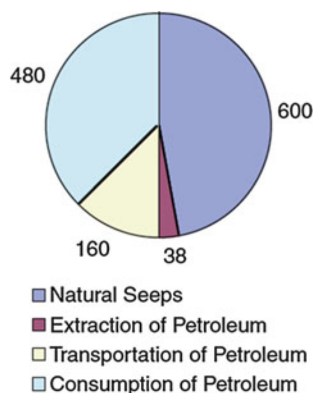


Fig. 2 Relative contribution of average, annual releases (1990–1999) of petroleum hydrocarbons (in kilotons) from natural seeps and activities associated with the extraction, transportation, and consumption of crude oil or refined products to the marine environment. (After NAS 2003)

As sources of new oil reservoirs on land became scarcer over the last 20 years, offshore oil production had dramatically increased. Though the production costs were much greater than wells on land, since the oil reservoirs being tapped were deeper, the value of the product was higher due to its lighter nature and higher value as fuel. However, the DWH blowout in deep water created a cautionary reevaluation and more risk assessment studies. In addition, unconventional oil production and shale gas production increased by more than 702% since 2007. This in turn has caused many oil companies to abandon offshore oil production in deep marine basins, including leases and exploration. “Global oil discoveries fell to a record low in 2016 as companies continued to cut spending and conventional oil projects sanctioned were at the lowest level in more than 70 years,” according to the International Energy Agency (IAE 2017). “The offshore sector, which accounts for almost a third of crude oil production and is a crucial component of future global

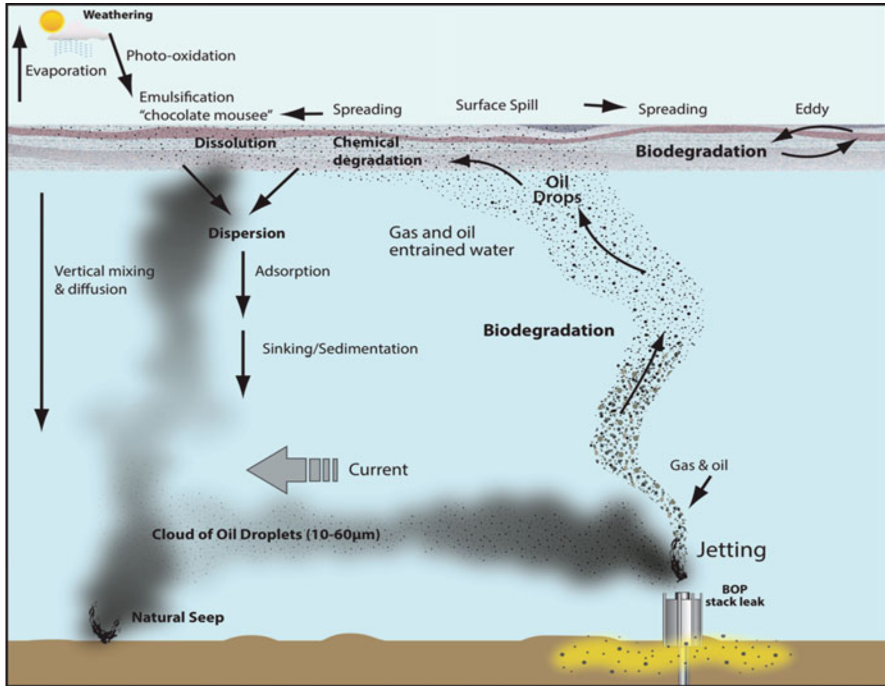


Fig. 3 Fate of oil spills in deep marine basins (*BOP* BlowOut Prevention device)

supplies, has been particularly hard hit by the industry's slowdown. In 2016, only 13% of all conventional resources sanctioned were offshore, compared with more than 40% on average between 2000 and 2015" (IAE 2017).

Nine important hydrocarbon (oil) basins where offshore petroleum leases have been licensed is compared (Fig. 4).

2 Deep Marine Bacterial Oil Degraders

Deep marine basins in the water column and sediments have members of all of the major oil-degrading phyla found in other environments (Fig. 1). In the ***Gulf of Mexico*** water from the 1100 m plume during the *Deepwater Horizon* spill contained 16 subfamilies in the gamma-proteobacteria that were significantly enriched in the plume out of 951 subfamilies detected in 62 bacteria phyla (Fig. 5) (Hazen et al. 2010). Sediment at 1500 m below the deepwater plume was dominated by *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *and Actinobacteria* (Kimes et al. 2013; Mason et al. 2014), whereas typically deep-sea sediments are dominated by *Euryarchaeota*, *Proteobacteria*, *Firmicutes*, *and Chloroflexi* (Biddle et al. 2008, 2011).

Fig. 4 Nine important hydrocarbon (oil) basins where offshore petroleum leases have been licensed

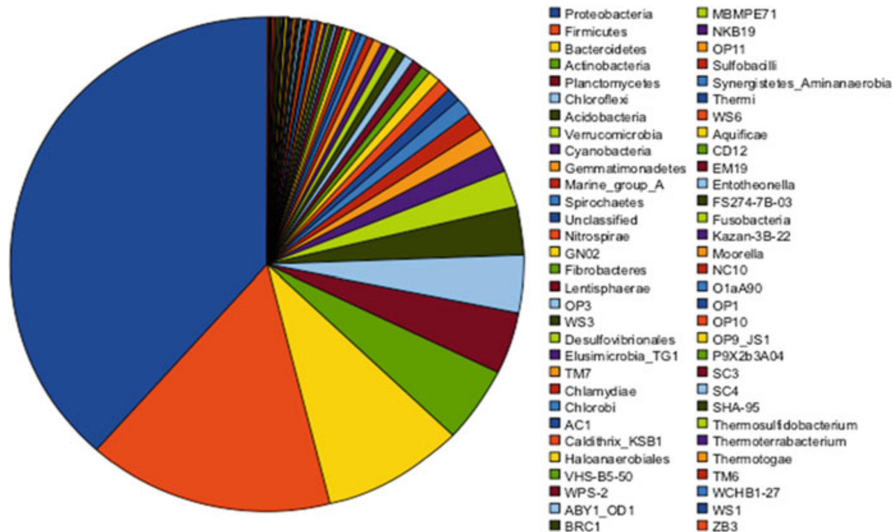


Fig. 5 Deepwater microbial communities during *Deepwater Horizon* oil spill (951 subfamilies were detected in 62 bacterial phyla. Only 16 subfamilies in Gammaproteobacteria significantly enriched in plume). (After Hazen et al. 2010)

The ***Caspian Sea*** has many natural seeps and oil production throughout the basin. It is estimated that the Caspian receives between 70 and 90 tonnes of petroleum each year (Chicherina et al. 2004). Total petroleum levels in the Caspian range between 0.067 and 2 mg/L (Korshenko and Gul 2005). The highest levels of petroleum hydrocarbons were found in the Southern Caspian. These concentrations range from 0.17 to 0.07 mg/L. Caspian Sea water from 200–600 m is dominated by *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria*, and *Thaumarchaeota*. Fungi capable of oil degradation have also been isolated from the Caspian Sea water column (Salmanov 2006; Lein et al. 2010). Studies on the Caspian Sea indicate that the surface of deepwater sediments with low oxygen levels was dominated by *Gammaproteobacteria*; however, surface sediments with bottom waters under hypoxic conditions were dominated by *Deltaproteobacteria*. The ammonia-oxidizing *Thaumarchaeota* was dominant in all surface sediments (Mahmoudi et al. 2015).

Eastern Mediterranean's Nile Deep-Sea Fan has numerous natural hydrocarbon seeps (Heijs et al. 2008; Mastalerz et al. 2009; Omoregie et al. 2009; Felden et al. 2013). Deep water from 400 to 1200 m is dominated by SAR406, *Gammaproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, *Actinobacteria*, *Chloroflexi*, and *Thaumarchaeota* (Techtmann et al. 2015). The microbial community in the deep water was significantly correlated with inorganic phosphate, silicate, nitrate, and depth. Deep sediments were dominated by *Actinobacteria*, *Bacilli*, *Chloroflexi*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, and

Epsilonproteobacteria and the archaea *Methanosarcinales*, *Thermoplasmales*, *Halobacteriales*, and *Crenarchaea* (Heijs et al. 2008). Sulfate reduction, aerobic and anaerobic methanotrophy, aerobic sulfide oxidation, and aerobic and anaerobic heterotrophy were the dominant metabolic processes in the deep sediment (Heijs et al. 2008).

The ***Central Mediterranean and the Sirte Basin*** were largely the same for both water and sediment microbial communities as the Eastern Mediterranean. However, the microbial community structure in the water column was driven by dissolved oxygen, temperature, and salinity (Techtmann et al. 2017). The levels of hydrocarbon-degrading bacteria in coastal waters at locations in the Mediterranean have been determined to be $10–10^2$ cells/ml of water (Youssef et al. 2010). In many locations, the addition of oil enriches a robust community of oil-degrading microbes (Moursy and El-Abagy 1982; Santas et al. 1999; Zrafi-Nouira et al. 2009; Ibraheem 2010; Youssef et al. 2010; Chekroud et al. 2011; Farag and Soliman 2011). Cyanobacteria have been found to be a part of this community (Ibraheem 2010). The authors suggest that their data supports either the ability of these cyanobacteria to degrade hydrocarbons or a mutualism between these cyanobacteria and aerobic hydrocarbon-degrading bacteria. This community also contains hydrocarbon-degrading fungi from the genus *Candida* (Farag and Soliman 2011).

The ***Great Australian Bight*** (GAB) has only a few natural seeps but is of interest for oil production (Logan et al. 2010). Over the years, there have been a number of studies aimed at identifying the presence of hydrocarbon seeps within Australia's margins (Logan et al. 2010). To date, the only naturally occurring hydrocarbon seeps identified are located in Northern Australia's carbonate-rich shelf in the Timor Sea (Rollet et al. 2006; Wasmund et al. 2009; Logan et al. 2010). In Southern Australia, the presence of naturally occurring bitumen asphaltites within the GAB suggests that a naturally occurring seep may be present off of Australia's southern margin. However, there is no direct evidence and despite surveys of the area, no natural seeps within the GAB have been recorded (Struckmeyer et al. 2002; Logan et al. 2010). The GAB is very oligotrophic and one of the deepest basins being considered for petroleum exploration. In the water column, the *Thaumarchaeota* are the dominant microorganism below 185 m in depth (Techtmann et al. 2017). Other groups found are *Prochlorococcus*, *Synechococcus*, SAR11, *Rhodobacteriales*, *Oceanospirillales*, *Alteromonadales*, and *Bacteroidetes* (Wilkins et al. 2013). Microbial diversity in sediments near a methane seep has revealed novel aerobic methanotroph diversity (Wasmund et al. 2009). Within hydrocarbon seeps in the Timor Sea, phylogenetic analysis revealed the presence of sequences affiliated with *Gammaproteobacteria*, *Deltaproteobacteria*, *Alphaproteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Firmicutes*, and *Nitrospira*. Additionally, sequences associated with aerobic methanotrophs were identified, while sequences related to methanotrophic Archaea were found to be absent. Analysis of genes within the porewater revealed the absence of the methanogenic functional gene, methyl coenzyme M reductase, thus providing further evidence for the lack of methanotrophic assemblages at the seeps (Wasmund et al. 2009).

The ***Angola Basin*** (OSA) is a deepwater upwelling basin with a significant number of natural oil seeps (Berger et al. 1998). *Crenarchaeota* are the most dominant group of microbes in OSA below 400 m and are common in low-nutrient deep ocean environments worldwide. The *Crenarchaeota* are generally microaerophiles that are known to be ammonium oxidizers, which gives them a competitive advantage in low-nutrient environments. The microbial community in the OSA is driven by total organic carbon, dissolved oxygen, salinity, and temperature (Hazen et al. 2016). Other microbes in the water included *SAR406*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Euryarchaeota*, and *Thaumarchaeota.* The dominant sediment organisms are *Gammaproteobacteria*, particularly psychrophilic *Enterobacteriaceae*, *Alteromonadaceae*, *Oceanospirillaceae*, and *Legionellaceae* (Schauer et al. 2010). Other proteobacteria, *Chloroflexi* and *Planctomyces*, were also found in the sediment.

In the ***North Sea*** temperature, salinity, and availability of nutrients are some of the drivers that can dictate the microbial community structure. Often in marine systems, movement of waters by currents and tides and stratification help to determine these factors. In the North Sea, the loosely defined regions of the shallow continental shelf of the southern and central North Sea, the Norwegian Trench, and the Faroe-Shetland Channel have distinct current systems and vertical stratification. Natural hydrocarbon seeps in the North Sea as well as anthropogenic inputs of hydrocarbons contribute the overall hydrocarbon load of the Sea. Riverine inputs carry with it anthropogenic hydrocarbons and are estimated to contribute between 40 and 80 kilotons of petroleum hydrocarbon per year into the North Sea (Bedborough et al. 1987). Another significant source of anthropogenic hydrocarbons is the offshore oil and gas industry. This industry has had a major presence in the North Sea since the 1960s. Some studies (Bedborough et al. 1987) estimate that the offshore petroleum industry contributes around 23 kilotons of petroleum per year to the North Sea. The phytoplanktonic communities of the North Sea have been the subjects of a large number of studies (Dale et al. 1999; Riegman and Kraay 2001; Kuipers et al. 2003; Loder et al. 2012). Another study examining the long-term shifts in community structure examined the microbial community of the Helgoland Roads during August over the course of a half-century (Vezzulli et al. 2012). As in other studies, this report finds that *Alpha*- and *Gammaproteobacteria* dominate the community over the course of these decades. *Oceanospirillaceae*, *Halomonadaceae*, and *Alteromonadales* increase over time. The most significant increase that was observed is in the *Vibrio* spp. This increase in *Vibrios* is strongly correlated with sea surface temperature. Therefore, the authors conclude that the warming of sea surface temperatures has resulted in drastic shifts in the microbial communities of the North Sea. The bacterial and archaeal communities of the North Sea are key players in various geochemical cycles within the sea. In the Tommeliten seep, no methanotrophs were found. However, other organisms were found that might serve as functional analogs for the degradation of complex organic matter. Sequences for a relative of *Desulfitobacterium anili* were found at the Tommeliten

site. This organism has previously been shown to oxidize various hydrocarbons such as naphthalene and xylene (Widdel et al. 2007). In addition, a 16 s rDNA clone for a member of the *Oceanospirillales* was recovered from the Tommeliten site.

The coast of ***Trinidad and Tobago*** is very complex environment where many water masses interact. This location is increasingly becoming an important region for oil and gas production. The complex environment of this region makes it an interesting site to examine the microbial communities present in the waters and sediments and their potential to degrade hydrocarbons. Some of the microbiological work in the region has involved examining the microbes that colonize the unique gradients associated with the riverine inputs into the Atlantic from the Orinoco and the Amazon Rivers. One such paper examines the levels and turnover times of methane (CH_4) and carbon monoxide (CO) in the waters of the Caribbean Sea surrounding Trinidad and Tobago (Jones and Amador 1993). In rivers methane is produced microbially by methanogenic archaea, and CO is produced primarily by the photooxidation of organic matter. CO is subsequently consumed through microbial oxidation of CO. An interesting trend was seen in the Gulf of Paria, where the CO and CH_4 concentrations reached a local maximum. This is most likely due to effects on the flow of riverine waters into the gulf. This work confirms that the waters around Trinidad and Tobago are highly influenced by inputs of both nutrients and organic matter from the Orinoco River. A large number of mud volcanoes have been discovered near Trinidad and Tobago in the Barbados Prism (Biju-Duval et al. 1982; Brown and Westbrook 1987, 1988; Brown 1990; Griboulard et al. 1991; Deville et al. 2003). These mud volcanoes are rather randomly distributed along the continental slope (Deville et al. 2006, 2010). These mud volcanoes have been shown to exude both methane and higher hydrocarbons (Le Pichon et al. 1990a, b; Henry et al. 1996). The microbiology of deep sediments adjacent to Trinidad and Tobago was investigated by Guezennec and Fiala-Medioni (1996). They used phospholipid ester-linked fatty acids (PLFA) to examine the bacterial abundance and diversity of a mud volcano in the Barbados trench near Trinidad and Tobago. Prior to this work, white and reddish mats were observed near these seep sites indicative of some bacterial colonization (Le Pichon et al. 1990a). PLFA analysis estimated the cell numbers in these mud volcanoes to be 1.5×10^9 cell/g of sediment. These cell numbers are similar to others reported in nearby sediments of the Venezuelan Basin (5×10^8 cells/g). The lipid profiles of sites near the white and reddish mats suggested the presence of sulfur-oxidizing bacteria. Further, lipid characteristics of both type I and type II methanotrophs were common at all sites sampled. This would follow the high levels of methane present at these sites. The authors conclude that type I methanotrophs are more abundant than type II in all of the sediments. Other lipids commonly found in both sulfate-reducing bacteria as well as alkane-degrading bacteria were found in these sediments. Archaeal lipids potentially belonging to methanogens were also found in these sediments. While this study does shed some light on the groups of bacteria present in these mud volcanoes, further work needs to be done to characterize these communities. For example, one of the groups of lipids

found at high levels could either be contributed from sulfate-reducing bacteria or alkane-degrading bacteria.

The ***Amazonian Deep-Sea Basin*** brings distinct hydrographic and geochemical features to the waters around Brazil and has a large effect on the microbial communities present in these waters. In particular the role that river inputs have on these waters drastically affects the microbial communities present. The complex water masses in the deep water also harbor unique niches for microbes to flourish. Amazonian Deep-Sea Basin water contained oil degraders in the bacteria (*Alteromonadaceae*, *Colwelliaceae*, and *Alcanivoracaceae*), archaea (e.g., *Halobacteriaceae*, *Desulfurococcaceae*, and *Methanobacteriaceae*), and eukaryotic microbes (e.g., *Microsporidia*, *Ascomycota*, and *Basidiomycota*) (Campeao et al. 2017). The sediments off the coast of Brazil are relatively unexplored in terms of their microbial diversity. Despite the active oil and gas industry, which has characterized many of the seafloor and sub-seafloor features, the microbial community of the seafloor is relatively unknown. Microbes have been shown to be present in relatively high numbers (Cragg et al. 1997). Work associated with the Ocean Drilling Program reported bacterial cell numbers around 10^9 cells/g of sediment in the surface sediments and decreasing to 10^6 cells/g in the deeper sediments. Due to the oligotrophic nature of the open ocean water, oxygen is able to penetrate fairly deeply into the sediments of this region (Wenzhofer et al. 2001). One study characterized the hydrocarbon-degrading community associated with sediments impacted by a catastrophic oil spill in the Guanabara Bay (Brito et al. 2006). Thirty-two bacterial strains were isolated from oil-enriched mesocosms of Guanabara Bay sediment. The majority of these strains were *Alpha*- and *Gammaproteobacteria.* Many of these strains were related to *Marinobacter* spp. and *Alcanivorax* spp. The *Alphaproteobacteria* were shown to be able to degrade many of the branched chain hydrocarbons. Another study investigated various methods to stimulate hydrocarbon degradation in coastal sediments (Silva et al. 2009). In these sediments, hydrocarbon degraders were shown to be a significant proportion of the heterotrophic bacterial population. The addition of fertilizer and biosurfactant helped to stimulate the removal of hydrocarbons from this system.

3 *Colwellia psychrerythraea* in Deep Marine Basins

Colwellia psychrerythraea are often found in cold, oil-contaminated marine environments both in the deep, near shore, and sediment. Recent in-depth genomic and phenotypic studies of identical isolates from distant basins suggest that even when they show the same 16S rRNA identity, they show differential salt tolerance and distinct carbon source utilization (Techtmann et al. 2016). Differences in genomic content were also shown to encode for different functional capacity. Large segments of the genome appear to be acquired by horizontal gene transfer. Some of these genes confer increased functionality and selective advantage; however, the majority of differences do not appear to be related to adaptation to different environmental lifestyles. This suggests that a mixture of natural selection and neutral evolution

has contributed to the divergence of these organisms and the great genetic and phenotypic diversity present within this species. This observation may well be the norm for oil degraders rather than the exception.

4 *Thaumarchaeota* in Deep Marine Basins (Canary in the Coal Mine)

The *Thaumarchaeota* dominate the microbial community in the water column in nearly all deep basins that have been studied. This is predominantly because these psychrophilic, microaerophilic, ammonia-oxidizers are very adapted to the oligotrophic environment that dominates the depths of these basins. Some *Thaumarchaeota* have also been reported to degrade oil at low concentrations. Recent studies of four of these basins demonstrated that there were significant differences in the abundance and diversity of *Thaumarchaeotes* between these four basins and that their distribution showed biogeographic patterning (Techtmann et al. 2017). These studies have also demonstrated that oil and other hydrocarbons will cause the *Thaumarchaeotes* to disappear in the water column and sediments since they cannot compete with other oil degraders. Thus, disappearance of *Thaumarchaeotes* in deep marine basin water columns could be a good indicator of oil and/or hydrocarbon presence, i.e., “canary in the coal mine.”

5 Cometabolic Biodegradation of Oil

The aerobic cometabolic biodegraders are dependent upon oxygenases, e.g., methane monooxygenase, toluene dioxygenase, toluene monooxygenase, and ammonia monooxygenase. These enzymes are extremely strong oxidizers, e.g., methane monooxygenase is known to degrade over 1000 different compounds. However, like any bioremediation process, the proper biogeochemical conditions are necessary to maximize and maintain biodegradation, e.g., maintaining oxygen levels or other terminal electron acceptors that the cometabolic biodegrader is dependent (Hazen 1997; Hazen et al. 2016), and ► Chap. 14, “Cometabolic Bioremediation” in this book. In addition, cometabolic biostimulation may require pulsing of electron donor or electron acceptor to reduce competitive inhibition between the substrate the microbe can use and the contaminant. Pulsing of methane was found to significantly improve biodegradation of TCE rates by methanotrophs (Hazen 2010). Indeed, during the *Deepwater Horizon* (DWH) leak (Hazen et al. 2010), there was evidence that in the Gulf of Mexico where episodic releases of methane have occurred for millions of years from natural seeps, this pulsing of methane may be degrading oil and other organics via cometabolic biodegradation. The methane oxidizers bloomed during the DWH leaked above 400 m once the well was capped (Reddy et al. 2012; Redmond and Valentine 2012; Dubinsky et al. 2013). This suggests that intrinsic cometabolic bioremediation or cometabolic natural attenuation may be a serious phenomenon in the ocean (Stackhouse et al. 2017). Methanotrophs,

methane-oxidizing bacteria, oxidize methane via a series of enzymes that are unique to this group. The primary enzyme in this oxidation chain is methane monooxygenase. Methane monooxygenase is an extremely powerful oxidizer, thus giving it the capability of oxidizing a wide variety of normally recalcitrant compounds including oil (Cardy et al. 1991). See ▶ Chap. 14, "Cometabolic Bioremediation" in this book.

6 Biogeochemistry and Oil Biodegradation

Following the *Deepwater Horizon* oil spill, a rapid enrichment of hydrocarbon-degrading microorganisms was observed in the water column, and surface and subsurface plume waters were dominated by known hydrocarbon-degrading bacteria (King et al. 2015). Perhaps because substrate availability was limited as the surface slicks coalesced into thick emulsified oil with much reduced surface area for microbial colonization, (Edwards et al. 2011) no increase in microbial biomass in the surface slick and that microorganisms exhibited enzymatic signs of phosphate stress consistent with previous observations that the Gulf of Mexico is limited by phosphate availability. Other studies (Hazen et al. 2010) found that microbial cell density in the deepwater oil plume was significantly higher than waters outside of the plume, $>5.5 \times 10^4$ cells/ml in the oil plume and $<2.7 \times 10^4$ cells/mL outside of the plume, but the increase was not as dramatic as might have been expected. This is likely due to the low concentrations of oil in the deepwater plume (<1 ppm). We note that the Gulf of Mexico has similar levels of nitrogen and phosphorus to several other deep marine basins undergoing active oil and gas exploration, but many of these other areas, such as the Eastern Mediterranean Sea, have a much lower phosphorus levels (Table 1). If a spill were to occur in this environment, the rates and extent of biodegradation may be initially much slower than in the Gulf of Mexico. On the other hand, as dispersed oil continues to dilute as it gets further

Table 1 Physical/chemical comparison of deep basin with oil biodegradation

Nutrient	Eastern Med	Central Med	Great Australian Bight	Caspian	North Sea	Angola Basin	Brazil Basin	Gulf of Mexico
Nitrate (μg/kg)	178.4	160.0	144.7	127.8	569.6	20	25	208
Phosphate (μg/kg)	39.7	29.3	153.5	56.9	33.5	1.6	1.1	205
Ammonia (μg/kg)	28.0	30.0	125	739.6	12.5	10.2	ND	78
Iron (μg/kg)	6.5	ND	5.8	4.7	ND	ND	ND	56
Sulfate (μg/kg)	4.7	ND	1.6	1.9	ND	ND	ND	ND
Salinity (psu)	38.9	38.6	34.8	11.3	33.7	36	37	35
Temp (°C)	13.8	13.7	2.5	6.8	3	4	5	4.8
Dissolved oxygen (mg/L)	6.4	5.5	5.0	0.5	ND	3.4	ND	4.9

ND no data

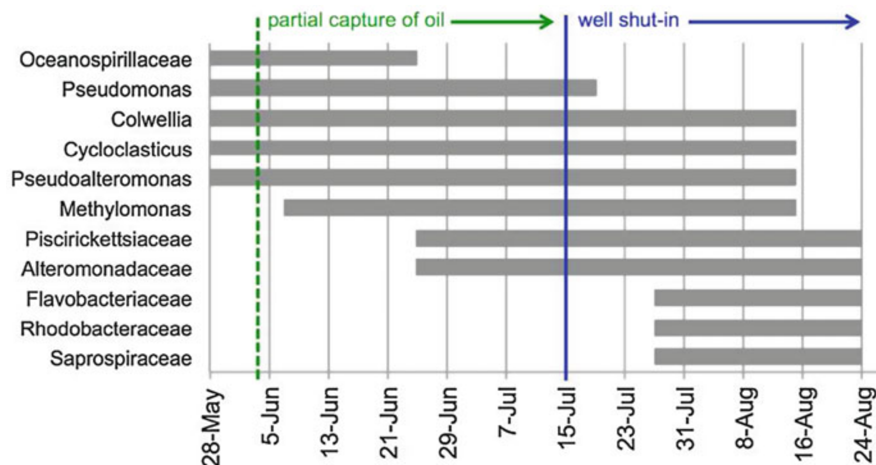


Fig. 6 Succession of the microbial community in the deep water of the Gulf of Mexico during the various phases of the response to the *Deepwater Horizon* oil spill. For each bar relative abundance was enriched over background in any sample (>2-fold mean nonplume intensity). (After Dubinsky et al. 2013)

from a spill site, it is likely that eventually the oil-to-available nutrient ratio will drop to a value where biodegradation can proceed, so if there are no immediate risk receptors, long-term biodegradation processes could remediate the spill. During DWH we also saw the total microbial biomass decrease quite slowly, since once the easy to degrade material was depleted and some of the oil degraders began to die, they became nutrients for organisms adapted to degrade their components (Dubinsky et al. 2013). This resulted in a temporal succession in the microbial community structure that was present so that microbes that could not degrade oil benefitted from the dead biomass as a resource (Fig. 6). This undoubtedly goes on in both spill scenarios and when there are natural episodic seeps of oil.

7 Environmental Systems and Synergistic Effects in Deep Marine Oil Biodegradation

Oil biodegradation in deep marine basins is best approached using multiple scales using environmental systems biology (Hazen et al. 2016; Hazen and Sayler 2016). Synergistic effects (Table 2) can result from combinations of oil droplet size, currents, oil type, degree of biodegradation, dissolution, cometabolic biodegradation, mineral fines, biosurfactants, temperature, gyres, and pressure (Hazen et al. 2016). Indeed, during the DWH spill, surface currents were to the NE, whereas the plume at 1100 m was moving to the SW; in addition there was a gyre at 1100 m that was causing recirculation from the well head to 15 km SW (Valentine et al. 2012). The droplet size was also demonstrated to greatly influence the biodegradation rate,

Table 2 Synergistic effects that impact biodegradation of oil. (After Hazen et al. 2016)

Factor working synergistically	Impact on biodegradation
Chemical dispersants + mineral fines	Individually each will promote dispersion of the oil. Combined, the formation and transfer of oil from the surface into the water column is enhanced
Autoinoculation + "memory response" of hydrocarbon degraders	Introduction of hydrocarbons to previously exposed water parcels leads to an increase in microbial abundance and accelerated hydrocarbon biodegradation
Oil droplet size + dispersion + biodegradation rates + dissolution	Enhances biodegradation, dissolution, and dispersion rates of oil hydrocarbons
Cometabolic biodegradation + dispersion + secondary electron donors	Enhances biodegradation, dissolution, and dispersion rates of oil hydrocarbons even when the oil itself cannot be a suitable electron donor
Biosurfactants from multiple microorganisms	Enhances bioavailability of poorly soluble compounds

whether caused by the jetting at the well head or the dispersant injected at the well head (Brakstad et al. 2014, 2015). Detailed microbiological models like Structured Learning in Microbial Ecology (SLiME) have also demonstrated that microbial community structure in deep marine basins can predict oil concentrations quite accurately (Smith et al. 2015). On board ship oil biodegradation simulations have also been shown to improve correlations with in situ collections.

8 Research Needs

To avoid and properly remediate disasters like the *Deepwater Horizon* spill, we need much more in-depth studies at an environmental systems biology level. This includes better models that take into account psychrophiles that can degrade oil and would not be predicted by simple Q10 formulas. More studies are also needed on dispersant usage with particular attention to realistic concentrations. On board ship studies and close attention to bottle effect, temperature and pressure have been found to critically effect conclusions of studies. The resources for doing these studies in deep basins are exceptionally high and need government commitments long term with a dynamic field test plan and experienced personnel for rapid deployment for any future spills.

References

Bedborough DR, Blackman RAA, Law RJ (1987) A survey of inputs to the North-Sea resulting from oil and gas developments. Philos Trans R Soc Lond Ser B-Biol Sci 316:495–509

Berger WH, Wefer G, Richter C, Lange CB, Giraudeau J, Hermelin O, Party SS (1998) The Angola-Benguela upwelling system: paleoceanographic synthesis of shipboard results from Leg 175. In: Proceedings of the ocean drilling program, initial reports. 175

Biddle JF, Fitz-Gibbon S, Schuster SC, Brenchley JE, House CH (2008) Metagenomic signatures of the Peru margin subseafloor biosphere show a genetically distinct environment. Proc Natl Acad Sci USA 105:10583–10588
Biddle JF, White JR, Teske AP, House CH (2011) Metagenomics of the subsurface Brazos-Trinity Basin (IODP site 1320): comparison with other sediment and pyrosequenced metagenomes. ISME J 5:1038–1047
Biju-Duval B, Le Quellec P, Mascle A, Renard V, Valery P (1982) Multibeam bathymetric survey and high resolution seismic investigations on the Barbados Ridge complex (Eastern Caribbean): a key to the knowledge and interpretation of an accretionary wedge. Tectonophysics 86:275–304
Brakstad OG, Daling PS, Faksness LG, Almas IK, Vang SH, Syslak L, Leirvik F (2014) Depletion and biodegradation of hydrocarbons in dispersions and emulsions of the Macondo 252 oil generated in an oil-on-seawater mesocosm flume basin. Mar Pollut Bull 84:125–134
Brakstad OG, Nordtug T, Throne-Hoist M (2015) Biodegradation of dispersed Macondo oil in seawater at low temperature and different oil droplet sizes. Mar Pollut Bull 93:144–152
Brito EMS, Guyoneaud R, Goñi-Urriza M, Ranchou-Peyruse A, Verbaere A, Crapez MAC, Wasserman JCA, Duran R (2006) Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara Bay, Brazil. Res Microbiol 157:752–762
Brown KM (1990) The nature and hydrogeologic significance of mud diapirs and diatremes for accretionary systems. J Geophys Res 95:8969–8982
Brown KM, Westbrook GK (1987) The tectonic fabric of the Barbados Ridge accretionary complex. Mar Pet Geol 4:71–81
Brown K, Westbrook GK (1988) Mud diapirism and subcretion in the Barbados Ridge accretionary complex: the role of fluids in accretionary processes. Tectonics 7:613–640
Campeao ME, Reis L, Leomil L, de Oliveira L, Otsuki K, Gardinali P, Pelz O, Valle R, Thompson FL, Thompson CC (2017) The deep-sea microbial community from the Amazonian basin associated with oil degradation. Front Microbiol 8:13
Cardy DLN, Laidler V, Salmond GPC, Murrell JC (1991) Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b. Mol Microbiol 5:335–342
Chekroud Z, Gouda MK, Houhamdi M (2011) Biodegradation of crude oil in marine medium. J Proteomics Bioinformatics 4:231–237
Chicherina OV, Leonov AV, Fashchuk DY (2004) Geographical and ecological characteristics of the Caspian Sea and modern tendencies in the evolution of its ecosystem. Water Resour 31:271–289
Cragg BA, Law KM, Cramp A, Parkes RJ (1997) Bacterial profiles in Amazon Fan sediments, Sites 934 and 940. In: Flood RD, Piper DJW, Klaus A, Peterson LC (eds) Proceedings of ODP, scientific results. Ocean Drilling Program, College Station, pp 565–571
Dale T, Rey F, Heimdal BR (1999) Seasonal development of phytoplankton at a high latitude oceanic site. Sarsia 84:419–435
Deville E, Battani A, Griboulard R, Guerlais S, Herbin JP, Houzay JP, Muller C, Prinzhofer A (2003) The origin and processes of mud volcanism: new insights from Trinidad. Geol Soc Lond Spec Publ 216:475–490
Deville E, Guerlais S-H, Callec Y, Griboulard R, Huyghe P, Lallemant S, Mascle A, Noble M, Schmitz J (2006) Liquefied vs stratified sediment mobilization processes: insight from the south of the Barbados accretionary prism. Tectonophysics 428:33–47
Deville É, Guerlais S-H, Lallemant S, Schneider F (2010) Fluid dynamics and subsurface sediment mobilization processes: an overview from Southeast Caribbean. Basin Res 22:361–379
Dubinsky EA, Conrad ME, Chakraborty R, Bill M, Borglin SE, Hollibaugh JT, Mason OU, Piceno YM, Reid FC, Stringfellow WT, Tom LM, Hazen TC, Andersen GL (2013) Succession of hydrocarbon-degrading bacteria in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. Environ Sci Technol 47:10860–10867
Edwards BR, Reddy CM, Camilli R, Carmichael CA, Longnecker K, Van Mooy BAS (2011) Rapid microbial respiration of oil from the Deepwater horizon spill in offshore surface waters of the Gulf of Mexico. Environ Res Lett 6:9

Farag S, Soliman NA (2011) Biodegradation of crude petroleum oil and environmental pollutants by *Candida tropicalis* strain. Braz Arch Biol Technol 54:821–830

Felden J, Lichtschlag A, Wenzhofer F, de Beer D, Feseker T, Ristova PP, de Lange G, Boetius A (2013) Limitations of microbial hydrocarbon degradation at the Amon mud volcano (Nile deep-sea fan). Biogeosciences 10:3269–3283

Griboulard R, Bobier C, Faugères JC, Vernette G (1991) Clay diapiric structures within the strike-slip margin of the southern leg of the Barbados prism. Tectonophysics 192:383–400

Guezennec J, Fiala-Medioni A (1996) Bacterial abundance and diversity in the Barbados trench determined by phospholipid analysis. FEMS Microbiol Ecol 19:83–93

Hazen TC (1997) Bioremediation. In: Amy P, Haldeman D (eds) Microbiology of the terrestrial subsurface. CRC Press, Boca Raton, pp 247–266

Hazen TC (2010) Cometabolic bioremediation. In: Timmis KN (ed) Handbook of hydrocarbon microbiology: microbial interactions with hydrocarbons, oils, fats and related hydrophobic substrates and products. Springer, Berlin

Hazen TC, Sayler GS (2016) Environmental systems microbiology of contaminated environments. In: Yates M, Nakatsu C, Miller R, Pillai S (eds) Manual of environmental microbiology, 4th edn. ASM Press, Washington, DC, pp 5.1.6-1–5.1.6-10

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Borglin SE, Fortney JL, Stringfellow WT, Bill M, Conrad ME, Tom LM, Chavarria KL, Alusi TR, Lamendella R, Joyner DC, Spier C, Baelum J, Auer M, Zemla ML, Chakraborty R, Sonnenthal EL, D'Haeseleer P, Holman HYN, Osman S, Lu ZM, Van Nostrand JD, Deng Y, Zhou JZ, Mason OU (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 330:204–208

Hazen TC, Prince RC, Mahmoudi N (2016) Marine oil biodegradation. Environ Sci Technol 50:2121–2129

Heijs SK, Laverman AM, Forney LJ, Hardoim PR, van Elsas JD (2008) Comparison of deep-sea sediment microbial communities in the Eastern Mediterranean. FEMS Microbiol Ecol 64:362–377

Henry P, Le Pichon X, Lallemant S, Lance S, Martin JB, Foucher J-P, Fiala-MÈdioni A, Rostek F, Guilhaumou N, Pranal V, Castrec M (1996) Fluid flow in and around a mud volcano field seaward of the Barbados accretionary wedge: results from Manon cruise. J Geophys Res 101:20297–20323

IAE (2017) Global oil discoveries and new projects fell to historic lows in 2016

Ibraheem IBM (2010) Biodegradability of hydrocarbons by cyanobacteria1. J Phycol 46:818–824

Jones RD, Amador JA (1993) Methane and carbon monoxide production, oxidation, and turnover times in the Caribbean Sea as influenced by the Orinoco River. J Geophys Res 98(C2):2353–2359

Kimes NE, Callaghan AV, Aktas DF, Smith WL, Sunner J, Golding BT, Drozdowska M, Hazen TC, Suflita JM, Morris PJ (2013) Metagenomic analysis and metabolite profiling of deep-sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill. Front Microbiol 4:17

King GM, Kostka JE, Hazen TC, Sobecky PA (2015) Microbial responses to the Deepwater Horizon oil spill: from coastal wetlands to the deep sea. Annu Rev Mar Sci 7(7):377–401

Korshenko A, Gul AG (2005) Pollution of the Caspian Sea. In: Kostianoy AG, Kosarev AN (eds) The Caspian environment, vol 5P. Springer, Berlin/Heidelberg, pp 109–142

Kuipers B, Witte H, van Noort G, Gonzalez S (2003) Grazing loss-rates in pico- and nanoplankton in the Faroe-Shetland channel and their different relations with prey density. J Sea Res 50:1–9

Le Pichon X, Foucher J-P, BoulËgue J, Henry P, Lallemant S, Benedetti M, Avedik F, Mariotti A (1990a) Mud volcano field seaward of the Barbados accretionary complex: a submersible survey. J Geophys Res 95:8931–8943

Le Pichon X, Henry P, Lallemant S (1990b) Water flow in the Barbados accretionary complex. J Geophys Res 95:8945–8967

Lein AY, Rusanov II, Klyuvitkin AA, Kravchishina MD, Zakharova EE, Veslopolova EF, Makkaveev PN, Ivanov MV (2010) Biogeochemical processes in the water column of the Caspian Sea in November 2008. Dokl Earth Sci 434:1381–1385

Loder MGJ, Kraberg AC, Aberle N, Peters S, Wiltshire KH (2012) Dinoflagellates and ciliates at Helgoland Roads, North Sea. Helgol Mar Res 66:11–23

Logan GA, Jones AT, Kennard JM, Ryan GJ, Rollet N (2010) Australian offshore natural hydrocarbon seepage studies, a review and re-evaluation. Mar Pet Geol 27:26–45

Mahmoudi N, Robeson MS 2nd, Castro HF, Fortney JL, Techtmann SM, Joyner DC, Paradis CJ, Pfiffner SM, Hazen TC (2015) Microbial community composition and diversity in Caspian Sea sediments. FEMS Microbiol Ecol 91:1–11

Mason OU, Scott NM, Gonzalez A, Robbins-Pianka A, Baelum J, Kimbrel J, Bouskill NJ, Prestat E, Borglin S, Joyner DC, Fortney JL, Jurelevicius D, Stringfellow WT, Alvarez-Cohen L, Hazen TC, Knight R, Gilbert JA, Jansson JK (2014) Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. ISME J 8:1464–1475

Mastalerz V, de Lange GJ, Dahlmann A (2009) Differential aerobic and anaerobic oxidation of hydrocarbon gases discharged at mud volcanoes in the Nile deep-sea fan. Geochim Cosmochim Acta 73:3849–3863

Moursy AS, El-Abagy MM (1982) Microbial degradation of hydrocarbons in Ismailia canal water. Environ Int 7:423–427

NAS (2003) Oil in the sea III: inputs, fates, and effects. The National Academies Press, Washington, DC

Omoregie EO, Niemann H, Mastalerz V, de Lange GJ, Stadnitskaia A, Mascle J, Foucher JP, Boetius A (2009) Microbial methane oxidation and sulfate reduction at cold seeps of the deep Eastern Mediterranean Sea. Mar Geol 261:114–127

Reddy CM, Arey JS, Seewald JS, Sylva SP, Lemkau KL, Nelson RK, Carmichael CA, McIntyre CP, Fenwick J, Ventura GT, Van Mooy BAS, Camilli R (2012) Composition and fate of gas and oil released to the water column during the Deepwater Horizon oil spill. Proc Natl Acad Sci USA 109:20229–20234

Redmond MC, Valentine DL (2012) Natural gas and temperature structured a microbial community response to the Deepwater Horizon oil spill. Proc Natl Acad Sci USA 109:20292–20297

Riegman R, Kraay GW (2001) Phytoplankton community structure derived from HPLC analysis of pigments in the Faroe-Shetland channel during summer 1999: the distribution of taxonomic groups in relation to physical/chemical conditions in the photic zone. J Plankton Res 23:191–205

Rollet N, Logan GA, Kennard JM, O'Brien PE, Jones AT, Sexton M (2006) Characterisation and correlation of active hydrocarbon seepage using geophysical data sets: an example from the tropical, carbonate Yampi Shelf, Northwest Australia. Mar Pet Geol 23:145–164

Salmanov MA (2006) Microbiological studies in the Deepwater area of the Southern Caspian Sea. Microbiology 75:206–212

Santas R, Korda A, Tenente A, Buchholz K, Santas P (1999) Mesocosm assays of oil spill bioremediation with oleophilic fertilizers: Inipol, F1 or both? Mar Pollut Bull 38:44–48

Schauer R, Bienhold C, Ramette A, Harder J (2010) Bacterial diversity and biogeography in deep-sea surface sediments of the South Atlantic Ocean. ISME J 4:159–170

Silva A, Oliveira FS, Bernardes D, França F (2009) Bioremediation of marine sediments impacted by petroleum. Appl Biochem Biotechnol 153:58–66

Smith MB, Rocha AM, Smillie CS, Olesen SW, Paradis C, Wu L, Campbell JH, Fortney JL, Mehlhorn TL, Lowe KA, Earles JE, Phillips J, Techtmann SM, Joyner DC, Elias DA, Bailey KL, Hurt RA Jr, Preheim SP, Sanders MC, Yang J, Mueller MA, Brooks S, Watson DB, Zhang P, He Z, Dubinsky EA, Adams PD, Arkin AP, Fields MW, Zhou J, Alm EJ, Hazen TC (2015) Natural bacterial communities serve as quantitative geochemical biosensors. MBio 6:e00326-00315

Stackhouse B, Lau MCY, Vishnivetskaya T, Burton N, Wang R, Southworth A, Whyte L, Onstott TC (2017) Atmospheric CH4 oxidation by Arctic permafrost and mineral cryosols as a function of water saturation and temperature. Geobiology 15:94–111

Struckmeyer HIM, Wiliams AK, Cowley R, Totterdell JM, Lawrence G, O'Brien GW (2002) Evaluation of hydrocarbon seepage in the Great Australian Bight. AAPEA J 42:371

Techtmann SM, Fortney JL, Ayers KA, Joyner DC, Linley TD, Pfiffner SM, Hazen TC (2015) The unique chemistry of Eastern Mediterranean water masses selects for distinct microbial communities by depth. PLoS One 10:e0120605
Techtmann SM, Fitzgerald KS, Stelling SC, Joyner DC, Uttukar SM, Harris AP, Alshibli NK, Brown SD, Hazen TC (2016) *Colwellia psychrerythraea* strains from distant deep sea basins show adaptation to local conditions. Front Environ Sci 4:33
Techtmann SM, Mahmoudi N, Whitt KT, Campa MF, Fortney JL, Joyner DC, Hazen TC (2017) Comparison of thaumarchaeotal populations from four deep sea basins. FEMS Microbiol Ecol 93:10
Tissot BP, Welte DH (1984) Petroleum formation and occurrence. Springer, Berlin
Valentine DL, Mezic I, Macesic S, Crnjaric-Zic N, Ivic S, Hogan PJ, Fonoberov VA, Loire S (2012) Dynamic autoinoculation and the microbial ecology of a deep water hydrocarbon irruption. Proc Natl Acad Sci USA 109:20286–20291
Vezzulli L, Brettar I, Pezzati E, Reid PC, Colwell RR, Hofle MG, Pruzzo C (2012) Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. ISME J 6:21–30
Wasmund K, Kurtboke DI, Burns KA, Bourne DG (2009) Microbial diversity in sediments associated with a shallow methane seep in the tropical Timor Sea of Australia reveals a novel aerobic methanotroph diversity. FEMS Microbiol Ecol 68:142–151
Wenzhofer F, Holby O, Kohls O (2001) Deep penetrating benthic oxygen profiles measured in situ by oxygen optodes. Deep-Sea Res Part I-Oceanogr Res Pap 48:1741–1755
Widdel F, Musat F, Knittel K, Galushko A (2007) Anaerobic degradation of hydrocarbons with sulphate as electron acceptor. In: Barton LL, Hamilton WA (eds) Sulphate-reducing bacteria: environmental and engineered systems. Cambridge University Press, Cambridge, UK
Wilkins D, Lauro FM, Williams TJ, Demaere MZ, Brown MV, Hoffman JM, Andrews-Pfannkoch C, McQuaid JB, Riddle MJ, Rintoul SR, Cavicchioli R (2013) Biogeographic partitioning of Southern Ocean microorganisms revealed by metagenomics. Environ Microbiol 15:1318–1333
Youssef M, El-Taweel G, El-Naggar A, El-Hawary SE, El-Meleigy M, Ahmed S (2010) Hydrocarbon degrading bacteria as indicator of petroleum pollution in Ismailia Canal, Egypt. World Appl Sci J 8:1226–1233
Zrafi-Nouira I, Guermazi S, Chouari R, Safi NMD, Pelletier E, Bakhrouf A, Saidane-Mosbahi D, Sghir A (2009) Molecular diversity analysis and bacterial population dynamics of an adapted seawater microbiota during the degradation of Tunisian zarzatine oil. Biodegradation 20:467–486

6 Biostimulation Strategies for Enhanced Bioremediation of Marine Oil Spills Including Chronic Pollution

Maria Nikolopoulou and Nicolas Kalogerakis

Contents

1 Introduction 90
2 Oil Spill Weathering Processes 90
3 Biostimulation 91
3.1 Water Soluble Inorganic Nutrients 91
3.2 Slow Release Fertilizers 92
3.3 Oleophilic Fertilizers 93
3.4 Oxygen as a Rate Limiting Substrate 94
4 Biostimulation Strategies for Chronically Polluted Sites 95
5 Research Needs 96
References 97

Abstract

Biostimulation offers an excellent strategy for combating oil spills following first response actions. Bioremediation rates can be enhanced significantly through the successful stimulation of indigenous degraders with suitable nutrients. The conditions under which biostimulation leads to increased effectiveness are reviewed and strategies for successful biostimulation applications to open sea and near shore environments including chronically polluted sites are suggested.

M. Nikolopoulou (✉) · N. Kalogerakis
School of Environmental Engineering, Technical University of Crete, Chania, Greece
e-mail: mnikol80@gmail.com; nicolas.kalogerakis@enveng.tuc.gr

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_7

1 Introduction

As long as society keeps on relying on petroleum hydrocarbons to cover its energy needs, despite the stricter environmental regulations that have been adopted by most countries, oil spills will remain a serious risk to marine ecosystems. Although conventional methods, such as physical removal with booms, skimmers, and absorbent materials, are the first response option, they rarely achieve complete cleanup of oil spills and must be deployed soon after the spill occurs. Chemical methods, particularly dispersants, although they have been routinely used in many countries as a response action, are only allowed when the coastline depth is more than 15 m. In addition, due to their potential toxicity effects on marine organisms they can be applied only under certain conditions. On the other hand, enhanced bioremediation has emerged as a promising technology for combating marine oil spills following first response actions. Intrinsic bioremediation can be enhanced by either of the two complementary approaches, bioaugmentation and biostimulation. In bioaugmentation, the addition of oil-degrading bacteria boosts bioremediation rates whereas in biostimulation, the growth of indigenous hydrocarbon degraders is stimulated by the addition of nutrients (mainly N and P) or other growth-limiting nutrients. Increased addition of N and P sources can result in eutrophication of the marine environment. The common strategy is to design biostimulants which target the oil droplets in the sea water and are not readily diluted and washed out by the wave action. In this work, we present a quick review of existing approaches and current research actions for open sea, near shore, or chronically polluted marine areas by petroleum hydrocarbons.

2 Oil Spill Weathering Processes

Whenever oil is introduced into the marine environment, a series of physical, chemical, and biological processes start taking place that alter the composition and properties of the original oil. As bioremediation is a rather slow process used after conventional cleanup has been applied, the residual oil is often highly weathered before enhanced bioremediation strategies are applied.

The weathering processes include spreading, evaporation, dissolution, photo-oxidation, dispersion, emulsification, biodegradation as well as adsorption onto suspended particulates, sedimentation, and tar ball formation. The combined result of these processes creates a high variability in field studies and difficulties in the evaluation of the efficacy of bioremediation enhancing agents.

This problem can be overcome through the use of biomarkers – practically nonbiodegradable components present in crude oil. The extent of biodegradation is estimated by evaluating the concentration ratio of a target hydrocarbon to one of these recalcitrant biomarkers. Several substances have been used as biomarkers (e.g., pristine and phytane; hopanes and alkylated PAHs isomers) although hopanes have emerged as the best choice (Prince et al. 1994). Hopane normalization is an effective

way to distinguish biodegradation from the effects of the physical washout and sand/ sediment exchange (Venosa et al. 1996).

3 Biostimulation

Biostimulation refers to the addition of one or more rate-limiting nutrients to accelerate contaminant biodegradation rates. In most shoreline ecosystems that have been heavily contaminated with hydrocarbons, nutrients are likely the limiting factors in oil biodegradation. Spilled petroleum hydrocarbons represent a large carbon source whereas in most marine environments the presence of nitrogen and phosphorous is limited. Oxygen represents another very significant and potentially rate limiting nutrient that should be kept in mind before embarking on a biostimulation field application.

3.1 Water Soluble Inorganic Nutrients

Most laboratory experiments have shown that addition of growth limiting nutrients, namely nitrogen and phosphorus, enhances the rate of oil biodegradation and the optimum ratio of carbon to nitrogen to phosphorus is about 100:10:1. The actual amount of N and P needed for biodegradation of the released hydrocarbons is site-specific as it is associated with the type of oil components and the background value of nutrients in the marine environment.

Xia et al. (2006) studied the effects of different forms of N in seawater polluted by diesel. They found that the addition of NO_3-N was more successful than that of NH_4-N in accordance with previous studies by Wrenn et al. (1994) where in poorly buffered seawater polluted with Arabian light crude oil, nitrate was found as a better nitrogen source than ammonia. This is attributed to acid production associated with ammonia metabolism which inhibits oil biodegradation. When the culture pH is controlled, the performance of oil biodegradation is similar for both amendments with a shorter lag time for ammonia. With no control of pH, nitrate was found to have the most pronounced effect in stimulating oil degradation when using pristane as a biomarker (Ramstad and Sveum 1995).

Prevailing seawater temperature affects oil biodegradation. Coulon et al. (2007) found that when increasing temperature from 4 °C to 20 °C had a significant effect in all microcosm treatments and the maximum degradation of TPH was observed at 20 °C. Furthermore, addition of N and P resulted in the greatest hydrocarbon degradation. However, these results do not exclude bioremediation as a treatment in polluted arctic environments, as Wrabel and Peckol (2000) showed the effectiveness of nutrients application at coastline temperatures of the western North Atlantic. Biostimulation has been tested and applied successfully to enhance oil biodegradation in cold Arctic, alpine, and Antarctic environments where psychrophilic bacteria are plentiful (Margesin and Schinner 1999).

Commonly used water-soluble nutrient products include mineral nutrient salts (e.g., KNO_3, $NaNO_3$, NH_3NO_3, K_2HPO_4, $MgNH_4PO_4$, $Ca(H_2PO_4)_2$, $Na_5P_3O_{10}$) and many commercial inorganic fertilizers (e.g., the 23:2 N:P garden fertilizer used in the Exxon Valdez case). Typically, they are applied in the field by spraying aqueous nutrient solutions or by spreading dry granules. This approach has been effective in enhancing oil biodegradation in many field trials (Roling et al. 2004; Swannell et al. 1996; Venosa et al. 1996) including Arctic environments (Prince et al. 2003). However, the problem that still remains is that water soluble nutrients are easily washed by wave and tide action, and thus enhanced biodegradation is difficult to achieve in nonsheltered marine environments or medium to high energy shorelines.

3.2 Slow Release Fertilizers

Considerable effort has been devoted to the development of nutrient delivery systems that overcome the washout problems characteristic of open sea and intertidal environments. Use of slow release fertilizers can provide a continuous source of nutrients to oil contaminated areas overcoming the requirement for multiple nutrient applications in the field and resulting in cost benefits compared to water-soluble nutrients due to less frequent application. Slow release fertilizers consist typically of inorganic nutrients in solid form coated with a hydrophobic compound like paraffin or vegetable oil. The most well-known slow-release fertilizer Customblen (vegetable oil coated calcium phosphate, ammonium phosphate, and ammonium nitrate) performed well on some of the shorelines of Prince William Sound, particularly in combination with an oleophilic fertilizer (Atlas 1995; Swannell et al. 1996).

Kasai et al. (2002) investigated the effects of slow release fertilizers (solid granular nitrogen fertilizer (Super IB) and slow-release solid granular phosphorous fertilizer (Linstar 30)) on oil biodegradation. The addition of fertilizers promoted the degradation of certain components of crude oil: more than 90% of *n*-alkanes (C15–C30) and more than 60% of (alkyl)naphthalenes were degraded within 30 days, whereas the degradation of three-ring aromatics (phenanthrene, anthracene, fluorene, and their alkylsubstituted derivatives) was less extensive, being between 30% and 40%. In contrast, Maki et al. (2002, 2003) found that alkanes degraded to a lesser extent than naphthalenes or fluorenes and to almost the same extent as dibenzothiophenes and phenanthrenes in field experiments performed in sand and cobble stone beaches of Japan after Nakhodka oil spill. However, in both laboratory and field experiments the final degradation efficiencies for each oil component in the fertilized sections were not significantly different from those in the unfertilized sections, and the degradation of each oil component had almost ceased after 6 weeks. It was concluded that excessive amounts of macronutrients are required to accelerate oil biodegradation and under these conditions fertilization is only effective in the early stages.

The challenge that still remains in applying slow release fertilizers is to control the release rates so that suitable nutrient concentrations can be maintained over longer periods of time in the marine environment. Fast release rates do not provide a

long-term source of nutrients, whereas very slow release rates are insufficient to enhance biodegradation rates. For example, Sveum and Ramstad (1995) tested Max Bac, a slow release fertilizer similar to Customblen, and found that it failed to enhance oil biodegradation significantly due to its slow release rate. On the other hand, if one uses a mixture of water soluble and slow release fertilizers in one application better results are obtained.

3.3 Oleophilic Fertilizers

An alternative strategy to overcome the problem of quick dilution and wash out of water-soluble nutrients containing nitrogen and phosphorus is the use of oleophilic biostimulants. The application of N and P sources in oleophilic form is considered to be a more effective nutrient application method, since oleophilic additives remain dissolved in the oil phase and thus are available at the oil-water or oil-sediment interface where they enhance bacterial growth and metabolism (Santas and Santas 2000).

The most well-known oleophilic fertilizer is Inipol EAP22, a microemulsion containing urea as N-source, lauryl phosphate as P-source, 2-butoxy-1-ethanol as a surfactant, and oleic acid to give the mixture its hydrophobicity. This fertilizer has been subjected to extensive studies under various shoreline conditions and was successfully used in oil bioremediation on the shorelines of Prince William Sound (Swannell et al. 1996; Zhu et al. 2001). Another oleophilic fertilizer that was used extensively at the Prestige heavy fuel oil spill is S200 which differs from Inipol EAP22 only in the formulation of the surfactant component (Díez et al. 2005; Jiménez et al. 2006). Díez et al. (2005) observed enhanced biodegradation of the Prestige fuel oil in microcosms containing S200 compared with those containing inorganic phosphorous and nitrogenous salts. These results led to a bioremediation field assay at a cobblestone mixed with sand and gravel beach on the Cantabrian coast (north Spain) using S200. A rigorous control of biodegradation of aliphatic and aromatic hydrocarbons using internal conservative molecular markers for 220 days showed an acceleration of biodegradation at 30–60 days and an enhancement of biodegradation, especially of the heavier n-alkanes (C25–C35) and the alkylated PAHs (Jiménez et al. 2006). Other oleophilic fertilizers include polymerized urea and formaldehyde, and organic fertilizers derived from natural products such as fishmeal, meat meal, lecithin, and uric acid. Uric acid which is the major component of guano fertilizer has been effectively used for crude oil degradation in a simulated open system resulting in 70% petroleum degradation (Knezevich et al. 2006). In a recent study, Gertler et al. (2015) investigated metabolic processes and microbial community changes in a series of microcosms where it was observed that about 80% of uric acid was converted to ammonium within the first few days of the experiment. Experimental data suggested that strains related to *Halomonas* spp. converted uric acid into ammonium, which stimulated the growth of hydrocarbon degrading microbial consortia.

The effectiveness of oleophilic fertilizers depends on the characteristics of the site such as type of sediment or high/low energy wave action and tide. From early on it was shown that oleophilic fertilizers can be more effective than water-soluble fertilizers when the spilled oil resided in the intertidal zone (Sveum et al. 1994); however, no enhancement of biodegradation rates was observed in zones of limited water transport. Variable results have also been produced regarding the persistence of oleophilic fertilizers. Some studies showed that Inipol EAP22 can persist in a sandy beach for a long time under simulated tide and wave actions (Santas and Santas 2000; Swannell et al. 1995); however, experience from very high energy shorelines even oleophilic fertilizers can be rapidly washed out. It is noted that addition of rhamnolipid biosurfactants alone had little effect on biodegradation; however, in combination with water soluble nutrient additions, provoked a significant increase (McKew et al. 2007; Nikolopoulou et al. 2013a) and even greater increase when combined with oleophilic fertilizers (Nikolopoulou et al. 2013b). Sole biosurfactant addition is warranted only to increase bioavailability of weathered petroleum components in situations where background levels of N and P are relatively high.

Many researches have compared the effectiveness of these nutrient products to stimulate oil biodegradation rates. The variable results from laboratory and filed studies indicate the importance of prevailing local conditions. Water-soluble fertilizers are likely more cost-effective in low-energy and fine-grained shorelines and generally sheltered sites where wash out is limited. On the other hand, slow-release fertilizers may be ideal nutrient sources if the nutrient release rates can be well controlled and the nondissolved particles cannot be washed out by the wave action. Finally, oleophilic fertilizers may be more suitable for use in higher-energy, coarse-grained beaches and generally exposed sites and open sea environments. Biostimulation with nutrients and biosurfactants enables naturally occurring microbes to adapt better and faster to the oil spill environment resulting in shorter lag phase and faster crude oil degradation (Nikolopoulou and Kalogerakis 2008), thus making it an effective tool for combating oil spills.

3.4 Oxygen as a Rate Limiting Substrate

Despite the apparent effectiveness of oleophilic fertilizers or mixed products, no enhancement of oil biodegradation rates should be expected if they are added to an anoxic marine environment. In several instances, the concentration of dissolved oxygen can be close to zero leading to practically zero aerobic biodegradation rates. It should be noted that although anaerobic biodegradation of hydrocarbons has been documented in marine environments, the actual rate is particularly low. Although oxygen can be successfully delivered (in various forms) to hydrocarbon-contaminated soils and groundwater enhancing biodegradation rates, this is not the case in marine environments as it is very difficult to implement such technologies in the field. Tiling is essentially the only option in aerating the top layers of contaminated sediments during low tide.

4 Biostimulation Strategies for Chronically Polluted Sites

What should our action be when faced with a chronically polluted marine site? Before proceeding with the addition of slow release or oleophilic fertilizers, one should make sure that such an action will lead to an increase in biodegradation rates. To answer this question, one must consider the following cases:

1. Is it an old pollution event or is there continuous seeping of petroleum products into the chronically polluted site? The later is often encountered near petroleum refineries or crude/refined oil storage tanks sites located on the shoreline where we may have intermittent seeping (following rainfall patterns and changes in groundwater levels) of petroleum products that are present as free product (typically as LNAPLs) on top of contaminated groundwater bodies into the seawater through seeps at the bottom of the sea in close proximity to the shore. If this area is not exposed to high waves, there is little mixing action, and the petroleum contamination appears to be permanent. In such cases, addition of fertilizers is expected to reduce the visible pollution; however, it will not solve the problem. The source of the contamination should be contained and the free product recovered using well known and tested technologies (e.g., funnel and gate systems, bioslurping and hydraulic walls) before it reaches the seawater. Then, biostimulation with mixture of water soluble and slow release fertilizers should help in the complete bioremediation of the site. Such sites are often low energy environments, and hence there is no need for oleophilic formulations.
   Another situation that needs to be considered is the continuous release of transportation fuel from a shipwreck at the bottom of the sea. Again, if we have a chronically polluted site, it must be a low energy environment where the floating oil phase is not quickly dispersed by the wave action and it may accumulate on the shoreline. In such an environment, again biostimulation with water soluble slow release fertilizers is a good option to increase biodegradation rates.
2. If the chronic pollution is due to an old incident, there is no continuous source of hydrocarbons and the site appears to have very minimal recovery, then one must consider (i) lack of a rate limiting nutrient, (ii) excessive toxicity due to the presence of other organic compounds or heavy metals, or (iii) limited bioavailability of the contaminants. The second is often related to sheltered marine environments (low energy wave areas) like commercial harbors near industrial zones. The management of contaminated sediments from a variety of chemicals is a complex issue that should not aim on just meeting simple chemical thresholds, but instead it should aim at a basin-scale good ecological status and should include ecological risk assessment of sediment-bound chemicals on aquatic biota (Apitz et al. 2005). Bioremediation of contaminated sediments is beyond the scope of this review. However, biostimulation can be an important tool when dealing with chronically polluted sediments from petroleum hydrocarbons due to an oil spill. The role of biosurfactants can be significant to increase the bioavailability of the heavier hydrocarbons (Banat et al. 2010; Antoniou et al. 2015).

In addition, one should consider the type of shoreline in question, e.g., fine-grained or coarse-grained sand beach, gravel beach, exposed tidal flat, sheltered rocky shore, sheltered tidal flat, or the most sensitive salt marshes and mangroves. The least sensitive exposed rocky shores tend to recover from oil spills within a few months, whereas the very sensitive salt marshes can maintain petroleum contamination for many years. Tidal pumping enhances oil penetration into the sediments. Permeability and porosity of the sediments dictate the rate and depth of oil penetration. On coarse-grained beaches, oil can penetrate deeper and remain longer (when it is trapped below the limit of wave action) compared to finer grained sediments such as silts and clay. However, oil is more easily removed by water flushing from coarse-grained sediments (Zhu et al. 2001). From the above, we can conclude that chronic petroleum pollution can only occur on beaches where the wave/tidal action is not strong. The addition of nutrients is only warranted when oxygen is not a limiting substrate. If oxygen is limiting, no significant gains are expected through biostimulation.

There are no viable options available to oxygenate sediments except simple tilling which is a physical method used routinely in "landfarming" for the management of petroleum sludges. As an alternative, solid peroxygen materials like calcium or magnesium peroxide (CaO_2 and MgO_2) or calcium superoxide (CaO_4) which contains higher percentage of stored oxygen than CaO_2 can be applied. These techniques appear to be the only low cost means of aeration of the top layer of sediments. If measured concentrations of N and P are very low, biostimulation should also be employed.

The distribution of hydrocarbon degraders is strongly related to the historical exposure of the environment to hydrocarbons. Marine environments with chronic oil contamination will have a much higher percentage of hydrocarbon degraders than nonpolluted sites. In pristine environments, hydrocarbon degraders are extremely low in concentration; however, several studies have shown that starting from pristine seawater, addition of petroleum hydrocarbons with suitable addition of N and P sources leads to an explosion in the population of hydrocarbon degraders in a short number of days [e.g., Cappello et al. 2007].

5 Research Needs

Although the problem of combating marine oil spills has been studied extensively for several decades, there are several areas that could benefit from further research developments. These are:

- Development of low cost oxygenation systems for aerobic bioremediation of contaminated anoxic sediments.
- Development of novel biostimulants that are nontoxic to the marine environment, for example, by increasing in situ production of rhamnolipids at the oil-water interface.

- Development of novel oleophilic amendments with better transport characteristics for application in cold shoreline environments.
- Further increase our understanding of the function of microbial biofilms that develop around an oil droplet and its relationship to different types of biostimulants.

Acknowledgments This work was funded by EU FP-7 PROJECT KILL•SPILL – "Integrated Biotechnological Solutions for Combating marine Oil Spills" (Grant agreement No. 312139).

References

Antoniou E, Fodelianakis S, Korkakaki E, Kalogerakis N (2015) Biosurfactant production from marine hydrocarbon-degrading consortia and pure bacterial strains using crude oil as carbon source. Front Microbiol 6:274. https://doi.org/10.3389/fmicb.2015.00274

Apitz SE, Davis JW, Finkelstein K, Hohreiter DW, Hoke R et al (2005) Assessing and managing contaminated sediments: Part I, developing an effective investigation and risk evaluation strategy. Integr Environ Assess Manag 1:2–8

Atlas RM (1995) Bioremediation of petroleum pollutants. Int Biodeter Biodegr 35:317–327

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L et al (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87:427–444. https://doi.org/10.1007/s00253-010-2589-0

Cappello S, Caruso G, Zampino D, Monticelli LS, Maimone G, Denaro R, Tripodo B, Troussellier M, Yakimov M, Giuliano L (2007) Microbial community dynamics during assays of harbour oil spill bioremediation: a microscale simulation study. J Appl Microbiol 102:184–194

Coulon F, McKew BA, Osborn AM, McGenity TJ, Timmis KN (2007) Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. Environ Microbiol 9:177–186

Díez S, Sabaté J, Viñas M, Bayona JM, Solanas AM, Albaigés J (2005) The prestige oil spill. I. Biodegradation of a heavy fuel oil under simulated conditions. Environ Toxicol Chem 24:2203–2217

Gertler C, Bargiela R, Mapelli F, Han X, Chen J, Hai T, Amer RA, Mahjoubi M, Malkawi H, Magagnini M, Cherif A, Abdel-Fattah YR, Kalogerakis N, Daffonchio D, Ferrer M, Golyshin PN (2015) Conversion of uric acid into ammonium in oil-degrading marine microbial communities: a possible role of Halomonads. Microbial ecology 70:724–740

Jiménez N, Viñas M, Sabaté J, Díez S, Bayona JM, Solanas AM, Albaiges J (2006) The prestige oil spill. II. Enhanced biodegradation of a heavy fuel oil by the use of an oleophilic fertilizer under field conditions. Environ Sci Tech 40:2578–2585

Kasai Y, Kishira H, Sasaki T, Syutsubo K, Watanabe K, Harayama S (2002) Predominant growth of Alcanivorax strains in oil-contaminated and nutrient-supplemented sea water. Environ Microbiol 4:141–147

Knezevich V, Koren O, Ron EZ, Rosenberg E (2006) Petroleum bioremediation in seawater using guano as the fertilizer. Biorem J 10:83–91

Maki H, Utsumi M, Koshikawa H, Hiwatari T, Kohata K, Uchiyama H, Suzuki M, Noguchi T, Yamasaki T, Furuki M, Watanabe M (2002) Intrinsic biodegradation of heavy oil from nakhodka and the effect of exogenous fertilization at a coastal area of the sea of Japan. Water Air Soil Pollut 145:123–138

Maki H, Hirayama N, Hiwatari T, Kohata K, Uchiyama H, Watanabe M, Yamasaki F, Furuki M (2003) Crude oil bioremediation field experiment in the Sea of Japan. Mar Pollut Bull 47:74–77

Margesin R, Schinner F (1999) Biological decontamination of oil spills in cold environments. J Chem Technol Biotechnol 74:381–389

McKew BA, Coulon F, Yakimov M, Denaro R, Genovese M, Smith CJ, Osborn AM, Timmis KN, McGenity TJ (2007) Efficacy of intervention strategies for bioremediation of crude oil in marine systems and effects on indigenous hydrocarbonoclastic bacteria. Environ Microbiol 9:1562–1571

Nikolopoulou M, Kalogerakis N (2008) Enhanced bioremediation of crude oil utilizing lipophilic fertilizers combined with biosurfactants and molasses. Mar Pollut Bull 56:1855–1861

Nikolopoulou M, Eickenbusch P, Pasadakis N, Venieri D, Kalogerakis N (2013a) Microcosm evaluation of autochthonous bioaugmentation to combat marine oil spills. N Biotechnol 30:734–742

Nikolopoulou M, Pasadakis N, Kalogerakis N (2013b) Evaluation of autochthonous bioaugmentation and biostimulation during microcosm- simulated oil spills. Mar Pollut Bull 72:165–173

Prince RC, Elmendorf DL, Lute JR, Hsu CS, Haith CE, Senius JD, Dechert GJ, Douglas GS, Butler EL (1994) 17α(H), 21β(H)-Hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ Sci Tech 28:142–145

Prince RC, Bare RE, Garrett RM, Grossman MJ, Haith CE, Keim LG, Lee K, Holtom GJ, Lambert P, Sergy GA, Owens EH, Guenette CC (2003) Bioremediation of stranded oil on an Arctic Shoreline. Spill Sci Technol Bull 8:303–312

Ramstad S, Sveum P (1995) Bioremediation of oil-contaminated shorelines: effects of different nitrogen sources. In: Hinchee RE et al (eds) Applied bioremediation of petroleum hydrocarbons. Battelle Press, Columbus, pp 415–422

Roling WFM, Milner MG, Jones DM, Fratepietro F, Swannell RPJ, Daniel F, Head IM (2004) Bacterial Community dynamics and hydrocarbon degradation during a field-scale evaluation of bioremediation on a mudflat beach contaminated with buried oil. Appl Environ Microbiol 70:2603–2613

Santas R, Santas P (2000) Effects of wave action on the bioremediation of crude oil saturated hydrocarbons. Mar Pollut Bull 40:434–439

Sveum P, Ramstad S (1995) Bioremediation of oil-contaminated shorelines with organic and inorganic nutrients. In: Hinchee RE et al (eds) Applied bioremediation of petroleum hydrocarbons. Battelle Press, Columbus, pp 201–217

Sveum P, Faksness LG, Ramstad (1994) Bioremediation and of oil-contaminated shorelines: the role of carbon in fertilizers. In: Hinchee RE et al (eds) Hydrocarbon bioremediation. Lewis Publishers, Boca Raton, pp 163–174

Swannell RPJ, Croft BC, Grant AL, Lee K (1995) Evaluation of bioremediation agent in beach microcosms. Spill Sci Technol Bull 2:151–159

Swannell RPJ, Lee K, McDonagh M (1996) Field evaluations of marine oil spill bioremediation. Microbiol Rev 60:342–365

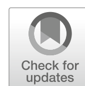
Venosa AD, Suidan MT, Wrenn BA, Strohmeier KL, Haines JR, Eberhart BL, King DW, Holder E (1996) Bioremediation of experimental oil spill on the shoreline of Delaware Bay. Environ Sci Tech 30:1764–1775

Wrabel ML, Peckol P (2000) Effects of bioremediation on toxicity and chemical composition of No. 2 fuel oil growth responses of the Brown Alga *Fucus vesiculosus*. Mar Pollut Bull 40:135–139

Wrenn BA, Haines JR, Venosa AD, Kadkhodayan M, Suidan MT (1994) Effects of nitrogen source on crude oil biodegradation. J Ind Microbiol 13:279–286

Xia WX, Li JC, Zheng XL, Bi XJ, Shao JL (2006) Enhanced biodegradation of diesel oil in seawater supplemented with nutrients. Eng Life Sci 6:80–85

Zhu X, Venosa AD, Suidan MT, Lee K (2001) Guidelines for the bioremediation of marine shorelines and freshwater wetlands. US Environmental Protection Agency, Cincinnati, OH



7 Weathered Hydrocarbon Biotransformation: Implications for Bioremediation, Analysis, and Risk Assessment

S. Cipullo, K. J. Brassington, S. J. T. Pollard, and F. Coulon

Contents

1 Introduction 100
2 Weathered Petroleum Hydrocarbon Analysis in Soil 101
3 Risk Assessment for Weathered Hydrocarbons 106
4 Use of Fugacity Modeling to Parametrize and Conceptualize Fate and Behavior of Petroleum Hydrocarbons 107
5 Bioavailability Complexity 108
6 Bioremediation of Weathered Hydrocarbons 109
7 Research Needs/Knowledge Gaps 111
References 112

Abstract

Weathered petroleum hydrocarbons are highly complex and important soil contaminants, which, despite 40 years of petroleum research, are still not sufficiently understood or appropriately characterized for informing conta.minated land risk assessments. Improved insights into biotransformation of these contaminants and their residual toxicity are essential for improving risk assessments, bioremediation strategies, and effective regeneration of previously contaminated land. The remediation of land contaminated with weathered hydrocarbons has long been limited by inappropriate analytical methodology, the absence from risk assessment frameworks, reduced stakeholder confidence, lack of ecotoxicological analysis in risk assessments, and a distinct paucity of information regarding weathered hydrocarbon toxicity, distribution, transport, and availability in the

S. Cipullo · K. J. Brassington · S. J. T. Pollard · F. Coulon (✉)
School of Water, Energy and Environment, Cranfield University, Cranfield, UK
e-mail: s.cipullo@cranfield.ac.uk; kjbrassington@aol.com; s.pollard@cranfield.ac.uk; f.coulon@cranfield.ac.uk

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_4

environment. Recent research has resulted in the development of a robust analytical method for identification of hydrocarbon residues (weathered hydrocarbons) which are the principal source of the organic carcinogens or suspected carcinogens that drive quantitative risk assessment (e.g., benzo[*a*]pyrene), development of a tool kit for contaminated sites incorporating ecotoxicological consideration, and an improved understanding of weathered hydrocarbon toxicity and biotransformation chemistry. However, knowledge gaps still remain, and additional implications for bioremediation practitioners have been identified concerning remedial methodology at previously remediated sites.

1 Introduction

Petroleum hydrocarbons are common environmental contaminants. They are components of crude oil and products derived from it and are consequently found on a variety of sites including refineries, chemical materials and by-products storage sites, and manufactured gas production sites. They may also be present as a result of spills and leaks during transportation. They are a highly complex mixture of aliphatic and aromatic hydrocarbons with minor amounts of other heterogenic compounds such as nitrogen, sulfur, and oxygen (Farrell-Jones 2003). Once released to the environment, they are subject to physical, chemical, and biological processes that further change their composition, toxicity, availability, and distribution (partitioning) within the environment. Such degradation processes (weathering processes) include adsorption, volatilization, dissolution, biotransformation, photolysis, oxidation, and hydrolysis (Brassington et al. 2007; Pollard et al. 1994, 2005). The extent of weathering experienced is particularly important when characterizing petroleum contamination prior to remediation (Wang et al. 1998), especially the heavy oils, which have high viscosity (ca. 50–360 mPa s), high boiling point (ca. 300– $>$ 600 °C), and carbon number ranges in excess of C_{20}. These weathering processes shift their chemical composition toward recalcitrant, asphaltenic products of increased hydrophobicity.

Typical concentration of total petroleum hydrocarbons (TPH) of weathered oils is below 5000 mg kg^{-1}, volatile compounds such as benzene, toluene, ethylbenzene, and xylenes (BTEX) are not detected or less than 1 ppm, and the chloride concentration is less than 250 mg kg^{-1}. The aliphatic and aromatic fractions of weathered oils are usually ranging from C_{12} to C_{40} (Table 1). These fractions are commonly less bioavailable within the soil due to their unfavorable physicochemical properties (e.g., solubility, volatility, and K_{ow} water/octanol partitioning coefficient) which restricts further microbial attack and degradation (Pollard et al. 1994). However, attempts to improve the bioavailability of the aliphatic and aromatic fractions of weathered hydrocarbons to microorganisms during bioremediation activities may result in increased human exposure. These residual fractions are the principal source of the organic carcinogens or suspected carcinogens that drive quantitative risk assessment (e.g., benzo[*a*]pyrene, benz[*a*]anthracene, chrysene) at contaminated sites (Environment Agency 2005).

Table 1 Petroleum hydrocarbon fractions (based on equivalent carbon number[a]) for use in UK human health risk assessment. Hydrocarbon fractions usually identified for weathered oils are in bold (Environment Agency 2005)

Aliphatic fraction	Avg EC	Aromatic fraction	Avg EC
EC > 5–6	5.5	EC > 5–7	6.5
EC > 6–8	7.0	EC > 7–8	7.5
EC > 8–10	9.0	EC > 8–10	9.0
EC > 10–12	11	EC > 10–12	11
EC > 12–16	14	**EC > 12–16**	14
EC > 16–35	25.5	**EC > 16–21**	18.5
EC > 35–44	39.5	**EC > 21–35**	28.5
		EC > 35–44	39.5
EC > 44–70			57

[a]The equivalent carbon (EC) number of a hydrocarbon is related to its boiling point (b.p.) normalized to the boiling point of an n-alkane series or its retention time on a nonpolar b.p. gas chromatographic (GC) column. For hydrocarbons where the boiling points are known, an EC can be calculated. Hexane contains six carbon atoms and has a boiling point of 69 °C and an EC number of 6. Benzene also contains six carbon atoms and has a boiling point of 80 °C. Based on benzene's b.p. and its retention time on a b.p. GC column, benzene's EC number is 6.5. This approach has been recognized more appropriate differentiation technique than the actual carbon number of the chemical. For hydrocarbons with higher relative carbon number indices, the disparity (in terms of EC) between aliphatic and aromatic hydrocarbons is substantial (Brassington et al. 2007)

Although these important qualitative and quantitative differences between weathered and non-weathered petroleum hydrocarbons are widely acknowledged (see, for review, Brassington et al. 2007), weathered hydrocarbons are not sufficiently understood or appropriately characterized for assessing risk at contaminated sites.

Measuring the total concentration of petroleum hydrocarbons (TPH) in soil does not give a useful basis for the evaluation of the potential risks to human and the environment. The variety of physical-chemical properties, and thus differences in the migration and fate of individual compounds, and the toxicity of different fractions and compounds in oil products must be taken into account in human health risk assessments.

2 Weathered Petroleum Hydrocarbon Analysis in Soil

While there is a range of methods available for the analysis of weathered hydrocarbons including gravimetric analysis, infrared spectrometry (IR), gas chromatography with flame ionization detector (GC-FID), and gas chromatography coupled to mass spectrometry (GC-MS), method choice may partially be restricted or influenced by the risk assessment being used during the remediation of contaminated land (API 2001; ARCADIS Geraghty and Miller International Inc. 2004). In addition, economics also frequently play an important role in method choice. Many of the risk assessments now used during the remediation of contaminated sites incorporate

analytical guidance and reference methods (Table 2). Variations in protocols between frameworks may however give rise to inter-framework variation, affecting the remedial goals set as a result. Indeed, the comparison of reference analytical methods used for petroleum risk assessment protocols highlights the need for practical and simple extraction procedures that allow a better characterization of both aliphatic and aromatic hydrocarbon fractions within oil-contaminated samples, including soil and sediment samples with high moisture levels (Brassington et al. 2007; Environment Agency 2005). In addition to this the Environment Agency of England and Wales notes that currently adopted methods for petroleum hydrocarbon analysis may not be suitable for the heavier compounds ($>C_{16}$) and questions whether it is practical or relevant for analyzing weathered hydrocarbons (Brassington et al. 2007; Risdon et al. 2008).

Various extraction techniques for petroleum hydrocarbons exist within the open literature; however many suffer from inter-method variation and both positive and negative bias (Buddhadasa et al. 2002; Environment Agency 2003; Whittaker et al. 1995). Historically, Soxhlet extraction has been the benchmarked method, due to its exhaustive nature, high recoveries, and ability to be easily standardized (Risdon et al. 2008; Shu et al. 2003). However Soxhlet suffers from long extraction times, a need to concentrate samples, high solvent use, and the degradation of thermally liable compounds (Risdon et al. 2008; Shu et al. 2003). Consequently this has resulted in investigations into and development of the alternative methods (Hawthorne et al. 2000; Hollender et al. 2003; Risdon et al. 2008; Whittaker et al. 1995). Alternative methods to Soxhlet include ultrasonication (Banjoo and Nelson 2005; Risdon et al. 2008; Sanz-Landaluze et al. 2006), pressurized liquid extraction (Hawthorne et al. 2000), supercritical fluid extraction (Hawthorne et al. 2000), subcritical water extraction (Hawthorne et al. 2000), and microwave-assisted extraction (Saifuddin and Chua 2003). Although some of the alternative methods offer improvements over Soxhlet extraction, most of these methods need further refinement and optimization, as there have been conflicting results from different investigations into the same method. For example, Heemken et al. (1997), Sun et al. (1998), Banjoo and Nelson (2005), and Sporring et al. (2005) demonstrated ultrasonic methods that had equivalent or better extraction efficiencies than Soxhlet, whereas investigations by Song et al. (2002) and Hollender et al. (2003) gave worse extraction efficiencies.

Recently, a novel and robust ultrasonic extraction method for contaminated soils with weathered hydrocarbons has been developed and optimized (Risdon et al. 2008). The method covers the determination of TPH between nC_8 and nC_{40} and subranges of hydrocarbons including diesel range organic compounds, kerosene range organic compounds, and mineral oil range organic compounds in soils. Further modifications to the TPH carbon banding may be made as requested for risk assessment including ranges known as Texas Risk banding (TPH C_8–C_{10}, C_{10}–C_{12}, C_{12}–C_{16}, C_{16}–C_{21}, and C_{21}–C_5) as well as separation of the aliphatic and aromatic fractions as defined in the UK regulatory framework (Environment Agency 2005). The method can be routinely used for measuring hydrocarbons down to 10 mg kg^{-1} in soil. Detection limits may vary for individual carbon ranges calculated on the percentage of the full range they cover. With an extraction efficiency and

Table 2 Summary of different analytical methods developed for risk assessment frameworks (Modified from Brassington et al. (2007))

	Massachusetts Department of Environmental Protection (MaDEP 1994)	Total Petroleum Hydrocarbon Criteria Working Group *(TPHCWG* 1997a, b, 1998a, b*;* 1999*)*	Canadian Council of Ministers of the Environment *(CCME* 2000*)*	New Zealand *(Ministry for the Environment* 1999*)*	New South Wales *(National Environment Protection Council* 1999*)*	Risdon et al. (2008)
Extraction technique	Use of two methods. Volatile petroleum hydrocarbon (VPH) method (MaDEP 2004b) and extractable petroleum hydrocarbon (EPH) method developed by MaDEP. VPH method uses purge and trap with methanol. EPH method uses DCM for extraction and solvent exchanges into hexane. Using one of several US EPA solvent	Vortex or shaker method using n-pentane	Purge and trap for C_6 to C_{10} range using methanol. Soxhlet for the C_{10} to C_{50} range	Purge and trap is used for the C_6 to C_9 range. For the C_{10} to C_{36} range, any methods that meet set performance criteria are used	US EPA methods 3540B (US EPA 2005) or 3540C (US EPA 1996a) (Soxhlet extraction), 3550B (US EPA 1996b) (sonication extraction) or sequential bath sonication and agitation described by NEPC (National Environment Protection Council 1999)	Sequential ultrasonic solvent extraction for the nC8 to nC40 using 1:1 acetone/hexane mixture

(*continued*)

Table 2 (continued)

	Massachusetts Department of Environmental Protection (MaDEP 1994)	Total Petroleum Hydrocarbon Criteria Working Group *(TPHCWG* 1997a, b, 1998a, b*;* 1999*)*	Canadian Council of Ministers of the Environment *(CCME* 2000*)*	New Zealand *(Ministry for the Environment* 1999*)*	New South Wales *(National Environment Protection Council* 1999*)*	Risdon et al. (2008)
	extraction methods (MaDEP 2004a)					
Evaporation	The EPH method uses those specified by the US EPA. However, after fractionation the use of a gentle stream of air or nitrogen is recommended to bring the sample to the required volume. Evaporation is not applicable to the VPH method	N/A	Uses an evaporation vessel after extraction for the C_{10} to C_{50} range. After silica gel cleanup, rotary evaporator is used to reach the required sample volume	Use of any method that meets set of performance criteria	US EPA methods specified for extraction using Kuderna-Danish (K-D) evaporation	Not required but can be used to achieve lower limits

Cleanup/ fractionation	Silica gel cleanup for EPH method. Not applicable to VPH method	Extract fractionation using alumina or silica	One of two specified cleanup steps for C_{10} to C_{50} range, not fractionated	Cleanup steps and fractionation are optional as this may not be required for each sample/ analytical approach	Solvent exchange into hexane followed by K-D evaporation and treated with silica gel as described in US EPA method 1664 (US EPA, 2005; National Environment Protection Council 1999)	Not necessary; however is used to fractionate samples. Uses microscale silica gel column chromatography after a water partition step
Analysis technique	EPH uses GC-FID[a]. VPH may use either GC/FID[a] or GC/ PID[b]	GC-FID[a]	GC-FID[a]	For the C_{10} to C_{36} range, GC-FID[a] is used, and for the C_6 to C_9 range, GC-MS[c] is used	GC-MS[c], or GC-FID[a]; however the use of GC/MS[c] to identify unusual mixtures is noted as being necessary when analyzing by GC-FID[a]	Combination of GC-FID and GC-MS depending upon level of analysis

[a]GC-FID refers to gas chromatography with flame ionization detection
[b]GC-PID refers to gas chromatography with photoionization detection
[c]GC-MS refers to gas chromatography with mass spectroscopy detection

recovery between 95% and 99%, this method can be easily positioned as a good alternative to Soxhlet extraction and shows a good potential for implementation as a standard method potentially providing further insight to the contaminated land sector. The method has been accredited ISO17025 for TPH analysis, banding, and class separation.

3 Risk Assessment for Weathered Hydrocarbons

Risk assessment is an established requirement for the management of contaminated land (ARCADIS Geraghty and Miller International Inc. 2004) and now a widely used support tool for environmental management decisions. It is employed as a means of assessing and managing potential impacts to human and ecosystem health (Vegter et al. 2002). Several risk-based frameworks for petroleum hydrocarbons in soil have been published under the auspices of the Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG 1999), the American Society for Testing and Materials (ASTM 1994), the Massachusetts Department of Environmental Protection (MADEP 2002), the Environment Agency of England and Wales (Environment Agency 2005), the American Petroleum Institute (API 2001), and the Canadian Council of Ministers of the Environment (CCME 2000), each reflecting national legislation, a range of expert judgments, and socioeconomic issues. Typically these frameworks adopt a three-tiered approach with increasingly sophisticated levels of data collection and analysis, as an assessor moves through the tiers. However, these frameworks, and the exposure assessment methods embedded within them, do not specifically address weathered hydrocarbons, although some acknowledge that petroleum products released to the environment will have undergone some degree of degradation (API 2001; Environment Agency 2005; MADEP 2002; TPHCWG 1998a).

Assessing the risk of weathered hydrocarbons that they pose at contaminated sites is complicated because the profile of compounds present in weathered oil can be very different from the composition of fresh oil. However, many hydrocarbon compounds would be sufficiently similar in structure to expect that they might have similar toxicities and endpoints. In view of these factors, the UK approach to human health risk assessment of petroleum hydrocarbons favored the adoption of a combined indicator and fraction approach within a tiered risk-based framework (Environment Agency 2005). Specific indicator compounds (genotoxic carcinogens and noncarcinogens) should be assessed because these are often the key risk drivers at petroleum-contaminated sites. Genotoxic carcinogens are assumed not to have a threshold concentration as even very small concentrations (or doses) are assumed to pose some (albeit small) risk of cancer. There are cases in which carcinogenicity can be assumed to occur only after some dose or threshold concentration is reached, depending on the mode of action by which the contaminant is thought to cause cancer. The assessment of fractions should facilitate a more representative picture of risk at sites where the origin of the petroleum hydrocarbons contamination may be unclear. The fractions are typically used to consider threshold health effects (Environment Agency 2005).

Table 3 List of potential indicator compounds for weathered hydrocarbons

Potential indicator compounds for weathered hydrocarbon-contaminated soils	
Naphthalene	Aromatic > EC10–EC12
Acenaphthene	Aromatic > EC12–EC16
1-Methylnaphthalene	
Pyrene	Aromatic > EC16–EC21
Phenanthrene	
Fluoranthene	Aromatic > EC21–EC35
Benz[*a*]anthracene[a]	
Benzo[*b*]fluoranthene[a]	
Benzo[*k*]fluoranthene[a]	
Benzo[*a*]pyrene[a]	
Benzo[*ghi*]perylene	
Chrysene[a]	
Dibenz[*a,h*]anthracene[a]	
Indeno[1,2,3-*c,d*]pyrene	
5-Methylchrysene	

[a]Non-threshold indicator compound, as known to possess some genotoxic carcinogenic potential

Recent work conducted by the research consortium PROMISE on optimizing biopile processes for weathered hydrocarbons within a risk management framework identified suitable potential hydrocarbon fractions and indicator compounds for weathered hydrocarbons (Tables 1 and 3), the adoption of which is essential to the remediation of weathered hydrocarbons in soil (Coulon 2008). Within the aliphatic fraction, only the carbon ranges C_{12}–C_{16} has been identified as threshold health indicator fractions for weathered hydrocarbons. Additionally the atypically high threshold toxicity, relative to the other hydrocarbon compounds, of 2-methylnaphthalene (oral exposure) and naphthalene (inhalation exposure) indicated that they should be viewed as potential indicator compounds at contaminated sites.

As shown in Tables 1 and 3, use of the combined indicator and fraction approach instead of measuring the total concentration of TPH in weathered oils provides a better insight of the carbon range compounds and the key risk drivers within each fraction. Access to this detailed information is important for assessing human and environmental risks and effective remediation at contaminated sites.

4 Use of Fugacity Modeling to Parametrize and Conceptualize Fate and Behavior of Petroleum Hydrocarbons

Understanding the environmental fate of a contaminant is a key requirement when estimating potential risks to human health. To achieve this, meaningful information on a substance's behavior and distribution, toxicity, concentration, and potential exposure at a site is essential (Allan et al. 2006; Environment Agency 2003).

The overriding dominance of the nonaqueous phase liquid (NAPL) for hydrophobic contaminants is theoretically established but rarely incorporated into the exposure assessment tools used to derive soil screening levels and guideline values (Pollard et al. 2008). This oversight is likely to have a marked influence on soil assessment criteria at hydrocarbon-contaminated sites. Its significance comes into play when one considers the residual risk posed by posttreatment residues. Level I and II fugacity models were developed comprising four phases within the soil matrix, namely, air, water, mineral soil, and NAPL. The implications of the fugacity modeling developed by Pollard et al. 2008 are important for risk analysts and remediation engineers. The fugacity modeling confirmed the propensity for risk critical compounds to be preferentially partitioned to the NAPL and soil phases. However, modeled depletion times for contaminants in the context of authentic soils are immaterial, and thus research efforts should be focused on the likely exposures of humans and other receptors to residual saturation at hydrocarbon-contaminated sites. The results indicate clearly the need for modifications to the exposure assessment models used to generate soil screening guidelines or guideline values, so as to better represent contaminant fate in the multimedia systems.

5 Bioavailability Complexity

Bioavailability has been recognized as a key component in risk assessment, but it has not been widely implemented yet (Harmsen and Naidu 2013). Bioavailability of contaminants can be influenced by a wide range of factors including sorption rate, partitioning, mineral speciation, pH, ageing, soil structure, and soil organic content. The physical-chemical characteristics of soil and contaminants are not the only factors affecting bioavailability in soil. In fact, chemical-biological processes such as microbial degradation also contribute in making contaminants less available for uptake. The relationship between the percentage of bioavailable and non-bioavailable fractions is represented in Fig. 1. While bioavailable fraction is decreasing over time the non-bioavailable fraction is increasing. For example, weathered hydrocarbon residues pose negligible risks to human health, and this should be reflected in posttreatment remedial objectives. Bioavailability is directly related with exposure and risk estimates and inversely related to risk-based cleanup levels. Higher cleanup levels are required when bioavailability is high, mainly due to increased exposure and risk estimates.

Given the multiple variables affecting the availability of chemicals in the soil, we should look at bioavailability not as a fixed value (concentration), but as a dynamic process between an organism and the chemical uptake over time (Lanno et al. 2004). However the evaluation of contaminant-soil matrix interactions, that might partially be the cause of the non-accessibility, is still a challenge (Wu et al. 2013, 2014). Research should focus on understanding and accurately representing the bioavailable fraction and ensuring that this parameter is correctly quantified in the risk assessment.

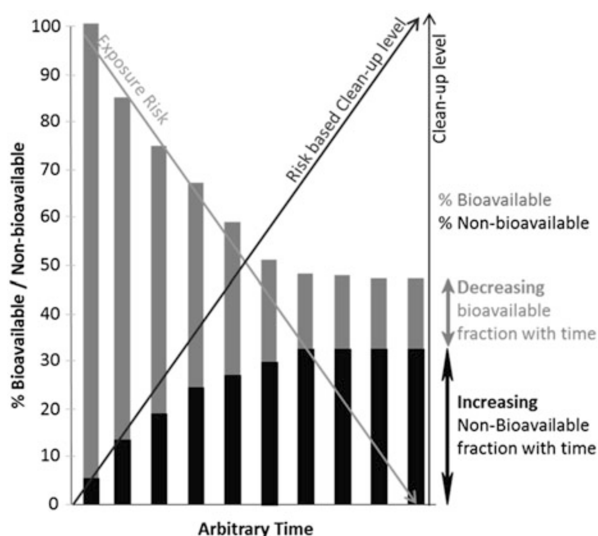


Fig. 1 Relationship between the percentage of bioavailable and non-bioavailable fractions, exposure risks, and risk-based cleanup level; (Adapted from Reid et al. 2000)

6 Bioremediation of Weathered Hydrocarbons

There is a plethora of approaches for the remediation of contaminated land, encompassing physical, chemical, and biological methods which can contain, destroy, or separate the contaminants. Implementation of the EU Landfill Directive (The Council of the European Union 1999) encourages the development and implementation of alternative remediation techniques such as bioremediation in a move away from the mainstay method of excavation and disposal. The organic nature of petroleum hydrocarbons makes these contaminants highly suited to bioremediation techniques as such, and due to the widespread, health, and ecological hazards posed by petroleum hydrocarbons, greater interest has been directed at these contaminants. Bioremediation is a well-established method that works well for remediating petroleum hydrocarbon-contaminated soil (Flathman et al. 1994; Hyman and Dupont 2001). Bioremediation methods are often optimized, using biostimulation and bioaugmentation to enhance biotransformation, reduce cost, and process duration.

Typically, biotransformation is rapid in the initial stages of bioremediation, with rates seen to asymptote toward the end of remediation treatments (Ellis 1994; Fogel 1994; Wood 1997) as the proportion of less bioavailable and recalcitrant compounds increase. Weathered hydrocarbons generally display relatively low bioavailability and are more recalcitrant than their non-weathered counterparts. As such optimization of bioremediation can prove essential to the successful remediation of weathered hydrocarbons (Giles et al. 2001; Guerin 2000).

Recently, Brassington (2008) demonstrated that significant biotransformation of weathered hydrocarbons in soil can be achieved. After 112 days of bioremediation

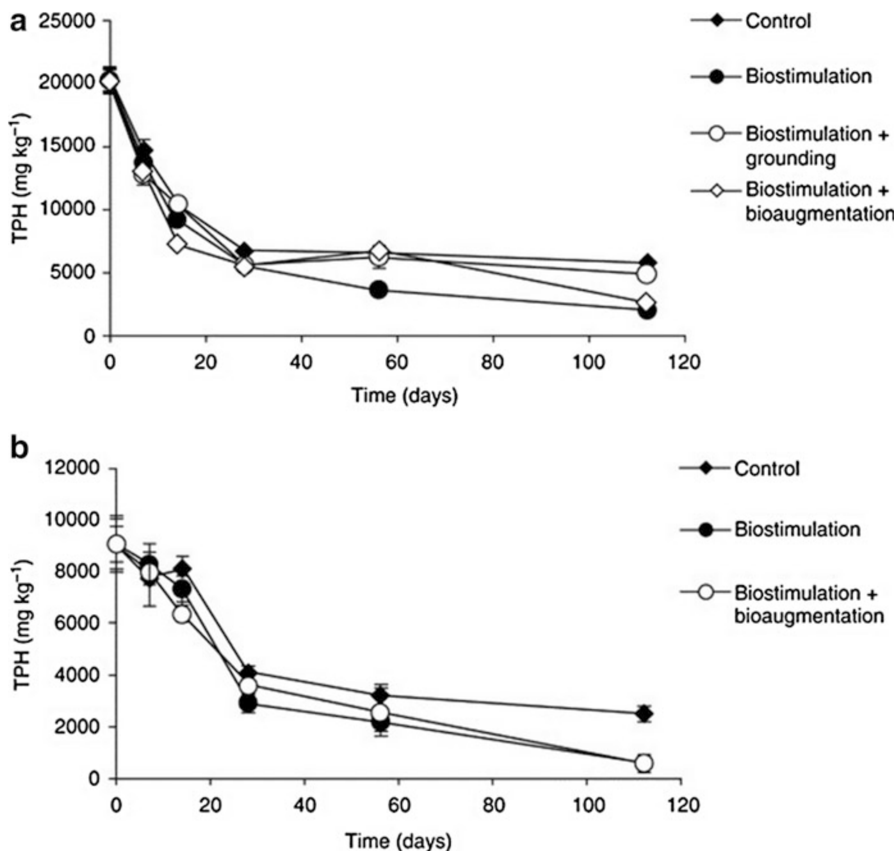


Fig. 2 Change in mean (± se) total petroleum hydrocarbon (TPH) concentration (mg kg^{-1}) over 112 days of treatment for soil **a** and soil **b**

treatments, using biostimulation and bioaugmentation methods (Fig. 2), the residual fraction of weathered petroleum hydrocarbons of two soils (A and B) was degraded up to 85% and 92%, respectively, of its initial content. Soil A was a hydrocarbon-contaminated sandy soil that has previously undergone remediation, and soil B was a clay soil-contaminated with weathered hydrocarbons.

Further, on the basis of physicochemical parameters, the study suggested that the success of bioremediation considering both biostimulation and bioaugmentation approaches was largely dependant of the oil contaminant and soil structure characteristics. For the majority of the contaminated soils investigated, mineral nutrients played an essential role without which in some cases bioremediation could not occur. Slow-release fertilizers were shown to be an important alternative to liquid fertilizers, in mitigating issues arising from the addition of liquid fertilizers. Combining bioaugmentation strategies with biostimulation may improve the rate and extent of weathered hydrocarbon degradation, while the potential benefit of bioaugmentation

Table 4 Ecotoxicological tests used for weathered hydrocarbons (selected species are in bold)

Test	Species considered
Earthworm survival	*Eisenia fetida, Lumbricus terrestris*, *Lumbricus rubellus*
Seed germination	*Brassica alba* **mustard white**, *Triticum aestivum* (Consort) wheat, *Pisum sativum*, pea
Luminescence-based bacterial biosensors	Metabolic: *Vibrio fischeri, Escherichia coli* HB101, *Pseudomonas putida* **F1 Tn5** Catabolic: *Escherichia coli* **HMS174**, *Escherichia coli* DH5α, *Pseudomonas putida* TVA8

still needs further evidence. Ex situ bioremediation for treatment will allow greater control over soil temperature, water holding capacity, and leaching.

The design of an efficient bioremediation system always requires a careful site assessment. Consideration of the physical, chemical, and biological properties of the contaminated sites is essential in establishing appropriate response and recovery methods. Despite the ability of indigenous microorganisms to degrade petroleum hydrocarbons, there are still situations where the use of a microbial inoculum might enhance petroleum hydrocarbon biodegradation.

A diagnostic strategy toolbox for ecological hazard assessment of weathered hydrocarbons has been developed by the research consortium PROMISE (Brassington 2008; Dawson et al. 2007). The toolbox highlighted the role of a multiple-trophic view when considering both the hazard and remediation of weathered hydrocarbons. The selected bioassay techniques were used in combination with the chemical analysis to allow ecological relevance and a more focused understanding of hydrocarbon transformations (Table 4). The bioassays were selected on their ease of execution and representation of different ecological soil organisms.

The research demonstrated that a gross reduction of the hydrocarbons does not represent environmental nor sustainable improvement, as reported by the biological response. In order to integrate and rank the effectiveness of remediation treatments, the biological indicator data were transformed into a soil quality index (SQI) (Dawson et al. 2007). The results highlighted that biological receptor specificity defined the risk-based endpoint and reinforced the concepts of risk reduction within a dynamic and degrading oil matrix. Furthermore, the changes in toxicity and to some extent the bioavailability of fractions within the matrix reflect that some pollutants may be mobilized during remedial activities.

7 Research Needs/Knowledge Gaps

It is clear that successful bioremediation of land contaminated with weathered petroleum hydrocarbons depends upon the successful integration of effective analysis techniques, informed optimization of bioremediation, and an appropriate risk assessment protocol. Remediation of contaminated soils is limited by several factors including a lack of toxicological and environmental fate and behavior data, inadequate and variable chemical analyses, negative stakeholders' perception of

bioremediation techniques, and variable risk assessments that do not always consider weathered hydrocarbons. While the work of researchers has taken important steps toward addressing key knowledge gaps and methodological limitations within analysis and risk protocols, there is still work to be done.

Further investigation is still required to increase our knowledge on weathered hydrocarbon chemical, toxicological, and biological diagnostics as well as environmental fate and behavior. This combined diagnostic approach will significantly help to identify optimal remediation strategies and contribute to change the over-conservative nature of the current risk assessments.

References

Allan IJ, Semple KT, Hare R, Reid BJ (2006) Prediction of mono- and polycyclic aromatic hydrocarbon degradation in spiked soils using cyclodextrin extraction. Environ Pollut 144:562–571

API (2001) Risk-based methodologies for evaluating petroleum hydrocarbon impacts at oil and natural gas E&P Sites, API Publication 4709, American Petroleum Institute Publishing Services, Washington, DC

ARCADIS Geraghty & Miller International Inc (2004) Risk assessment comparison study, 916830024, NICOLE/ISG

ASTM (1994) Emergency standard guide for risk-based corrective action applied at petroleum release sites, ES 48–94, ASTM, Philadelphia

Banjoo DR, Nelson PKJ (2005) Improved ultrasonic extraction procedure for the determination of polycyclic aromatic hydrocarbons in sediments. Chromatogr A 1066:9–18

Brassington KJ (2008) New insights into the biotransformation of weathered hydrocarbons in soil. PhD thesis, SAS, Cranfield University

Brassington KJ, Hough RL, Paton GI, Semple KT, Risdon GC, Crossley J, Hay I, Askari K, Pollard SJT (2007) Weathered hydrocarbon wastes: a risk management primer. Crit Rev Environ Sci Technol 37:199–232

Buddhadasa SC, Barone S, Bigger SW, Orbell JD (2002) Australian approaches to improving methods for the analysis of TPH contamination in soil. In: 17th WCSS, Thailand

CCME (2000) Canada-wide standards for petroleum hydrocarbons (PHCs) in soil: scientific rationale. CCME, Winnipeg

Coulon F (2008) Risk assessment for soils contaminated with weathered hydrocarbons. Brownfield briefing conferences, contaminated land risk assessment 2008, Propero House, Central London, 29–30 April 2008

Dawson JJC, Godsiffe EJ, Thompson IP, Ralebitso-Senior TK, Killham KS, Paton GI (2007) Application of biological indicators to assess recovery of hydrocarbon impacted soils. Soil Biol Biochem 39:164–177

Ellis B (1994) Reclaiming contaminated land: in situ/ex situ remediation of creosote-and petroleum hydrocarbon-contaminated sites. In: Flathman P et al (eds) Bioremediation field experience. Lewis Publishers, Boca Raton, pp 107–143

Environment Agency (2003) Principles for evaluating the human health risks from petroleum hydrocarbons in soils: a consultation paper, R & D technical report P5–080/TR1, Environment Agency, Bristol

Environment Agency (2005) The UK approach for evaluating human health risks from petroleum hydrocarbons in soils, Science report P5–080/TR3, Environment Agency, Bristol

Farrell-Jones J (2003) Petroleum hydrocarbons and polyaromatic hydrocarbons. In: Thompson CK, Nathanail PC (eds) Chemical analysis of contaminated land. Blackwell, Oxford, pp 132–176

Flathman PE, Jerger DE, Exner JH (1994) Bioremediation field experience. Lewis Publishers, Boca Raton

Fogel S (1994) Full-scale bioremediation of No. 6 fuel oil-contaminated soil: 6 months of active and 3 years of passive treatment. In: Flathman P et al (eds) Bioremediation field experience. Lewis Publishers, Boca Raton, pp 161–175
Giles WRJR, Kriel KD, Stewart JR (2001) Characterization and bioremediation of weathered oil sludge. Environ Geosci 8:110–122
Guerin TF (2000) Long-term performance of a land treatment facility for the bioremediation of non-volatile oil wastes, Resour. Conserv Recycl 28:105–120
Harmsen J, Naidu R (2013) Bioavailability as a tool in site management. J Hazard Mater 261:840–846. https://doi.org/10.1016/j.jhazmat.2012.12.044
Hawthorne SB, Grabanski CB, Martin E, Miller DJ (2000) Comparisons of soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: recovery, selectivity and effects on sample matrix. J Chromatogr A 892:421–433
Heemken OP, Theobald N, Wenclawiak BW (1997) Comparison of ASE and SFE with Soxhlet, sonication, and methanolic saponification extractions for determination of organic micro-pollutants in marine particulate matter. Anal Chem 69:2171–2180
Hollender J, Koch B, Lutermann C, Dott W (2003) Efficiency of different methods and organic solvents for the extraction of polycyclic aromatic hydrocarbons from soils. Int J Environ Anal Chem 83:21–32
Hyman M, Dupont RR (2001) Groundwater and soil remediation: process design and cost estimating of proven technologies. American Society of Civil Engineers Press, Reston
Lanno R, Wells J, Conder J, Bradham K, Basta N (2004) The bioavailability of chemicals in soil for earthworms. Ecotoxicol Environ Saf 57(1):39–47. https://doi.org/10.1016/j.ecoenv.2003.08.014
MADEP (1994) Interim final petroleum report: development of health-based alternative to the total petroleum hydrocarbon (TPH) parameter, Massachusetts Department of Environmental Protection, Executive Office of Environmental Affairs, Commonwealth of Massachusetts, Boston
MADEP (2002) Draft updated petroleum hydrocarbon fraction toxicity values for the VEP/EPH/APH methodology, Massachusetts Department of Environmental Protection, Executive Office of Environmental Affairs, Commonwealth of Massachusetts, Boston
MADEP (2004a) Method for the determination of extractable petroleum hydrocarbons (EPH), Revision 1.1, Massachusetts Department of Environmental Protection, Executive Office of Environmental Affairs, Commonwealth of Massachusetts, Boston
MADEP (2004b) Method for the determination of volatile petroleum hydrocarbons (VPH), Revision 1.1, Massachusetts Department of Environmental Protection, Executive Office of Environmental Affairs, Commonwealth of Massachusetts, Boston
Ministry for the Environment (1999) Guidelines for assessing and managing petroleum hydrocarbon contaminated sites in New Zealand. Ministry for the Environment, Wellington
National Environment Protection Council (1999) Schedule B (3): guideline on laboratory analysis of potentially contaminated soils. National Environment Protection Council. Available from. http://www.ephc.gov.au/pdf/cs/cs_03_lab_analysis.pdf. Accessed 11 Dec 2008)
Pollard SJT, Hrudey SE, Fedorak PM (1994) Bioremediation of petroleum- and creosote-contaminated soils: A review of constraints. Waste Manag Res 12:173–194
Pollard SJT, Hough RL, Brassington KJ, Sinke A, Crossley J, Paton GI, Semple K, Risdon G, Jackman SJ, Bone B, Jacobsen C, Lethbridge G (2005) Optimising the biopiling of weathered hydrocarbons within a risk management framework – PROMISE. In: CL:AIRE and FIRST FARADAY joint conference on contaminated land, International Convention Centre, Birmingham, 27–28 April 2005
Pollard SJT, Hough RL, Kim K-H, Bellarby J, Paton G, Semple K, Coulon F (2008) Fugacity modelling to predict the distribution of organic contaminants in the soil: Oil matrix of constructed biopiles. Chemosphere 71:1432–1439
Reid BJ, Jones KC, Semple KT (2000) Bioavailability of persistent organic pollutants in soils and sediments a perspective on mechanisms, consequences and assessment. Environ Pollut 108:103–112
Risdon G, Pollard SJT, Brassington KJ, McEwan JN, Paton G, Semple K, Coulon F (2008) Development of a novel and robust analytical procedure for weathered hydrocarbon contaminated soils within a UK risk based framework. Anal Chem 80:7090–7096

Saifuddin N, Chua KH (2003) Extraction of Tetrachloroethylene from weathered soils: A comparison between soxhlet extraction and microwave-assisted extraction. Malays J Chem 5:30–33

Sanz-Landaluze J, Bartolome L, Zuloaga O, González L, Dietz C, Cámara C (2006) Accelerated extraction for determination of polycyclic aromatic hydrocarbons in marine biota. Anal Bioanal Chem 384:1331–1340

Shu YY, Lai TL, Lin H-S, Yang TC, Chang C-P (2003) Study of factors affecting on the extraction efficiency of polycyclic aromatic hydrocarbons from soils using open-vessel focused microwave-assisted extraction. Chemosphere 52:1667–1676

Song YF, Jing X, Fleischmann S, Wilke B-M (2002) Comparative study of extraction methods for the determination of PAHs from contaminated soils and sediments. Chemosphere 48: 993–1001

Sporring S, Bøwadt S, Svensmark B, Björklund E (2005) Comprehensive comparison of classic Soxhlet extraction with Soxtec extraction, ultrasonication extraction, supercritical fluid extraction, microwave assisted extraction and accelerated solvent extraction for the determination of polychlorinated biphenyls in soil. J Chromatogr A 1090:1–9

Sun F, Littlejohn D, Gibson MD (1998) Ultrasonication extraction and solid phase extraction clean-up for determination of US EPA 16 priority pollutant polycyclic aromatic hydrocarbons in soils by reversed-phase liquid chromatography with ultraviolet absorption detection. Anal Chim Acta 364:1–11

The Council of the European Union (1999) Council directive 1999/31/EC of 26 April 1999 on the landfill of waste, Official Journal of the European Communities, Luxembourg, available at http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A31999L0031 (accessed on 4/03/2017)

TPHCWG (1997a) Selection of representative TPH fractions based on fate and transport considerations, Total petroleum hydrocarbon criteria working group series, vol 3. Amherst Scientific, Amherst

TPHCWG (1997b) Development of fraction specific reference doses (RfDs) and reference concentrations (RfCs) for total petroleum hydrocarbons (TPH), Total petroleum hydrocarbon criteria working group series, vol 4. Amherst Scientific, Amherst

TPHCWG (1998a) Analysis of petroleum hydrocarbons in environmental media, Total petroleum hydrocarbon criteria working group series, vol 1. Amherst Scientific, Amherst

TPHCWG (1998b) Composition of petroleum mixtures, Total petroleum hydrocarbon criteria working group series, vol 2. Amherst Scientific, Amherst

TPHCWG (1999) Human health risk-based evaluation of petroleum release sites: implementing the working group approach, Total petroleum hydrocarbon criteria working group series, vol 5. Amherst Scientific, Amherst

USEPA (1996a) SW-846 test method 3540C: Soxhlet extraction, United States Environmental Protection Agency, available at https://www.epa.gov/hw-sw846/sw-846-test-method-3540c-soxhlet-extraction (accessed on 04/03/2017)

USEPA (1996b) Method 3550B - Ultrasonic extraction, United States Environmental Protection Agency, available at http://www.cromlab.es/Articulos/Metodos/EPA/3000/3550B.PDF (accessed on 04/03/2017)

USEPA (2005) Hazardous waste test methods / SW-846, United States Environmental Protection Agency, available at https://www.epa.gov/hw-sw846 (accessed on 04/03/2016)

Vegter J, Lowe J, Kasamas H (2002) Sustainable management of contaminated land: an overview. Austrian Federal Environment Agency, Vienna. , on behalf of CLARINET

Wang Z, Fingas M, Blenkinsopp S, Sergy G, Landriault M, Sigouin L, Folght J, Semple K, Westlake DWS (1998) Comparison of oil composition changes due to biodegradation and physical weathering in different oils. J Chromatogr A 809:89–107

Whittaker M, Pollard SJT, Fallick TE (1995) Characterization of refractory wastes at heavy oil-contaminated sites: a review of conventional and novel analytical. Methods Environ Technol 16:1009–1033

Wood PA (1997) Remediation methods for contaminated sites. In Issues in environmental science and technology: contaminated land and its reclamation. Cambridge: The Royal Society of Chemistry, pp. 47–71.

Wu G, Kechavarzi C, Li X, Wu S, Pollard SJT, Sui H, Coulon F (2013) Machine learning models for predicting PAHs bioavailability in compost amended soils. Chem Eng J 223:747–754

Wu G, Li X, Kechavarzi C, Sakrabani R, Sui H, Coulon F (2014) Influence and interactions of multi-factors on the bioavailability of PAHs in compost amended contaminated soils. Chemosphere 107:43–50

Role of Biosurfactants

8

Eliora Z. Ron and E. Rosenberg

Contents

1 Introduction 117
2 Utilizing Biosurfactants for Bioremediation 120
3 Research Needs 120
References 120

Abstract

Oil is a mixture of a large number of components, all of which are hydrophobic. The high molecular weight fraction, especially the polyaromatic hydrocarbons, has a low water solubility that significantly reduces their availability to bioremediating bacteria. The addition of biosurfactants is critical for increasing the bioavailability of these hydrocarbons, and encouraging results were obtained with the use of high molecular weight biosurfactants.

1 Introduction

One of the limiting factors in oil bioremediation is the bioavailability of many fractions of the oil, mainly those with lower water solubility, such as polycyclic aromatic hydrocarbons. The hydrocarbon-degrading microorganisms produce biosurfactants of diverse chemical nature and molecular size to enhance the uptake of hydrocarbons, including the ones with low water solubility. In general, bacteria produce low molecular weight biosurfactants that lower surface and interfacial tensions, and high molecular weight biosurfactants bind tightly to surfaces (Desai

E. Z. Ron (✉) · E. Rosenberg
Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Tel Aviv, Israel
e-mail: eliora@post.tau.ac.il; eros@post.tau.ac.il

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_6

and Banat 1997; Ron and Rosenberg 2001, 2002; Rosenberg et al. 1998a). These surface-active high molecular weight materials increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation (Ron 2000; Ron and Rosenberg 2002).

The **low molecular weight biosurfactants** are generally glycolipids in which carbohydrates are attached to a long-chain aliphatic acid or lipopeptides. Glycolipid bioemulsifiers, such as rhamnolipids, trehalose lipids, and sophorolipids, are disaccharides that are acylated with long-chain fatty acids or hydroxy fatty acids. One of the best-studied glycolipids is rhamnolipid, produced by several species of *Pseudomonads* that consists of 2 mol of rhamnose and 2 mol of β-hydroxydecanoic acid (Yakimov et al. 1998). Recently, a new class of glycolipids, glucose lipids, produced by *Alcanivorax borkumensis* has been described (Yakimov et al. 1998) that consists of an anionic glucose lipid with a tetrameric oxyacyl side chain.

The **high molecular weight bacterial surfactants** are produced by a large number of bacterial species from different genera and are composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers (Bonilla et al. 2005; Gutierrez et al. 2007; Lim et al. 2007; Mahanty et al. 2006; Maneerat et al. 2006; Rosenberg and Ron 1999). The high molecular weight surfactants are less effective in reducing interfacial tension, but are efficient at coating the oil droplets and preventing their coalescence. These are highly efficient emulsifiers that work at low concentrations (0.01–0.001%), representing emulsifier-to-hydrocarbon ratios of 1:100 to 1:1,000. These high molecular weight bioemulsifiers exhibits considerable substrate specificity. For example, some of them emulsify efficiently only mixtures of aliphatic and aromatic (or cyclic alkane) hydrocarbons but not pure aliphatic, aromatic, or cyclic hydrocarbons; others can also emulsify pure hydrocarbons but only of a high molecular weight. The best-studied biosurfactants are the bioemulsans produced by different species of *Acinetobacter* (Rosenberg and Ron 1998). Among them are the RAG-1 emulsan, which is a complex of an anionic heteropolysaccharide and protein, the surface activity of which is due to the presence of fatty acids that are attached to the polysaccharide backbone via O-ester and N-acyl linkages. *A. calcoaceticus* BD4, initially isolated and characterized by Taylor and Juni (1961), produces a surface-active extracellular polysaccharide-protein complex. Alasan, produced by a strain of *A. radioresistens*, is a complex of an anionic polysaccharide and protein with a molecular weight of approximately 1×10^6 (Navon-Venezia et al. 1995). The polysaccharide component of alasan is unusual in that it contains covalently bound alanine. The protein component of alasan appears to play an important role in both the structure and activity of the complex (Navon-Venezia et al. 1998; Toren et al. 2001). One of the alasan proteins, with an apparent molecular mass of 45 kDa, was studied at the molecular level. This protein has an amino acid sequence homologous to that of *Escherichia coli* OmpA and is highly effective in stabilizing oil-in-water emulsions and in solubilizing hydrocarbons, including polycyclic aromatic hydrocarbons (Toren et al. 2002a, b).

Biosurfactants are involved in bioremediation by increasing the surface area of hydrophobic water-insoluble substrates and increasing the bioavailability of hydrophobic compounds**.** The low water solubility of many hydrocarbons – especially the polycyclic aromatic hydrocarbons (PAHs) is believed to limit their availability to microorganisms, which is a potential problem for bioremediation of contaminated sites. It has been assumed that surfactants would enhance the bioavailability of hydrophobic compounds. Several nonbiological surfactants have been studied, and both negative and positive effects of the surfactants on biodegradation were observed. For example, the addition of the surfactant Tergitol NP-10 increased the dissolution rate of solid-phase phenanthrene and resulted in an overall increase in the growth of a strain of *P. stutzeri* (Grimberg et al. 1996). Enzymatically synthesized lauroyl glucose emulsified different hydrophobic substrates and enhanced degradation of crude oil by a known oil-degrading Rhodococcus species (Kelkar et al. 2007). A similar effect was obtained by the addition of Tween 80 to two *Sphingomonas* strains – the rate of fluoranthene mineralization was almost doubled. In contrast, the same surfactant inhibited the rate of fluoranthene mineralization by two strains of *Mycobacterium* (Van Delden et al. 1998), and no stimulation was observed in other studies using several surfactants (Bruheim et al. 1997; Bruheim and Eimhjellen 1998).

Biosurfactants are more effective than chemical surfactants in increasing the bioavailability of hydrophobic compounds. In addition, they are selective, environmentally friendly, and generally less stable than most synthetic surfactants. The high molecular weight bioemulsifier alasan was recently shown to significantly increase the rate of biodegradation of several PAHs (Rosenberg et al. 1998a, b).

One of the major reasons for the prolonged persistence of high molecular weight hydrophobic compounds is their low water solubility that increases their sorption to surfaces and limits their availability to biodegrading microorganisms. When organic molecules are bound irreversibly to surfaces, biodegradation is inhibited (van Loosdrecht et al. 1990). Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Marcoux et al. 2000). Surfactants that lower interfacial tension dramatically are particularly effective in mobilizing bound hydrophobic molecules and making them available for biodegradation. Low molecular weight biosurfactants that have low CMCs increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Miller and Zhang 1997). Data have been reported that indicate that biosurfactants can stimulate, inhibit, or have no effect on biodegradation of hydrocarbons, as reviewed by Bruheim et al. (1997). In this regard, it has been reported that rhamnolipid-producing strains of *P. aeruginosa* are involved in the degradation of PAHs by a bacterial community (Arino et al. 1998; Holden et al. 2002). Much less is known on how polymeric biosurfactants increase apparent solubilities of hydrophobic compounds. It has also been demonstrated that alasan increases the apparent solubilities of PAHs 5–20 fold and significantly increases their rate of biodegradation (Barkay et al. 1999; Rosenberg et al. 1998a).

2 Utilizing Biosurfactants for Bioremediation

Bioremediation involves the acceleration of natural biodegradative processes in contaminated environments by improving the availability of materials (e.g., nutrients and oxygen), conditions (e.g., pH and moisture content), and prevailing microorganisms. Thus, bioremediation usually consists of application of nitrogenous and phosphorous fertilizers, adjusting the pH and water content, if necessary, supplying air, and often adding bacteria. The addition of emulsifiers is advantageous when bacterial growth is slow – in cold conditions and at high concentrations of pollutants – or when the pollutants consist of compounds that are difficult to degrade – such as PAHs, halogenated PAHs, or xenobiotics. Bioemulsifiers can be applied as an additive to stimulate the bioremediation process. The recent advances in genomics and genetic technologies will probably result in constructing bacterial strains that will overproduce emulsifiers. Such bacteria can be used for more efficient and cost-effective production of emulsifiers. Moreover, it is expected that the increase in bioemulsifier concentration during bioremediation would be achieved by the addition of such bacteria.

The bacteria that overproduce bioemulsifiers can also participate in oil degradation. In addition, they can function in a bacterial consortium, supplying the emulsifier for other bacteria that carry out the degradation of the hydrocarbons. In the latter case, the bioemulsifier can diffuse in the soil or can even be transferred to the other bacteria on close contact, such as in biofilms. In fact, horizontal transfer of capsule polysaccharide has been demonstrated in bacteria (Osterreicher-Ravid et al. 2000) resulting in bacteria coated with emulsifying polysaccharide capsule produced by bacteria of another species. In addition, there are data indicating that several Acinetobacter strains secrete protein emulsifiers into the environment (Walzer et al. 2006). The effect of these phenomena on oil bioremediation remains to be further investigated. Clearly, optimization of this process would involve selecting the best oil degrading microorganisms, the most suitable biosurfactant, the best bioemulsifier producers, and the most effective combination of these.

3 Research Needs

There are research needs in two areas:

1. Identification of additional biosurfactants with high activity in increasing the bioavailability of high molecular weight hydrocarbons, especially polycyclic aromatics
2. Development of production/fermentation technologies that will make the use of biosurfactants cost effective

References

Arino S, Marchal R, Vandecasteele JP (1998) Involvement of a rhamnolipid-producing strain of *Pseudomonas aeruginosa* in the degradation of polycyclic aromatic hydrocarbons by a bacterial community. J Appl Microbiol 84:769–776

Barkay T, Navon-Venezia S, Ron EZ, Rosenberg E (1999) Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl Environ Microbiol 65:2697–2702
Bonilla M, Olivaro C, Corona M, Vazquez A, Soubes M (2005) Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. J Appl Microbiol 98:456–463
Bruheim P, Eimhjellen K (1998) Chemically emulsified crude oil as substrate for bacterial oxidation: differences in species response. Can J Microbiol 44:195–199
Bruheim P, Bredholt H, Eimhjellen K (1997) Bacterial degradation of emulsified crude oil and the effect of various surfactants. Can J Microbiol 43:17–22
Desai JD, Banat M (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64
Grimberg SJ, Stringfellow WT, Aitken MD (1996) Quantifying the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16 in the presence of a nonionic surfactant. Appl Environ Microbiol 62:2387–2392
Gutierrez T, Mulloy B, Bavington C, Black K, Green DH (2007) Partial purification and chemical characterization of a glycoprotein (putative hydrocolloid) emulsifier produced by a marine bacterium Antarctobacter. Appl Microbiol Biotechnol 76:1017–1026
Holden PA, LaMontagne MG, Bruce AK, Miller WG, Lindow SE (2002) Assessing the role of *Pseudomonas aeruginosa* surface-active gene expression in hexadecane biodegradation in sand. Appl Environ Microbiol 68:2509–2518
Kelkar DS, Kumar AR, Zinjarde SS (2007) Hydrocarbon emulsification and enhanced crude oil degradation by lauroyl glucose ester. Bioresour Technol 98:1505–1508
Lim DJ, Kim JD, Kim MY, Yoo SH, Kong JY (2007) Physicochemical properties of the exopolysaccharides produced by marine bacterium *Zoogloea sp*. KCCM10036. J Microbiol Biotechnol 17:979–984
Mahanty B, Pakshirajan K, Dasu VV (2006) Production and properties of a biosurfactant applied to polycyclic aromatic hydrocarbon solubilization. Appl Biochem Biotechnol 134:129–141
Maneerat S, Bamba T, Harada K, Kobayashi A, Yamada H, Kawai F (2006) A novel crude oil emulsifier excreted in the culture supernatant of a marine bacterium, *Myroides sp*. strain SM1. Appl Microbiol Biotechnol 70:254–259
Marcoux J, Deziel E, Villemur R, Lepine F, Bisaillon JG, Beaudet R (2000) Optimization of high-molecular-weight polycyclic aromatic hydrocarbons' degradation in a two-liquid-phase bioreactor. J Appl Microbiol 88:655–662
Miller RM, Zhang Y (1997) Measurement of biosurfactant-enhanced solubilization and biodegradation of hydrocarbons. Methods Biotechnol 2:59–66
Navon-Venezia S, Zosim Z, Gottlieb A, Legmann R, Carmeli S, Ron EZ, Rosenberg E (1995) Alasan: a new bioemulsifier from *Acinetobacter radioresistens*. Appl Environ Microbiol 61:3240–3244
Navon-Venezia S, Ron EZ, Banin E, Rosenberg E (1998) The bioemulsifier alasan: role of protein in maintaining structure and activity. Appl Microbiol Biotechnol 49:382–384
Osterreicher-Ravid D, Ron EZ, Rosenberg E (2000) Horizontal transfer of an exopolymer complex from one bacterial species to another. Environ Microbiol 2:366–372
Ron EZ (2000) Microbial life on petroleum. In: Seckbach J (ed) Journey to diverse microbial worlds. Kluwer Academica Publishers, Dordrecht, pp 303–315
Ron EZ, Rosenberg E (2001) Natural roles of biosurfactants. Environ Microbiol 3:229–236
Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Biotechnol 13:249–252
Rosenberg E, Ron EZ (1998) Surface active polymers of Acinetobacter. In: Kaplan D (ed) Biopolymers from renewable sources. Springer, Berlin, pp 281–291
Rosenberg E, Ron EZ (1999) High- and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162
Rosenberg E, Barkay T, Navon-Venezia S, Ron EZ (1998a) Role of Acinetobacter bioemulsans in petroleum degradation. In: Fass R et al (eds) Novel approaches for bioremediation of organic pollution. Kluwer Academic/Plenum Publishers, New York, pp 171–180

Rosenberg E, Navon-Venezia S, Zilber-Rosenberg I, Ron EZ (1998b) Rate-limiting steps in the microbial degradation of petroleum hydrocarbons. In: Rubin H, Narkis N, Carberry J (eds) Soil and aquifer pollution. Springer, Berlin, pp 59–172

Taylor WH, Juni E (1961) Pathways for biosynthesis of a bacterial capsular polysaccharide. I. Carbohydrate metabolism and terminal oxidation mechanisms of a capsuleproducing coccus. J Bacteriol 81:694–703

Toren A, Navon-Venezia S, Ron EZ, Rosenberg E (2001) Emulsifying activities of purified Alasan proteins from *Acinetobacter radioresistens* KA53. Appl Environ Microbiol 67:1102–1106

Toren A, Orr E, Paitan Y, Ron EZ, Rosenberg E (2002a) The active component of the bioemulsifier alasan from *Acinetobacter radioresistens* KA53 is an OmpA-like protein. J Bacteriol 184:165–170

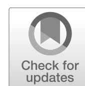
Toren A, Ron EZ, Bekerman R, Rosenberg E (2002b) Solubilization of polyaromatic hydrocarbons by recombinant bioemulsifier AlnA. Appl Microbiol Biotechnol 59:580–584

Van Delden C, Pesci EC, Pearson JP, Iglewski BH (1998) Starvation selection restores elastase and rhamnolipid production in a *Pseudomonas aeruginosa* quorum-sensing mutant. Infect Immun 66:4499–4502

van Loosdrecht MC, Lyklema J, Norde W, Zehnder AJ (1990) Influence of interfaces on microbial activity. Microbiol Rev 54:75–87

Walzer G, Rosenberg E, Ron EZ (2006) The Acinetobacter outer membrane protein A (OmpA) is a secreted emulsifier. Environ Microbiol 8:1026–1032

Yakimov MM, Golyshin PN, Lang S, Moore ER, Abraham WR, Lunsdorf H, Timmis KN (1998) *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int J Syst Bacteriol 48(Pt 2):339–348



Plant-Endophyte Partnerships to Assist Petroleum Hydrocarbon Remediation

9

S. Thijs, N. Weyens, P. Gkorezis, and J. Vangronsveld

Contents

1 Introduction 124
2 Ecology of Endophytic Bacteria 125
2.1 Critical Factors in the Isolation of Endophytic Bacteria 126
2.2 Endophytic Communities Identified by Culture-Independent Tools 127
2.3 Colonization 128
3 Beneficial Effects of Plant-Microbe Partnerships 131
3.1 Direct Promotion of Plant Growth 132
3.2 Indirect Promotion of Plant Growth 137
4 Catabolic Potential of Endophytic Bacteria 139
5 Phytoremediation of Petroleum Hydrocarbons 140
5.1 Plant-Bacteria Partnerships 142
5.2 Plant-Endophyte Interactions for Petroleum Hydrocarbon Degradation 142
5.3 Potential of Meta-Omics Approaches to Elucidate Microbial Functions 143
5.4 Critical Factors, Success Factors, and Future Outlook 144
6 Conclusions 145
7 Research Needs 146
References 146

Abstract

Petroleum hydrocarbons (PHs) are the most widespread organic contaminants in soil and groundwater worldwide. The financial, environmental, and health impacts of the contaminants are considerable. Regulations require the remediation of contaminated sites and encourage the use of biological methods such as phytoremediation, whereby plants and their associated microorganisms are used.

S. Thijs (✉) · N. Weyens · P. Gkorezis · J. Vangronsveld
Centre for Environmental Sciences, Hasselt University, Diepenbeek, Belgium
e-mail: sofie.thijs@uhasselt.be; nele.weyens@uhasselt.be; panos.gkorezis@uhasselt.be; jaco.vangronsveld@uhasselt.be

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_9

Whilst being cheap and sustainable, there are few elements like the efficiency and predictability that has retarded its implementation into commercial-wide applications. Endophytic bacteria living inside plant tissues are fundamental to plant health and many of them can break-down contaminants taken-up by the host, thereby reducing phytotoxicity. Sequencing of genomic DNA of pure strains and endophytic microbial communities provides critical new opportunities for phytoremediation applications to restore PH contaminated soils. In this chapter, we discuss on a number of beneficial effects of plant-endophyte partnerships, and highlight how new insights from genomics and metagenomics can assist soil remediation to enhance plant growth and ecosystem services of reclamated soil.

1 Introduction

The industrialization of modern societies and the ever-increasing demand for energy generation for heat, our transportation networks, and electricity generation has resulted in the extensive exploitation of petroleum hydrocarbons, which are the most widespread class of organic contaminants worldwide (Kästner 2008). Underground leaking of storage tanks are still one of the major sources of toxic hydrocarbon spills. For example, about 435,000 underground tanks with petroleum hydrocarbons have leaked in the United States (Kansas State University, 2005) and nearly 1.5 million underground storage tanks have been closed since 1984, but a large number of sites are still in need of remediation. In Europe, 60% of soil contamination contains mineral oil and trace elements (Panagos et al. 2013). The negative impacts of PH pollution on human health and the environment are of concern for the scientific community, policy makers and the public (Tang et al. 2011). Prolonged exposure to PHs can result in respiratory system disfunction, central nervous system disruption, and increase in the probability of lung, skin, bladder, liver and kidney cancers (Aguilera et al. 2010; Rodríguez-Trigo et al. 2010). Often PH pollution is left untreated, however restored lands are increasingly valuable for the production of edible crops, biofuels and fibers. Generally, conventional physical and chemical in situ and ex situ clean–up technologies for petroleum hydrocarbon remediation such as excavation, air sparging, removal and off-site treatment in biopiles, slurry- and solid phase reactors amongst others (Kulik et al. 2006; Moldes et al. 2011) are environmental unfriendly and invasive, often only result in incomplete removal of the pollutants, and are frequently expensive. In the next decades it is estimated that an average 125,000 dollars will be spent to treat each PH contaminated site by conventional techniques and costs can be upwards to billions when groundwater is polluted (Panagos et al. 2013).

Consequently, a significant amount of research is now focusing on alternative technologies to supplement and/or replace traditional approaches (Gerhardt et al. 2009; Cook and Hesterberg 2013). While the synergistic action of plants and their related microorganisms to remove and degrade petroleum compounds is

considered to be advantageous in terms of cost, due to low capital expenditure, sustainability, and flexibility for in situ implementation, there are still numerous aspects about the mechanisms involved and the elements of uncertainty and efficiency that remain the subject of research and debate among members of the scientific community.

Endophytic bacteria living inside plant tissues have attracted major interest for increasing phytoremedation efficiency and predictability (Weyens et al. 2009a; Rylott 2014; Zhu et al. 2014; Ijaz et al. 2015). One of the main reasons is their lifestyle, endophytic bacteria dwelling the internal tissues of plants (roots, stems, leaves) overcome the competition for nutrients and space, and are physically protected from unfavorable environmental conditions which may enhance their survival and catabolic gene expression (Schulz and Boyle 2006). Moreover a number of reports have shown that endophytic bacteria have a better capacity to enhance PH phytoremediation than rhizosphere or soil bacteria (Barac et al. 2004; Andria et al. 2009; Weyens et al. 2010; Yousaf et al. 2011).

The purpose of this chapter is to give a detailed overview of the use endophytes in PH phytoremediation. We will focus on the ecology of endophytic bacteria, the mechanisms of colonisation, and diverse aspects of plant growth promotion (PGP) mechanisms. Furthermore, we will discuss their catabolic behaviour, and examples of endophyte-stimulated phytoremediation. Throughout the discussions we show how developments in genomics has been instrumental for understanding the plant and microbial catabolic mechanisms, directing management and monitoring of phytoremediation of petroleum hydrocarbon sites. Finally, we describe the future applications of endophytes and genomics, and the necessary steps to integrate the data into comprehensive management of PH contaminated sites.

2 Ecology of Endophytic Bacteria

For a long time endophytes were largely ignored or considered contaminants, now it is widely recognised that many endophytes are important plant symbionts without imposing visible sign of infection or negative effect on their host (De Bary 1866; Schulz and Boyle 2006; Reinhold-Hurek and Hurek 2011). After the first definition by De Bary (1866), endophytes were defined as “all organisms occurring within plant tissues,” though later, various researchers used different definitions for endophytes depending on the research context and need (Kogel et al. 2006; Rosenblueth and Martinez-Romero 2006; Schulz and Boyle 2006). Since endophytes can proliferate inside the plant tissue, they can interact very closely with their host, face less competition for nutrients, and are better protected from adverse changes in the environment when compared to bacteria in the rhizosphere and phyllosphere (Andria et al. 2009). These elements increased the attractiveness of using endophytes in many phytoremediation studies (Weyens et al. 2009a, b; Rylott 2014; Ijaz et al. 2015) with often great successes (Barac et al. 2004). Evidence of the occurrence of endophytes has come from culture-dependent and culture-independent analyses, next to fluorescence in situ hybridisation-confocal laser scanning microscopy

(FISH-CLSM). In the following paragraphs we give an overview of the diversity and ecology of endophytes as assessed by the culture-dependent and independent techniques.

Hyperaccumulating alpine pennycress (*Thlaspi caerulescens*) (Lodewyckx et al. 2002a), tall fescue (Malinowski et al. 2000), *Arabidopsis* seeds (Truyens et al. 2015a, b, 2016) and different grass species (Dalton et al. 2004; Thijs et al. 2014a) to woody tree species such as oak and ash (Weyens et al. 2009c), sycamore (Thijs et al. 2014b), poplar (Porteous Moore et al. 2006; Van der Lelie et al. 2009), *Mimosa pudica* (Pandey et al. 2005), pine seeds (Cankar et al. 2005), and other forest trees as reviewed (Pirttilä and Frank 2011). These reports illustrate that endophytic bacteria show a tremendously high diversity not only in plant hosts but also in bacterial taxa. Detailed information for host species with their associated endophytic bacterial diversity is available in earlier reviews (Lodewyckx et al. 2002b; Reinhold-Hurek and Hurek 2011; Hardoim et al. 2015). Most isolates belong to the Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes phyla of the domain bacteria (Bulgarelli et al. 2013). For example, the cultivable endophytic bacterial community associated with English oak was dominated by Actinobacteria (65.1%), with *Frigobacterium* spp. (45.0%) and *Okibacterium* spp. (13.0%) forming the majority (Weyens et al. 2009c). Proteobacteria represented 23.1% and were dominated by gamma-Proteobacteria (17.9%) including *Pseudomonas* spp. (9%), *Xanthomonas* spp. (4.6%), *Enterobacter* spp. (3.4%) and *Erwinia* (0.8%). The remaining part of the endophytes associated with English oak were Firmicutes (11.8%) with 8.8% Bacillaceae and 3.0% Paenibacillaceae (Weyens et al. 2009c). The possible application of these cultivable plant-associated bacteria for improving phytoremediation will be discussed below.

2.1 Critical Factors in the Isolation of Endophytic Bacteria

The isolation procedure is a critical and essential step when investigating endophytic bacterial communities. Commonly used procedures for isolation of endophytes combine surface sterilization of the plant part with subsequently either maceration of the plant tissue and plating onto nutrient agar, or plating of small surface sterilized plant parts onto nutrient agar (Eevers et al. 2015). In general, surface sterilization consists of: (1) thorough washing of the plant tissue, (2) surface sterilization, (3) several aseptic rinses and (4) a sterility check. Considering the high variety in procedures of isolation, comparison between different studies should be made carefully. In our laboratory, sterilization procedures were optimized for the isolation of endophytic bacteria from different parts of poplar (Porteous Moore et al. 2006; Beckers et al. 2016), willow (Weyens et al. 2012), yellow lupine (Weyens et al. 2014), alpine pennycress (*Thlaspi caerulescens*) (Lodewyckx et al. 2002a), rapeseed (Croes et al. 2013), tobacco (Mastretta et al. 2009) and *Arabidopsis* (Truyens et al. 2015a). In addition to the sterilization procedure, another very critical step in the isolation procedure is the choice of the growth medium; it directly affects the number and type of strains that can be isolated. Different growth media have been developed

and improved over time (Bacon and White 1994; Schulz et al. 2006; Govindasamy et al. 2014). The addition of plant extract to the medium can improve the culturability of endophytes and regrowth of stored endophytes (Eevers et al. 2015). In adition, substantial progress has been made in culturing new groups of organisms through co-culture, and in situ incubation using diffusion systems such as the iChip (Nichols et al. 2010), see for a review here (Stewart 2012).

However, even after optimization of these critical steps in the isolation procedure, cultivation-dependent techniques still strongly underestimate the bacterial numbers, as they do not record the majority of viable but non-cultivable bacteria. Cultivation-independent methods for exploring microbial communities in natural habitats have suggested that cultivable isolates represent less than 1–10% of the bacterial taxa present in a plant (Torsvik et al. 2002). Therefore, in order to obtain a more complete insight in the composition of plant associated microbial communities, cultivation-independent methods based on rRNA gene identification or total environmental DNA or RNA sequencing should be employed. It must be mentioned that we also observed many times that even after their initial isolation and growth in culture medium, many endophytic strains were difficult or could not be further propagated under laboratory conditions, supporting the importance of the analysis of their communities with molecular cultivation-independent techniques. Moreover, mining the yet-to-be-cultured majority provides access to an immense reservoir of unexploited microbial diversity by cultivation.

2.2 Endophytic Communities Identified by Culture-Independent Tools

Insights on the total microbial community level have been obtained using culture-independent 16S rRNA-based methods such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and 16S rRNA clone libraries, which provided a more detailed overview of the in situ communities than culture-based studies (Su et al. 2012). Since the advent of next-generation sequencing platforms, culture-independent molecular methods have greatly improved to provide high-resolution microbial community profiles (Sloan and Lebeis 2015). Using these molecular sequencing techniques such as pyrosequencing, Illumina and Ion Torrent, the first examples of a high-resolution analysis of endophytic root microbiomes were obtained of the model plant *Arabidopsis* (Bulgarelli et al. 2012; Lundberg et al. 2012; Bodenhausen et al. 2013; Schlaeppi et al. 2014), and also rice (Sessitsch et al. 2012), willow (Yergeau et al. 2014), poplar (Beckers et al. 2016), and *Acer pseudoplatanus* (Thijs et al. submitted). These studies showed that soil type was a main determinant of the root endophytic community composition by acting as a "seed bank" of microorganisms that can be recruited into the root-associated community. Despite major differences in soil and microbial community compositions, it has been shown that root endophytic microbiomes are composed of a core set of microbial taxa. Bulgarelli et al. 2013 suggested a two-selection step process to explain this. First, plant rhizodeposits

mediate a substrate-driven community shift in the rhizosphere, and second, the host-genotype innate immune system fine-tunes the microbial profile in the selection of root endophyte assemblages (Bulgarelli et al. 2013). Root-endophyte microbial communities generally display an order-of-magnitude reduction in species richness compared to rhizosphere and bulk soil communities and numbers progressively decrease towards the aerial parts (stem, leaves, flowers, seeds) (Bulgarelli et al. 2013; Afzal et al. 2014). Besides soil and air for leaf endophytes, other environmental factors may affect the composition of endophytic communities such as climate variables and interactions with other organisms (Sloan and Lebeis 2015). For leaf endophytic communities for example, the surrounding air and environmental growth conditions were considered important determinants next to host genotype (Kembel et al. 2014).

In most studies, a common trend is that the proportion of Proteobacteria and Actinobacteria are typically increased in the root-endosphere of plants compared to the bulk soil, while Acidobacteria are frequently underrepresented (Bulgarelli et al. 2012; Sessitsch et al. 2012; Beckers et al. 2016). Many Proteobacteria have been characterised for beneficial plant-growth promoting features, while Actinobacteria are famous for their antibiotic producing capacity. Therefore it is not unlikely that some intrinsice plant-microbe signalling can favour the prevalence of these groups. Even between plant species, significant overlap between the core set of microbial taxa have been reported, for example between sugarcane and *Arabidopsis* root endophytes, and common ivy phyllosphere and *Arabidopsis* phyllosphere (Stevens et al., submitted). This raised the interesting hypothesis that a number of bacterial families have had long association with plants (Schlaeppi et al. 2014). This warrants a larger survey of edophytic microbial communities from a wider range of plant species growing in different soil types and environments to gain novel insights in the ecology of microbial endophytes.

2.3 Colonization

As most endophytes derive from the rhizosphere, root-endophytic communities are the most diverse, and contain highest densities in the plant. Root colonization by rhizosphere bacteria occurs along the root axis and is a dynamic process with highest cell densities at root hairs and root tips (Fan et al. 2012). Root colonisation can be considered to involve four steps (Compant et al. 2010). The initial step consists of bacteria moving to the plant roots. This movement can either be passive via soil water fluxes, or active, via specific induction of flagellar activity by plant-released compounds (chemotaxis). In the second step, non-specific adsorption of bacteria to the roots is occurring with subsequent anchoring (third step), which represents the firm attachment of the bacterial cell to the root surface. Very often, bacteria form dense biofilms on the root surface and this happens more under stress conditions such as contamination and low nutrient levels (Ramey et al. 2004). Ultimately, a subset of the rhizosphere microbiota can enter the root tissues and establish as endophytes. Bulgarelli et al. (2013) listed different root exudate components that

are involved in these processes. Also border-like cell derived proteoglycans have been attributed to be involved in the attachment of bacterial cells, in particular *Rhizobium*, to root cells of non-leguminous plants (Santaella et al. 2008).

Since many facultative endophytic bacteria are also surviving in the rhizosphere, it is clear that there exists a close relationship between endophytes and bacteria colonizing the rhizosphere. The root is the primary site where endophytes gain entry into plants, with the exception of bacteria transmitted through the seeds, which are already present in the embryo at the time of germination (Porteous Moore et al. 2006; Truyens et al. 2015b). Bacterial entry into plants mainly occurs at locations of epidermal damage, that arise as a result of normal growth of the plant like formation of laterals, or through root hairs and at epidermal conjunctions (Compant et al. 2010). Furthermore, exudates leaking through these wounds are a source of nutrients for the colonizing bacteria and hence create favourable conditions. Several microscopic studies confirmed this route of colonization (Watt et al. 2006; Lagendijk et al. 2010; Fan et al. 2012). This was further supported by the analysis of the genome of *Enterobacter* sp. 638, a plant growth promoting endophyte from poplar (Taghavi et al. 2010) whose genome was sequenced by the JGI. This analysis revealed the presence of several gene clusters important for cell mobility including four operons for flagellar biosynthesis (*FlgNMABCDEFGHIJKL*, *flhEAB fimA yraIJ lpfD cheZYBR tap tar csuEDCAB int cheWA motBA flhCD, fliYZA fliCDSTEFGHJKLMNOPQR* and *fliEFHIJKLMNOPQR*). The *Enterobacter* sp. 638 genome further contained a number of genes associated with agglutination and cell adhesion, similar to those found in both animal and plant pathogens (Taghavi et al. 2009; Taghavi et al. 2010). Many of these genes were not found in *E. coli* K12, and are hypothezised to be important for plant colonization. Wounds and lateral root formation, however, are not absolutely required for the entrance of endophytic bacteria in their host plant. For instance, vector organisms (e.g., *Saccharicoccus sacchari*, arbuscular mycorrhizae, and insects) are possible candidates to assist potential endophytes to gain entrance to the apoplastic spaces and to colonize the host plant (Frey-Klett et al. 2011).

Several studies reported increased cellulase and pectinase activities during colonization of endophytes suggesting that active penetration is also an option (McCully 2001; Rosenblueth and Martinez-Romero 2006). Although *Enterobacter* sp. 638 was not able to grow on pectin (poly(1,4-D-galacturonate)) as a sole carbon source, its genome carries the genes involved in pectate degradation, a demethylated backbone of pectin (Taghavi et al. 2010). In addition, other regions on the genome of the same strain encode for carbohydrate uptake and metabolism. However, cellulose hydrolases were not found on the genome of *Enterobacter* sp. 638 (Taghavi et al. 2010).

Once inside the plant, endophytic bacteria either remain localized in a specific plant tissue like the root cortex or the xylem (Fig. 1), or continue colonizing the plant systematically by transport through the vascular system or the apoplast.

Except for particular cases such as *Azoarcus* spp. and *Rhizobium* in grasses, or *Alcaligenes faecalis* in rice, endophytic bacteria primarily colonize intercellularly (Hardoim et al. 2008). Highly specific adaptations are those of endosymbionts like

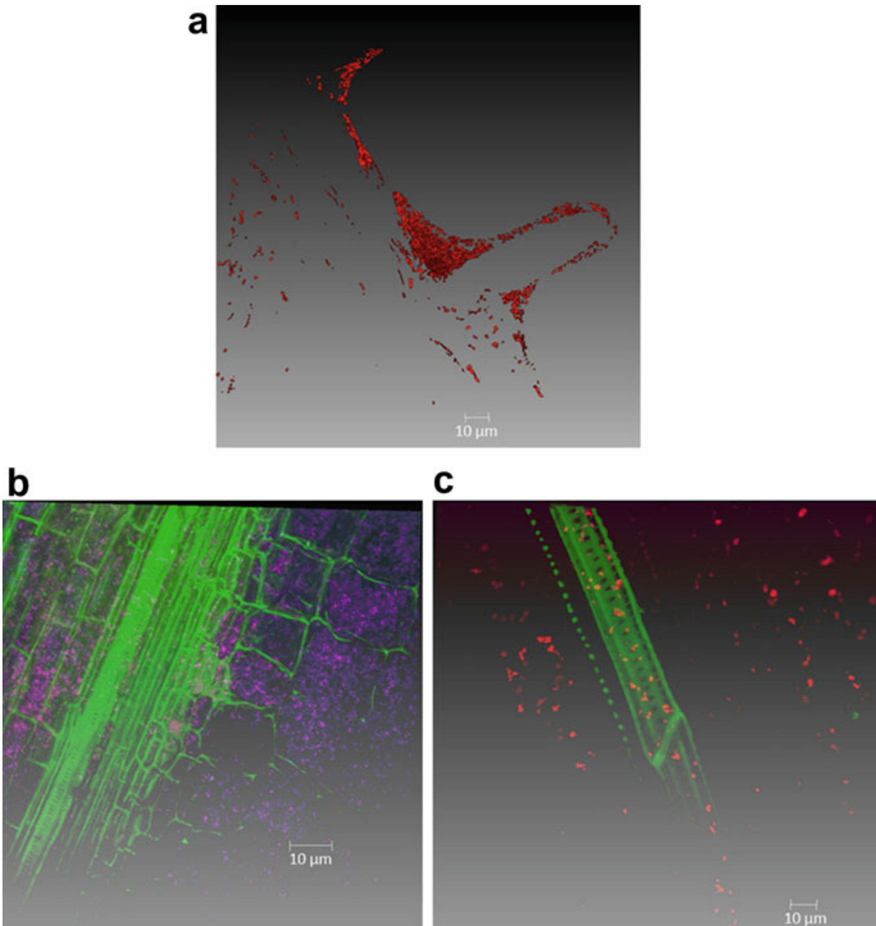


Fig. 1 *Methylobacterium* sp. CP3 mCherry-tagged micro-colonies on a root hair (**a**), intracellularly in root cortex cells (**b**), and in the xylem of *Crotalaria pumila* (**c**) as observed with confocal laser scanning microscopy (*CLSM*) (Sanchez-Lopez, Thijs et al. 2016, submitted)

rhizobia which live intracellularly in nodule cells of leguminous plants (Murray 2011). To better understand the modes and sites of entry of endophytic bacteria, *gfp* and *egfp* marked strains were inoculated in various plant species and their colonization was investigated by means of confocal microscopy (Bloemberg et al. 2000; Germaine et al. 2004; Gilbertson et al. 2007; Cardinale 2014).

Besides plant roots as entry for many endophytes, entry may also occur through the seeds and through natural holes in the phyllosphere such as leaf stomata and lenticels (pores for gas exchange in stems) (Kluepfel 1993), hydrathodes (water pores), and nectarthodes (opening in nectary glands of blossoms). A general model of leaf colonization by phyllobacteria consisting of 8 important steps was presented by Beattie and Lindow (1999): (1) bacterial immigration, (2) habitat modification,

(3) bacterial division, (4) microcolony formation, (5) formation of large aggregates, (6) entry into internal spaces, (7) habitat modification and bacterial division, and (8) egression onto the leaf surface (8). However, they acknowledged that these steps will vary in function of different bacterial species (Beattie and Lindow 1999). Recently, a bacterial bioreporter for fructose and sucrose used to quantify the availability of nutrients to individual cells in the phyllosphere, revealed that growth of *Erwinia herbicola* occurred at the expense of sugars but that there was a highly heterogenous availability of fructose to individual cells (Leveau and Lindow 2001).

3 Beneficial Effects of Plant-Microbe Partnerships

As reviewed by Lugtenberg et al. (2002), Ryu et al. (2005); Glick (2012), Bloemberg and Lugtenberg (2001), plant growth promotion has been intensively investigated for plant growth promoting rhizobacteria (PGPR). We already mentioned before that there exists a close relationship between rhizosphere and endophytic bacteria suggesting that, presumably, they all use similar mechanisms to benefit their host plant. Many mechanisms of plant growth promotion by plant-associated bacteria have been described, and were broadly grouped into two categories: direct and indirect (Spaepen et al. 2009). Many efforts have been made to elucidate both the direct and indirect mechanisms by which plant-associated bacteria improve plant growth (Lugtenberg and Kamilova 2009; Roca et al. 2013; Farrar et al. 2014). Mechanisms for direct plant growth promotion may involve nitrogen fixation especially diazotrophs, the supply of less available nutrients such as phosphorus and other essential nutrients (Lugtenberg and Kamilova 2009; Drogue et al. 2012), the production of plant growth regulators such as auxins, cytokinins and gibberellines (Somers et al. 2004; Gray and Smith 2005), and the inhibition of plant ethylene production due to bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Glick 2005). Indirect mechanisms for plant growth promotion are related mainly to the suppression of pathogenic and deleterious microorganisms through competition for space and nutrients, antibiosis, production of hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins, and induction of plant defence mechanisms (Compant et al. 2005; Couillerot et al. 2009; Zamioudis and Pieterse 2012). All these mechanisms are attributed more to plant health than direct growth promotion.

Recently, genomic insights of plant growth promoting endophytes (PGPE) has increased our understanding of the molecular mechanisms of plant growth promotion (Bruto et al. 2014). For example, based on a genome sequence analysis of 304 Proteobacteria, it was shown that the number of genes contributing to plant-beneficial functions increased along the continuum from animal pathogens, phytopathogens, saprophytes, endophytes/symbionts to PGPR, suggesting that the accumulation of these PGP-genes might be an intrinsic PGPR feature (Bruto et al. 2014). The best-studied examples of PGPR belong to diverse genera and include, amongst others, *Azospirillum, Bacillus, Burkholderia, Enterobacter, Klebsiella, Paenibacillus, Pseudomonas* and *Rhizobium*. A variety of PGPR and PGPE have

also been used as inoculants for the remediation of contaminants (Zhuang et al. 2007). In this respect, it is important to understand the mechanisms of plant growth promotion to predict how bacteria interact with plants and whether they can establish themselves in the plant environment after inoculation in the field.

In general, free living bacteria usually do not show one single mechanism of plant growth promotion, but they may involve a combination of the individual mechanisms. In addition, synergistic interactions between PGPR and other microbes such as mycorrhizal fungi have been reported (Artursson et al. 2006). Besides understanding the molecular mecchanisms of PGP of single individuals, it is important to also comprehend the environmental and physiological factors regulating the biosynthesis of growth promoting and antimicrobial compounds produced by plant-associated bacteria. This is an essential step towards improving the level and reliability of their growth promoting activity. Below, some important PGP mechanisms and individual contribution to plant growth promotion are discussed more in detail.

3.1 Direct Promotion of Plant Growth

3.1.1 Diazotrophy

Although 78% of the earth's atmosphere is nitrogen, this element often is a limiting factor for plant growth. This because of the fact that atmospheric nitrogen exists as dinitrogen (N_2), a form of the element that is inaccessible to all except a few specially adapted prokaryotic organisms including some eubacteria, cyanobacteria and actinomycetes. For all other organisms (also plants), nitrogen should occur under the form of ammonia or nitrate before it can be incorporated into organic molecules. Indeed, higher plants cannot carry out this process in the absence of associated diazotrophic bacteria. These bacteria are equipped with the enzyme nitrogenase, an O_2-sensitive enzyme catalyzing the reduction of atmospheric nitrogen to ammonia. Diazotrophic bacteria can be grouped into those that involve close associations with plants, often called symbioses and those that involve only loose associations, often called associative interactions (De Bruijn 2015).

Symbiotic diazotrophic bacteria show highly specific, intimate interactions leading to the induction of new organs or organ-like structures in the host. The intimacy of this type of association maximizes the transfer of fixed nitrogen to the host plant. The plant supplies the prokaryotic symbiont with energy-rich compounds, which is necessary to support the high energy demands of nitrogen fixation. The plant further provides the prokaryote a protected environment. Examples of symbiotic diazotrophs are (1) legume symbionts, which are Gram negative bacteria that form nodules on roots of leguminous plants, (2) members of the genus *Frankia*, which are Gram positive bacteria that form nodules on roots of woody, dicotyledonous trees and shrubs and (3) cyanobacteria that form mutualisms with many Pteridophytes. Originally, all legume symbionts were classified into the genus *Rhizobium*; currently these symbionts are classified into several genera with most species belonging to the genera *Rhizobium, Sinorhizobium, Mesorhizobium* and *Bradyrhizobium* (Dobbelaere et al. 2003).

Associative diazotrophic bacteria vary in how closely they interact with their host plant. It is tempting to speculate that increasing closeness in this association should correspond to an increasing capacity to transfer fixed nitrogen to the host plant (Ladha et al. 1983). It is reasonable to suppose that nitrogen fixed by free-living diazotrophs on plant surfaces is transferred less efficiently to the plant host and is subject to greater losses than when nitrogen is fixed by endophytic bacteria. Availability of energy-rich carbon compounds and the capability of the heterotrophic N_2-fixing bacteria to capture and use it efficiently are the key factors that determine the quantity of fixed nitrogen by associative nitrogen-fixing bacteria (De Bruijn 2015). Associative diazotrophic endophytes have been mainly isolated from grasses such as rice, sorghum, wheat, maize and sugarcane. The most profoundly investigated diazotrophic endophytes are *Azoarcus* spp. in Kallar grass, *Alcaligenes, Azospirillum, Bacillus, Enterobacter, Herbaspirillum, Klebsiella, Pseudomonas* and *Rhizobium* in rice and maize and *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. in sugarcane (Ladha et al. 1983; Bashan et al. 2004; Iniguez et al. 2004; Alves et al. 2014). Several results, such as from N-balance, ^{15}N isotope dilution and ^{15}N natural abundance, have supplied evidence that some plants can acquire at least part of their nitrogen from associative nitrogen fixation. This transfer of fixed nitrogen to the host plant can occur directly or indirectly due to the death and subsequent mineralization of the bacterial cells (De Bruijn 2015). Many reports proved a direct contribution of endophytic bacteria to plant nitrogen fixation, such as in rice, wheat, and sugarcane (Ladha et al. 2005). Certain Brazilian cultivars of sugarcane get over half their needs for nitrogen from biological nitrogen fixation (Ladha et al. 2005). It has been shown that up to 70% of the nitrogen in sugarcane grown in presence of diazotrophic bacteria can originate from biological nitrogen fixation (Ladha et al. 2005). In rice plants, inoculation with *Azospirillum* contributed to 66% of the total ammonium present in the plants. As poplar (*Populus* spp.) and willow (*Salix* spp.) are very appropriate tree species for phytoremediation purposes, Doty et al. (2009) investigated their associated endophytes and revealed that some of their endophytes appear to be fixing nitrogen (Doty et al. 2009).

3.1.2 Solubilization of Unavailable Nutrients

Second to nitrogen among the mineral nutrients that limit terrestrial plant growth, phosphorus is very important. It often is abundant in soil, but mostly occurs in an insoluble form. Even when soils are rich in phosphorous, most of the P is insoluble and only a very limited amount (≈0.1%) is available to plants (Rodrıguez and Fraga 1999). Phosphorous can only be taken up by plants when it is in its monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble form (Rodrıguez and Fraga 1999). In addition, 75% of the phosphate fertilizers applied to soils is rapidly immobilized and is unavailable to plants (Rodrıguez and Fraga 1999). Phosphate solubilizing and phosphate mineralizing bacteria that can improve plant growth commonly occur in the rhizosphere (Delvasto et al. 2009; Bianco and Defez 2010; Drogue et al. 2012) and include *Azotobacter chroococcum*, *Bacillus* spp., *Enterobacter agglomerans*, *Pseudomonas chlororaphis*, *Pseudomonas putida*, and *Rhizobium* and *Bradyrhizobium* spp.. Also endophytic bacteria have been reported to solubilize

immobilized mineral phosphate. It was suggested that during initial colonization, endophytic bacteria could enhance phosphate availability to soybean plants (Rodrıguez and Fraga 1999). This suggestion was further supported by Kuklinsky-Sobral et al. (2004), showing that 52% of the endophytic bacteria isolated from soybean were able to solubilize mineral phosphate (Kuklinsky-Sobral et al. 2004).

It is evident that phosphate mobilization will especially be important in the rhizosphere. Therefore it is not surprising that the rhizosphere strain *P. putida* KT2440, possesses three ABC-type phosphate transport operons compared to only one in the closely related endophytic strain *P. putida* W619 (Loper et al. 2012). Consistently, other endophytic bacteria like *Enterobacter* sp. 638 and *Serratia proteamaculans* 568 were found to possess only one ABC phosphate transport operon (Taghavi et al. 2009).

Iron in soils is, like phosphorous, often occurring in an insoluble form, more specifically the highly insoluble ferric hydroxide form. Many bacteria produce organic molecules, called siderophores, which bind Fe^{3+} and render it available for conversion to the preferred form, Fe^{2+}. Radzki et al. (2013) reviewed the plant-microbe interactions involved in the regulation of siderophore production and their role in mediating competition for iron in the rhizosphere. Bacterial Fe^{3+}-siderophore complexes may not only facilitate uptake of iron into bacteria. Evidence exists that several plant species can recognize and take up bacterial Fe^{3+}-siderophore complexes, and that, especially in calcareous soils, this process is crucial for the uptake of iron by plants (Kloepper et al. 1980a, b; Schwyn and Neilands 1987; Hider and Kong 2010; Radzki et al. 2013).

Bacteria developed several distinct mechanisms to compete against each other for iron resources, a concept applied by plant growth promoting bacteria in the protection of their host plant against pathogens. They can produce a high number of specific iron uptake transporters, secrete great numbers of diverse siderophores (which is energy costly), or synthesize siderophore receptors to utilize siderophores excreted by other bacteria (Crowley et al. 1988; Hider and Kong 2010).

During its adaptation to survive in the soil prior to colonization of a host plant, or as a plant growth promoting endophyte, *Enterobacter* sp. 638 has developed an intermediate solution to deal with iron uptake. It possesses two ferrous iron uptake systems (FeoAB, EfeUOB) and nine iron ABC transporters (Taghavi et al. 2010). This number is much higher than the four iron ABC transporters present in *E. coli* K12 or the three found in *P. putida* KT2440 (Nelson et al. 2002).

Similarly to *E.coli* K12, *Enterobacter* sp. 638 is also able to produce the siderophore enterobactin (EntD, EntF, EntC, EntE, EntB and EntA), to secrete it (EntS), recover the Fe-enterobactin complex making use of a ferric siderophore uptake system tonB-dependant (ExbDB), and to extract the iron using an enterobactin esterase (Fes) after internalization of the Fe-enterobactin complex. In addition, *Enterobacter* sp. 638 possesses 12 outer membrane ferric and ferric-related siderophore receptors (Taghavi et al. 2010). In contrast, *E. coli* only possesses 6 siderophore receptors, while *P. putida* KT2440 is equipped with 18 receptors (Nelson et al. 2002), which is consistent with the concept of a plant growth promoting rhizosphere bacterium that has to compete for the iron resources present in the environment.

3.1.3 Phytohormones and Plant Growth Promoting Compounds

Phytohormones produced by plant-associated bacteria often stimulate plant growth. However, this bacterial phytohormone production does not have a direct benefit for the bacteria itself and can be explained in an indirect way. The stimulation of plant growth that is induced will lead to more nutrients available to the plant-associated bacteria. Auxins, cytokinins and gibberellins can be considered as causal agents for improving plant growth and development (Pieterse et al. 2009; Blinkov et al. 2014; Spaepen 2015).

The most investigated phytohormone produced by plant-associated bacteria is the **auxin** indole-3-acetic acid (IAA) (Spaepen and Vanderleyden 2011). IAA produced by *Azospirillum brasilense*, *Aeromonas veronii*, *Agrobacterium* spp., *Alcaligenes piechaudii*, *Bradyrhizobium* spp., *Comamonas acidovorans*, *Enterobacter* spp., and *Rhizobium leguminosarum*, can contribute to plant growth promotion (López-Bucio et al. 2007). IAA production can improve root growth and root length; it also has been associated with proliferation and elongation of root hairs (Pitts et al. 1998; Remans et al. 2007; Nakayama et al. 2012). Depending on the bacterial strain, the production of IAA seems to follow different pathways. In plant beneficial bacteria, IAA is predominantly synthesized via indolepyruvic acid; however, in phytopathogenic bacteria, IAA is generally produced from tryptophan via the intermediate indoleacetamide (Spaepen and Vanderleyden 2011).

IAA-producing plant beneficial bacteria have been isolated from different plant species: lettuce (Barazani and Friedman 1999), wheat, banana, and cotton (Mohite 2013), rice (Mehnaz et al. 2001), sugarcane (Sajjad Mirza et al. 2001) and poplar (Taghavi et al. 2005; Taghavi et al. 2009).

Contrasting observations were made in different studies that were conducted to determine the specific role of IAA production in plant growth promotion. A mutant strain of *Pseudomonas putida* showing a fourfold increase in IAA production lost the capacity to induce root elongation in canola seedlings, despite the fact that its growth rate and production of 1-aminocyclopropane-1-carboxulate (ACC) deaminase and siderophores remained the same. A supra-optimal IAA concentration can be hypothesized. In other studies, positive effects of bacterial IAA production on plant growth and development have been reported. In *Brassica* spp., a positive correlation was found between auxin production by different PGPR strains and their ability to increase the grain yield and numbers of branches and pods per plant (Asghar et al. 2002). This observation was further supported by the positive relation between auxin production by PGPR and the increase in number of branches and oil content in *B. napus* inoculated with these PGPR (Asghar et al. 2004).

Concerning the role of hormones other than auxins, such as cytokinins and gibberellins, less detailed information is available. **Cytokinins** play a role in the stimulation of cell division, cell enlargement and tissue expansion in certain plant parts (Skoog and Armstrong 1970; Frébort et al. 2011). Cytokinin-producing PGPR have been isolated from rape and lettuce (Arkhipova et al. 2007), wheat (Kudoyarova et al. 2014), soybean (de Garcia Salamone et al. 2006), and pine (Bent et al. 2001). However, the extent to which the cytokinins produced by these

bacteria play a role in plant growth promotion is still not unraveled. **Gibberellins** (gibberellic acid) are known to be involved in the extension growth of plant tissue, in particular of the stem (Bottini et al. 2004). Although gibberellin production by plant-associated bacteria seems to be unusual, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* spp., *Bacillus pumilus* and *Bacillus licheniformis* spp. (Gutiérrez-Mañero et al. 2001) able to synthesize this phytohormone, were isolated and were shown to improve plant growth and yield.

Volatile compounds such as 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol are produced by rhizobacteria to enhance plant growth (Ryu et al. 2003b). The endophytic strains *Enterobacter* sp. 638 and *S. proteamaculans* 568 possess the genetic capability for the conversion of pyruvate to acetolactate (acetolactate synthase) (Taghavi et al. 2009; Taghavi et al. 2010). Acetolactate is further spontaneously converted either into diacetyl, or into acetoin (acetolactate decarboxylase). *Enterobacter* sp. 638 possesses an additional gene to convert diacetyl into acetoin (acetoin dehydrogenase), which can be released and converted into 2,3-butanediol (2,3-butanediol dehydrogenase) by the plant. Interestingly, the poplar genome contains the genes necessary for the conversion of acetoin into 2,3-butanediol, but not for the biosynthesis of acetoin itself. This could mean that poplar is relying on endophytic bacteria like *Enterobacter* sp. 638 for the production of the plant growth hormones acetoin and 2,3-butanediol (Taghavi et al. 2010).

3.1.4 Counteracting Stress-Induced Ethylene

Ethylene is a plant hormone that is known to be increased in plants in response to both abiotic and biotic stress conditions. Among its multiple effects on plant development (from seed germination, morphogenesis, flowering induction up to senescence), frequently investigated effects include the inbition of root elongation, lateral root growth and root hair formation (Gamalero and Glick 2012). Therefore, decreases in ethylene production should lead to an indirect promotion of root elongation. Bacteria can affect ethylene production via two main mechanisms (Mastretta et al. 2006): (1) some bacteria can balance ethylene production levels through auxin production; (2) however, the most commonly described mechanism to reduce ethylene production levels is bacterial ACC-deaminase activity. ACC-deaminase was observed in strains of *Alcaligenes* spp., *Bacillus pumilus*, *Enterobacter cloacae*, *Burkholderia cepacia*, *Pseudomonas putida, Pseudomonas* spp. and *Variovorax paradoxus* (Glick et al. 2007; Saleem et al. 2007; Rashid et al. 2012), but is not produced by the endophytic bacteria *Enterobacter* sp. 638, *S. proteamaculans* 568, *S. maltophilia* R551-3 and *P. putida* W619 (Taghavi et al. 2009). The working mechanism in plant roots is believed to be via cleavage of ACC, which is the immediate precursor of ethylene during ethylene biosynthesis, resulting in increased root growth (Glick 2014). Bacteria, originating from different soils and containing ACC deaminase activity, stimulated plant growth even in soils containing phytotoxic cadmium concentrations (Belimov et al. 2005).

3.2 Indirect Promotion of Plant Growth

3.2.1 Competition

Competition has been claimed as an important mechanism of biocontrol; both, pathogens and non-pathogenic plant-associated bacteria compete for similar niches and the same nutrients. However, experimental evidence for this claim still hardly exists.

Under iron-limiting conditions, many plant growth promoting bacteria, especially pseudomonads, produce high-affinity Fe^{3+} binding siderophores (Kloepper et al. 1980a; Miethke and Marahiel 2007). By binding available iron, these bacteria are depriving pathogenic bacteria and fungi of iron, which could limit their growth. Many authors have illustrated the importance of siderophores in the inhibition of both fungal and bacterial pathogens (Miethke and Marahiel 2007).

3.2.2 Antibiosis

Antibiosis is the production and release of compounds that kill or inhibit the growth of the target pathogen; it is the best-known mechanism by which microbes can control plant diseases (Raaijmakers and Mazzola 2012). The antibiotics generally produced by diverse antagonistic bacteria consist of ammonia, butyrolactones, 2,4-diacetyl phloroglucinol (DAPG), kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, and zwittermycin A (Kim et al. 1999; Raaijmakers et al. 2002; Raaijmakers and Mazzola 2012). Many of these antibiotics have a broad-spectrum activity; DAPG was reported to be the most effective and is the most extensively studied antibiotic (Raaijmakers and Mazzola 2012). Using mutation analysis, molecular genetic tools, and using purified antibiotic compounds, the role of individual antibiotic compounds in suppression of root pathogens has been clearly established. Structures and modes of action of many antimicrobial compounds have been extensively reviewed (Poole 2012; Raaijmakers and Mazzola 2012).

Also biosurfactants have been investigated as antimicrobial compounds (De Souza et al. 2003; Bais et al. 2004). As pathogens frequently form a biofilm on the root surface, it is interesting that some biosurfactants were reported to prevent biofilm formation and even degrade existing biofilms (Kuiper et al. 2002; Bais et al. 2004). For instance, *Pseudomonas fluorescens* produces cyclic lipopeptides surfactants such as viscosinamide and tensin which have antifungal activity against *Rhizoctonia solani* and *Pythium ultimum* (Haas and Keel 2003; Nielsen and Sorensen 2003).

3.2.3 Production of Hydrolytic Enzymes

Another potential mechanism for plant-associated bacteria to control fungal pathogens is cell wall lysis. It is well established in the biocontrol of fungal pathogens in the rhizosphere. Endophytic bacteria isolated from potato roots show high levels of hydrolytic enzymes such as cellulase, chitinase and glucanase (Krechel et al. 2002).

However, in the endophytic bacteria *Enterobacter* sp. 638, *S. proteamaculans* 568 and *P. putida* W619 no members of the cellulase/endoglucanase (GH5, GH9, GH44, GH48 and GH74), lichenase (GH16) and xylanase (GH10, GH11) families of glycosyl hydrolases (GH) were found (Taghavi et al. 2009). This observation is consistent with the non phytopathogenic behavior of these bacteria.

Among the hydrolytic enzymes, chitinases are of high importance since chitin is a main cell wall component in the majority of the phytopathogenic fungi (Hoster et al. 2005). The endophytic bacteria *S. proteamaculans* 568, *P. putida* W619 and *S. maltophilia* R551-3 are able to grow on chitin as a carbon source. Chitinases from these strains belong to the glycosyl hydrolase family 18 in *S. proteamaculans* 568 and *S. maltophilia* R551-3, and glycosyl hydrolase family 19 in *P. putida* W619 (Taghavi et al. 2009; Taghavi et al. 2010). Gluconases are another important group of hydrolytic enzymes since they degrade the β-1,3-glucans of the fungal cell walls. Fridlender et al., (1993) reported that the production of β-1,3-glucanases by *Burkholderia cepacia* (formarly *Pseudomonas cepacia*) caused an inhibition in rhizosphere proliferation of various phytopathogenic fungi including *Rhizoctonia solani*, *Serratia rolfsii* and *Phytium ultimum* (Fridlender et al. 1993). Furthermore, Tanaka and Watanabe (1995) observed that a synergistic action of a combination of chitinases and β-1,3-glucanases resulted in a more effective inhibition of fungal pathogens than by the individual enzymes (Tanaka and Watanabe 1995).

3.2.4 Inhibition of Pathogen-Produced Enzymes or Toxins

For the degradation of polymers in plant cell walls and so facilitate the fungal infection, pathogenic fungi produce extracellular hydrolytic enzymes including cellulases, pectolytic enzymes (exo- and endo- polygalacturonases, pectin lyases), and cutinase. A suppression of the activity of these enzymes correlates with a reduction in virulence (Beraha et al. 1983). For instance, Bertagnolli et al. (1996) reported that the activities of extracellular enzymes, such as cellulase, pectin lyase and pectinase produced by *Rhizoctonia solani*, were inhibited by *Bacillus megaterium* B 153-2-2 producing an extracellular endoproteinase (Bertagnolli et al. 1996).

3.2.5 Induction of Plant Defence Mechanisms

In a plant, contact with a necrotizing pathogen or a non-pathogenic biocontrol bacterium can induce a state of physiological immunity, protecting it against subsequent viral, bacterial or fungal attacks (Bakker et al. 2007). The induced resistance associated with the colonization of plant roots by certain plant growth promoting rhizobacteria (PGPR), has been referred as induced systemic resistance (ISR) (Conrath et al. 2002; Ryu et al 2003a; Domenech et al. 2007). ISR is characterized by remote action, long-lasting resistance, and protection against a large number of pathogens. Most of the resistance-inducing microbes reported hitherto are Gram-negative bacteria, with mainly *Pseudomonas* and *Serratia* strains (Bakker et al. 2007). However, also a number of Gram-positive bacteria were reported to induce resistance (Kloepper et al. 2004).

ISR can activate multiple potential defence mechanisms, including an increase in activity of peroxidases, chitinases, β-1,3-glucanases, and other pathogensis-related proteins, the formation of protective biopolymers, such as lignin, cellulose, and hydroyproline-rich glycoproteins, and accumulation of antimicrobial low-molecular-weight substances, such as phytoalexins (Conrath et al. 2002; Ryu et al. 2003b). In addition, a single inducing agent can control a wide variety of pathogens.

4 Catabolic Potential of Endophytic Bacteria

Not only soil and rhizospheric bacteria but also many endophytes are described to have the catabolic enzymes to degrade or transform petroleum hydrocarbons. Petroleum hydrocarbons are a complex mixture of linear, branched and cyclic alkanes and aromatic compounds that are not all as easy biodegradable. From high to low biodegradability they can be ranked as follows: n-alkanes $>$ branched-chain alkanes $>$ branched alkenes $>$ low-molecular-weight n-alkyl aromatics $>$ monoaromatics $>$ cyclic aromatics and polycyclic aromatic hydrocarbons (PAHs) (Chikere et al. 2011).

The most complete degradation occurs under aerobic conditions. Alkane-degradation by bacteria for example begins with an oxidative attack at the terminal methyl group with the formation of a fatty alcohol, aldehyde and fatty acid (Van Hamme et al. 2003; Kanaly and Harayama 2010). The carboxylic acid can then be combined with CoA that via acetyl-CoA can enter the tricarboxylic acid (TCA) cycle. Short-chain alkane monooxygenases, including methane monooxygenases (MMO), are the first enzymes involved in oxidising C1–C4 alkanes (Martin et al. 2014). Gaseous alkanes can be metabolized by strains expressing propane or butane monooxygenases that are related to methane monooxygenases, and these have been found for example in *Gordonia* sp. TY-5 (Kotani et al. 2003). Medium-chain length (C5–C16) alkanes may be oxidized by two main classes of enzymes: integral membrane non-heme iron alkane hydroxylases (alk system) and soluble cytochrome P450s. The most thoroughly characterized alkane degradation pathway is encoded by the OCT plasmid carried by *Pseudomonas putida* Gpo1 (van Beilen et al. 2001). In this system, the alkBFGHJKL operon encodes the enzymes necessary for converting alkanes into acetyl-coenzyme A (CoA), while alkST encode a rubredoxin reductase (AlkT) and the positive regulator for the alkBFGHJKL operon (AlkS). The genetic characterization in *P. putida* GPo1 of the alkane degradation pathway boosted the research on the field and since then more than 60 homologues of alkane hydroxylase gene (alkB) have been cloned and sequenced in both Gram-positive and Gram-negative bacteria such as *Acinetobacter* sp., *Mycobacterium* sp., *Rhodococcus* sp., *Pseudomonas putida* P1, *P. aureofaciens* and *P. fluorescens* (Palleroni et al. 2010; Wang et al. 2010a, b). Sequencing of the genomic DNA of pure bacterial cultures isolated from contaminated sites allows the detailed analyses of PH-degradation pathways. Currently, there are well-established and growing genomic databases for the genomes and pure cultures, such as the National Centre for Biotechnology Information (GenBank, RefSeq), the National Institute of Genetics

(DNA Data Bank of Japan), and the European Bioinformatics Institute (EMBL). The advantage is that these genomic sequences can now be obtained in a day, rather than years, and for a fraction of the cost. This has opened access to the so-called "uncultured majority." Advances in this field has already revealed a striking diversity in nucleotide and amino acid sequences of the AlkB gene (van Beilen et al. 2001; Wasmund et al. 2009; Wang et al. 2010a, b) and suggests horizontal gene transfer in many cases of duplicated gene clusters.

Also beyond genomics, the advances in environmental DNA shot-gun metagenomics and high-throughput metatranscriptomics, the in-depth characterisation of active degradative natural microbial communities, is possible (Bell et al. 2014a). Although data management is a great challenge, these technologies advance our understanding of the dominant roles that microorganisms play in the metabolism of contaminants in soil and *in planta*, and what they mean for the metabolism of plants. Plants are not longer viewed as "autonomous entities," but as a biomolecular network composed of the host and its associated microbiome "metaorganism" (Thijs et al. 2016). Exciting new discoveries of catabolic pathways are expected in the next decades. This information can be used in biotechnological applications and phytoremediation such as using bacteria to predict changes in biodegradation rates, microbe-based plant breeding for phytoremediation, and novel synthetic strains for improved in situ or ex situ degradation (Fig. 2), as also explained further.

5 Phytoremediation of Petroleum Hydrocarbons

Phytoremediation requires the selection of plants with increased pollutant tolerance, production of sufficient root and shoot biomass, suitability for various soil types, effective pollutant uptake mechanisms, appropriate metabolic capabilities to degrade organic pollutants, and association with active degradative microorganisms (Vangronsveld et al. 2009; Wenzel 2009; Fig. 2).

Initially, the response of plants to PHs present in soil includes uptake and translocation followed by accumulation or evapotranspiration. It is known that root uptake of PHs is strongly affected by PHs lipophilicity, a parameter which is expressed as the octanol-water partition coefficient (Kow) (Gerhardt et al. 2009). PHs with a log Kow < 1 are characterized by high water-solubility, and plant roots do not generally accumulate them at a rate surpassing passive efflux into the transpiration stream with subsequent translocation to the shoot, therefore, that practically means impassability to be taken up by the plant roots, whereas PHs with a log Kow > 3.5 cannot be taken up and translocated into the plant due to tight sorption onto the soil or root surfaces (Weyens et al. 2009b). After being transported inside the plant, PHs can be either sequestered in root tissue, or transported into shoots and then to leaves, where they can be stored in the vacuole or volatized into the atmosphere (Reichenauer and Germida 2008; Fig. 2).

Over the last years, evidence has accumulated that many plant species are suitable for PH remdiation such as Italian ryegrass (*Lolium perenne*), sorghum (*Sorghum bicolour*), maize (*Zea mays*), tall fescue (*Festuca arundinacea*), alfalfa (*Medicago

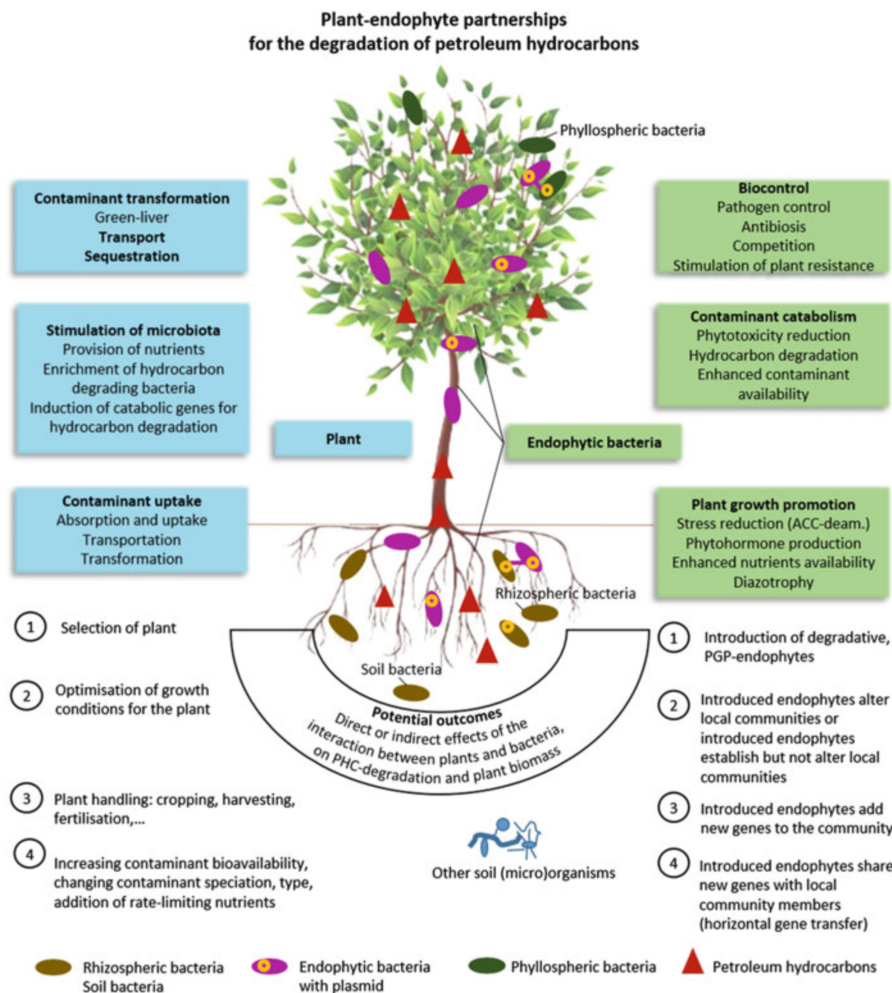


Fig. 2 Schematic overview of the plant-endophyte partnerships for petroleum hydrocarbon phytoremediation and plant growth. Plants can uptake, transform, transport and sequester the contaminants. In addition, plants assists endophytic bacteria by providing nutrients and space, enrichment of pollutant degrading bacteria and inducing catabolic gene expression for pollutant degradation. Plant-associated endophytic bacteria help the plant by decreasing toxicity of soil petroleum hydrocarbons through direct transformation and detoxification, increasing availability of nutrients (N, P, Fe), reducing phytotoxicity and evapotranspiration, producing plant growth promoting hormones, pathogen control, and enhancing the bioavailability of organic pollutants. Management options on the plant-level and endophytic community level are shown. The influence of these interventions on the plant-microbiome are diverse and can directly or indirectly effect plant growth and hydrocarbon degradation in soil

sativa var. Harpe), elephant grass (*Penninsetum purpureum*), bermuda grass (Cynodon dactylon), birdsfoot trefoil (*Lotus corniculatus* var. Leo), sunflower (*Helianthus annuus*), southern crabgrass (*Digitaria sanguinalis*), red clover (*Trifolium pretense*), beggar ticks (*Bidens cernua*), sedge species (*Cyperus rotundus*), leguminous plants, and willow (*Salix* spp.) (Kamath et al. 2004; Kaimi et al. 2007; Bell et al. 2014b; Yergeau et al. 2014). Although the plant may often metabolize or sequester environmental toxins, plants are at a significant disadvantage compared to microorganisms in two ways. Firstly, being photoautotrophic, plants do not rely on organic compounds as a source of energy or carbon. Secondly, plant metabolism of organic molecules (other than photosynthates) follows general transformations to more water-soluble forms, and sequestration processes to avoid build-up and potential toxicity to sensitive organelles (green-liver model) (Burken 2003), rather than mineralisation. Therefore, in order to develop a more efficient degradation of organic xenobiotics, plants depend significantly on their associated microorganisms.

5.1 Plant-Bacteria Partnerships

It is clear that plants have a positive effect on the microbial degradation of organic contaminants (Weyens et al. 2009a, b; Fig. 2). This positive effect can be explained by a higher microbial density and metabolic activity in the rhizosphere as a result of bacterial growth on carbon substrates (root exudates and death cells) provided by the plant roots (Segura et al. 2009). Moreover, diverse species of heterotrophic microorganisms are living together at high population densities in the rhizosphere, the phyllosphere and inside the plant (endophytes), which increases the possibilities for stepwise transformation of xenobiotics by consortia, or provides habitats that are conducive to genetic exchange and gene rearrangement (Fig. 2). Additionally, the plant's water evapotranspiration influences the transport of water soluble compounds by increasing their mass flow to the root surface where they can be acted upon by the rhizosphere microflora. After being taken up by the plant, endophytes can continue the degradation of organic xenobiotics (Weyens et al. 2009a, b). The importance of these plant-microbe partnerships in the remediation of organic pollutants was confirmed in studies at the level of the rhizosphere (Van Aken et al. 2010; Khan et al. 2013; Segura and Ramos 2013; Arslan et al. 2015), the phyllosphere (Scheublin et al. 2014) and inside the plant (Siciliano et al. 2001; Barac et al. 2004; Syranidou et al. 2016; Fig. 2).

5.2 Plant-Endophyte Interactions for Petroleum Hydrocarbon Degradation

In a pioneering study, it was shown that the enrichment of bacteria with the appropriate catabolic genes in the endophytic root compartment was correlated with the type and amount of contaminant and also on the genotype of the plant (Siciliano et al. 2001). Since then, a number of reports have confirmed that

endophytic bacteria, rather than rhizosphere or soil bacteria have a better capacity to enhance PH phytoremediation (Newman and Reynolds 2005; Andria et al. 2009; Weyens et al. 2009a; Afzal et al. 2014; Fig. 2).

Bacteria dwelling the internal tissues of plants (roots, stems, leaves) overcome the competition for nutrients and space, and are physically protected from unfavorable environmental conditions (Schulz and Boyle 2006). Some soil bacteria can penetrate into roots and move into shoots, indicating that these soil bacteria are a source for endophytic bacteria (Germaine et al. 2004; Germaine et al. 2009). In plant-endophyte associations, the plant has to confront the toxic nature of the hydrocarbons; therefore, an endophyte with the ability to mitigate the toxicity of the pollutant seems of utmost importance. Indeed some endophytic bacteria have the potential at first to tolerate and then mineralize hydrocarbons (Andria et al. 2009; Germaine et al. 2009; Yousaf et al. 2010; Gkorezis et al. 2015). Hence, this hydrocarbon degrading capacity of endophytic bacteria has been investigated aiming to enhance the remediation potential of trees, herbaceous plants and grasses (Afzal et al. 2014). In addition, other studies have demonstrated that endophytic bacteria with appropriate degradation pathways are metabolically active in the root and shoot of plants vegetated in diesel contaminated soils (Andria et al. 2009; Khan et al. 2013).

5.3 Potential of Meta-Omics Approaches to Elucidate Microbial Functions

Given the rich literature on PH-microbe interactions (Khan et al. 2013), there is also a tremendous scope for further integrating genomics and meta-omics to endophyte-stimulated site remediation (Fig. 2). Using metagenomics coupled to stable isotope probing (SIP), considerable progress has already been made to understand the mode of action by which soil microorganisms degrade organic contaminants, activate enzymes and whole metabolic pathways, interact with each-other and their environment, and for unraveling novel regulatory pathways.

Microbial communities have been characterized from hydrocarbon contaminated soils in the Arctic (Thomassin-Lacroix et al. 2002; Bell et al. 2011; Yergeau et al. 2012; Bell et al. 2013a, b), deepwater oil spills (Mason et al. 2014), in crude oil wells in Poland, and underground tank storage leakage sites (Gkorezis et al. 2015). Using carbon isotope (U-^{13}C) labeled naphthalene, phenanthrene, pyrene, fluoranthene and benz[a] anthracene added to polycyclic aromatic hydrocarbon (PAH) contaminated soil, diverse genera were discovered of which some were previously associated with the degradation of those compounds (*Pseudomonas, Burkholderia*) (Jones et al. 2011), but also newly associated sequences related to Pigmentiphaga and a group of yet-to-be cultured γ-Proteobacteria, identified as “Pyrene group 2” which attacked pyrene and benz[a] anthracene. A similar study using ^{13}C-labeled metagenomes recovered from samples in a long-term biphenyl- and polyaromatic hydrocarbon contaminated soil, showed a dominance of mainly Proteobacteria including *Burkholderia*, *Pandoraea*, *Dyella* and *Pseudomonas* and some yet-to-be cultured taxa. Some of these strains derived carbon

from naphthalene as well as biphenyl/benzoate, pointing out broader biodegradation abilities of some soil microbiota than previously known (Uhlik et al. 2012).

Metatranscriptomics studies have revealed complex responses to hydrocarbon pollution, providing evidence how bacteria and fungi catabolically adapt to changes in the chemical environment (Bell et al. 2011, 2014b; Yergeau et al. 2014). In addition, in some cases it appeared that the natural established plant-microbiome associations were not the most productive in terms of biodegradation efficiency (Bell et al. 2013b). When researchers added antibiotics (gentamicin and vancomycin) to a hydrocarbon-contaminated Arctic soil to inhibit distinct subgroups of the microbial community, the hydrocarbon degradation rates increased compared to the no-antibiotic control, while bacterial and fungal abundance were reduced (Bell et al. 2013b). These findings let the authors suggest that a large part of the microbial population is not actively involved in hydrocarbon degradation and rather compete with hydrocarbon degradative microorganisms for nutrients and space. The effect was again different when nutrients were added (Bell et al. 2013b). In this case, nutrient addition promoted a larger fungal population accounting for higher degradation rates (Bell et al. 2014b). Looking in the near future, data on gene, transcript, protein, metabolome, and interactome of plant-microbiota must be combined into holistic models in order to better appreciate the holobiont that is essential for phytoremediation (Fig. 2).

5.4 Critical Factors, Success Factors, and Future Outlook

Although phytoremediation of PHs and organics in general is very promising, it cannot be ignored that there are still some obstacles that need to be overcome, such as (1) the levels of pollutants tolerated by the plant, (2) the bioavailable fraction of the contaminants that often is limited and, (3) in some cases, the evapotranspiration of volatile organic pollutants to the atmosphere. A possible solution to overcome these limitations is the construction of plants specifically tailored for phytoremediation purposes by genetic manipulation (Maestri and Marmiroli 2011). However, since bacteria are much easier to manipulate than plants and natural gene transfer is possible (avoiding the limitations of the use of GMO), many studies focussed on the use of engineered plant-associated bacteria. Dzantor 2007 reviewed the state of the art of rhizosphere “engineering” for accelerated rhizodegradation of xenobiotic contaminants (Dzantor 2007). Even in case of an efficient rhizodegradation, compounds with a lipophilicity in the optimum range seem to enter the xylem before the soil and rhizosphere microflora can degrade them. Since the retention time of contaminants in the xylem ranges from several hours up to 2 days (Barac et al. 2004), (engineered) degrader endophytes colonizing the xylem are ideal candidates to prevent evapotranspiration of pollutants or intermediates through the leaves into the environment and to reduce phytotoxicity. Endophytic bacteria can be isolated, equipped with interesting characteristics and subsequently re-inoculated in the host plant (Fig. 2). Proof of this concept was provided by inoculating lupine plants (Barac et al. 2004) and poplar (Taghavi et al. 2005) with endophytic bacteria able to degrade toluene, which resulted in both, decreased toluene phytotoxicity and

a significant reduction of toluene evapotranspiration. As many catabolic pathways for hydrocarbons are found in soil bacteria where they are encoded on self-transferable plasmids or transposons, natural gene transfer offers great potential for the construction of endophytic strains with new catabolic functions. Moreover, heterologous expression of these catabolic functions might not be a major problem, especially when the donor strain and the recipient endophytic strain are closely related, as frequently is the case.

Although it is obvious that the application of engineered endophytic bacteria to improve phytoremediation of organic contaminants has several advantages, there are still some other factors that need optimisation (Newman and Reynolds 2005). Since phytoremediation projects can conceivably last decades, a major point of concern is the persistence and the stability of the engineered organisms and their degradation capabilities in association with plants growing in the field. As long as there is a selection pressure present, those community members possessing the appropriate degradation characteristics will have an advantage. Nevertheless, this is no guarantee that inoculated strains will become an integrated part of the natural endophytic community. However, instead of integrating a new strain in a stable community, the original community can also get adapted through horizontal gene transfer. Horizontal gene transfer has been shown to play an important role in the adaptation of a microbial community to a new environmental stress factor, including rhizosphere communities (van Elsas et al. 2003), and endophytic communities (Taghavi et al. 2005). Alltogether, the exploitation of endophytic bacteria, horizontal gene transfer, and environmental genomics should all be incorporated into processes at petroleum hydrocarbon contaminated sites to better predict and monitor on site processes.

6 Conclusions

Hydrocarbon contamination in soil and (ground)water is one of the major challenges we are facing today. The use of plants and microbes can drastically reduce the impact that contaminated sites have on the environment by improving the degradation at a low cost and sustainable manner. We have discussed that plants associate in diverse and intricate partnerships with microorganisms. Plants create specific favourable niches for their associated bacteria by providing them nutrients, and the plant-associated (endophytic) bacteria can improve growth and development of their host plant directly or indirectly. During phytoremediation of contaminated soils and (ground)water, endophytic bacteria possessing the appropriate degradative enzymes may reduce PH phytotoxcicity and enhance PH degradation thereby reducing the chance of evapotranspiration of volatile contaminants and/or degradation intermediates to the atmosphere. The integration of environmental genomics into plant-microbe sitmulated phytoremediaton may further improve the reliability and efficiency of the technique. Phytoremediation will require the use of molecular barcoding and sequencing for rapidly quantifying microbial communities to predict changes in degradation rates and future predictions. Besides using natural occuring endophytes, successful examples in

the past have shown the potential of using engineered endophytes, which have a high degradation capacity and ability to transfer their degradative genes to te indigenous microbial community by natural gene transfer. Because the new sequencing technologies are still in development, some risks to the environment may still exist related to the element of uncertainty and long-term performance. Nevertheless, great avenues and discoveries are anticipated in this exciting field in the years to come.

7 Research Needs

To address these challenges, cheaper and more sustainable solutions are needed to remove harmful pollutants from the environment. In this light, phytoremediation, the use of plants and associated microorganisms to degrade aromatic hydrocarbons, has gained increased interest by providing a green and economically beneficial solution.

References

Afzal M, Khan QM, Sessitsch A (2014) Endophytic bacteria: prospects and applications for the phytoremediation of organic pollutants. Chemosphere 117:232–242

Aguilera F, Méndez J, Pásaro E, Laffon B (2010) Review on the effects of exposure to spilled oils on human health. J Appl Toxicol 30:291–301

Alves GC, Videira SS, Urquiaga S, Reis VM (2014) Differential plant growth promotion and nitrogen fixation in two genotypes of maize by several Herbaspirillum inoculants. Plant Soil 387:307–321

Andria V, Reichenauer TG, Sessitsch A (2009) Expression of alkane monooxygenase (alkB) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* L.) grown in diesel contaminated soil. Environ Pollut 157:3347–3350

Arkhipova TN, Prinsen E, Veselov SU, Martinenko EV, Melentiev AI, Kudoyarova GR (2007) Cytokinin producing bacteria enhance plant growth in drying soil. Plant Soil 292:305–315

Arslan M, Imran A, Khan QM, Afzal M (2015) Plant-bacteria partnerships for the remediation of persistent organic pollutants. Environ Sci Pollut Res Int. https://doi.org/10.1007/s11356-015-4935-3

Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8:1–10

Asghar H, Zahir Z, Arshad M, Khaliq A (2002) Relationship between in vitro production of auxins by rhizobacteria and their growth-promoting activities in *Brassica juncea* L. Biol Fertil Soils 35:231–237

Asghar H, Zahir Z, Arshad M (2004) Screening rhizobacteria for improving the growth, yield, and oil content of canola (*Brassica napus* L.). Crop Pasture Sci 55:187–194

Bacon CW, White JF (1994) Stains, media and procedures for analyzing endophytes. In: Biotechnology of endophytic fungi of grasses. CRC PRess, Boca Raton, pp 47–56

Bais HP, Fall R, Vivanco JM (2004) Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Plant Physiol 134:307–319

Bakker PA, Pieterse CM, van Loon LC (2007) Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97:239–243

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, van der Lelie D (2004) Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nat Biotechnol 22:583–588

Barazani O, Friedman J (1999) Is IAA the major root growth factor secreted from plant-growth-mediating bacteria? J Chem Ecol 25:2397–2406

Bashan Y, Holguin G, de-Bashan LE (2004) Azospirillum-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). Can J Microbiol 50: 521–577

Beattie GA, Lindow SE (1999) Bacterial colonization of leaves: a spectrum of strategies. Phytopathology 89:353–359

Beckers B, Op De Beeck M, Weyens N, Van Acker R, Van Montagu M, Boerjan W, Vangronsveld J (2016) Lignin engineering in field-grown poplar trees affects the endosphere bacterial microbiome. Proc Natl Acad Sci U S A 113:2312–2317

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea L. Czern*.). Soil Biol Biochem 37:241–250

Bell TH, Yergeau E, Martineau C, Juck D, Whyte LG, Greer CW (2011) Identification of nitrogen-incorporating bacteria in petroleum-contaminated arctic soils by using [15N]DNA-based stable isotope probing and pyrosequencing. Appl Environ Microbiol 77:4163–4171

Bell TH, Yergeau E, F Juck D, G Whyte L, W Greer C (2013a) Alteration of microbial community structure affects diesel biodegradation in an Arctic soil. FEMS Microbiol Ecol 85:51–61

Bell TH, Yergeau E, Maynard C, Juck D, Whyte LG, Greer CW (2013b) Predictable bacterial composition and hydrocarbon degradation in Arctic soils following diesel and nutrient disturbance. ISME J 7:1200–1210

Bell TH, Joly S, Pitre FE, Yergeau E (2014a) Increasing phytoremediation efficiency and reliability using novel omics approaches. Trends Biotechnol 32:271–280

Bell TH, El-Din Hassan S, Lauron-Moreau A, Al-Otaibi F, Hijri M, Yergeau E, St-Arnaud M (2014b) Linkage between bacterial and fungal rhizosphere communities in hydrocarbon-contaminated soils is related to plant phylogeny. ISME J 8:331–343

Bent E, Tuzun S, Chanway CP, Enebak S (2001) Alterations in plant growth and in root hormone levels of lodgepole pines inoculated with rhizobacteria. Can J Microbiol 47:793–800

Beraha L, Wisniewski V, Garber E (1983) Avirulence and reduced extracellular enzyme activity in *Geotrichum candidum*. Bot Gaz 144:461–465

Bertagnolli B, Dal Soglio F, Sinclair J (1996) Extracellular enzyme profiles of the fungal pathogen *Rhizoctonia solani* isolate 2B-12 and of two antagonists, *Bacillus megaterium* strain B153-2-2 and *Trichoderma harzianum* isolate Th008. I. Possible correlations with inhibition of growth and biocontrol. Physiol Mol Plant Pathol 48:145–160

Bianco C, Defez R (2010) Improvement of phosphate solubilization and *Medicago* plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. Appl Environ Microbiol 76:4626–4632

Blinkov EA, Tsavkelova EA, Selitskaya OV (2014) Auxin production by the *Klebsiella planticola* strain TSKhA-91 and its effect on development of cucumber (*Cucumis sativus* L.) seeds. Microbiology 83:531–538

Bloemberg GV, Lugtenberg BJ (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4:343–350

Bloemberg GV, Wijfjes AH, Lamers GE, Stuurman N, Lugtenberg BJ (2000) Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. Mol Plant Microbe Interact 13:1170–1176

Bodenhausen N, Horton MW, Bergelson J (2013) Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PLoS One 8:e56329

Bottini R, Cassán F, Piccoli P (2004) Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Appl Microbiol Biotechnol 65:497–503

Bruto M, Prigent-Combaret C, Muller D, Moenne-Loccoz Y (2014) Analysis of genes contributing to plant-beneficial functions in plant growth-promoting Rhizobacteria and related Proteobacteria. Sci Rep 4:6261

Bulgarelli D, Rott M, Schlaeppi K et al (2012) Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488:91–95

Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807–838

Burken JG (2003) Uptake and metabolism of organic compounds:green liver concept. In: McCutcheon SC, Schnoor JL (eds) Phytoremediation: transformation and control of contaminants. Wiley, New Jersey, pp 59–83

Cankar K, Kraigher H, Ravnikar M, Rupnik M (2005) Bacterial endophytes from seeds of Norway spruce (*Picea abies* L. Karst). FEMS Microbiol Lett 244:341–345

Cardinale M (2014) Scanning a microhabitat: plant-microbe interactions revealed by confocal laser microscopy. Front Microbiol 5:94

Chikere CB, Okpokwasili GC, Chikere BO (2011) Monitoring of microbial hydrocarbon remediation in the soil. 3 Biotech 1:117–138

Compant S, Duffy B, Nowak J, Clement C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959

Compant S, Clément C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42:669–678

Conrath U, Pieterse CM, Mauch-Mani B (2002) Priming in plant-pathogen interactions. Trends Plant Sci 7:210–216

Cook RL, Hesterberg D (2013) Comparison of trees and grasses for rhizoremediation of petroleum hydrocarbons. Int J Phytoremediation 15:844–860

Couillerot O, Prigent-Combaret C, Caballero-Mellado J, Moenne-Loccoz Y (2009) *Pseudomonas fluorescens* and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens. Lett Appl Microbiol 48:505–512

Croes S, Weyens N, Janssen J, Vercampt H, Colpaert JV, Carleer R, Vangronsveld J (2013) Bacterial communities associated with *Brassica napus* L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microb Biotechnol 6:371–384

Crowley DE, Reid CP, Szaniszlo PJ (1988) Utilization of microbial siderophores in iron acquisition by oat. Plant Physiol 87:680–685

Dalton DA, Kramer S, Azios N, Fusaro S, Cahill E, Kennedy C (2004) Endophytic nitrogen fixation in dune grasses (*Ammophila arenaria* and *Elymus mollis*) from Oregon. FEMS Microbiol Ecol 49:469–479

De Bary A (1866) Morphologie und Physiologie Pilze, Flechten, und myxomyceten. W. Engelmann, Leipzig

De Bruijn F (2015) Biological nitrogen fixation. In: Lugtenberg B (ed) Principles of plant-microbe interactions Microbes for sustainable agriculture. Springer International Publishing, Switzerland, pp 215–225

de Garcia Salamone IE, Hynes RK, Nelson LM (2006) Role of cytokinins in plant growth promotion by rhizosphere bacteria. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Dordrecht, pp 173–195

De Souza JT, De Boer M, De Waard P, Van Beek TA, Raaijmakers JM (2003) Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. Appl Environ Microbiol 69:7161–7172

Delvasto P, Ballester A, Muñoz J, González F, Blázquez M, Igual J, Valverde A, García-Balboa C (2009) Mobilization of phosphorus from iron ore by the bacterium *Burkholderia caribensis* FeGL03. Miner Eng 22:1–9

Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit Rev Plant Sci 22:107–149

Domenech J, Ramos S, Probanza A, Lucas G, Gutierrez M (2007) Elicitation of systemic resistance and growth promotion of *Arabidopsis thaliana* by PGPRs from *Nicotiana glauca:* a study of the putative induction pathway. Plant Soil 290:43–50

Doty SL, Oakley B, Xin G, Kang JW, Singleton G, Khan Z, Vajzovic A, Staley JT (2009) Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47:23–33
Drogue B, Doré H, Borland S, Wisniewski-Dyé F, Prigent-Combaret C (2012) Which specificity in cooperation between phytostimulating rhizobacteria and plants? Res Microbiol 163:500–510
Dzantor EK (2007) Phytoremediation: the state of rhizosphere 'engineering' for accelerated rhizodegradation of xenobiotic contaminants. J Chem Tech Biot 82:228–232
Eevers N, Gielen M, Sánchez-López A, Jaspers S, White J, Vangronsveld J, Weyens N (2015) Optimization of isolation and cultivation of bacterial endophytes through addition of plant extract to nutrient media. J Microbial Biotechnol 8:707–715
Fan B, Borriss R, Bleiss W, Wu X (2012) Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. J Microbiol 50:38–44
Farrar K, Bryant D, Cope-Selby N (2014) Understanding and engineering beneficial plant-microbe interactions: plant growth promotion in energy crops. Plant Biotechnol J 12:1193–1206
Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P (2011) Evolution of cytokinin biosynthesis and degradation. J Exp Bot 62:2431–2452
Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A (2011) Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. Microbiol Mol Biol Rev 75:583–609
Fridlender M, Inbar J, Chet I (1993) Biological control of soilborne plant pathogens by a β-1, 3 glucanase-producing *Pseudomonas cepacia*. Soil Biol Biochem 25:1211–1221
Gamalero E, Glick BR (2012) Ethylene and abiotic stress tolerance in plants. In: Ahmad P, Prasad MNV (eds) Environmental adaptations and stress tolerance of plants in the Era of climate change. Springer, New York, pp 395–412
Gerhardt KE, Huang X-D, Glick BR, Greenberg BM (2009) Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges. Plant Sci: Int J Exp Plant Biol 176:20–30
Germaine K, Keogh E, Garcia-Cabellos G et al (2004) Colonisation of poplar trees by gfp expressing bacterial endophytes. FEMS Microbiol Ecol 48:109–118
Germaine KJ, Keogh E, Ryan D, Dowling DN (2009) Bacterial endophyte-mediated naphthalene phytoprotection and phytoremediation. FEMS Microbiol Lett 296:226–234
Gilbertson AW, Fitch MW, Burken JG, Wood TK (2007) Transport and survival of GFP-tagged root-colonizing microbes: implications for rhizodegradation. Eur J Soil Biol 43:224–232
Gkorezis P, Rineau F, Van Hamme J, Franzetti A, Daghio M, Thijs S, Weyens N, Vangronsveld J (2015) Draft genome sequence of acinetobacter oleivorans PF1, a diesel-degrading and plant-growth-promoting endophytic strain isolated from poplar trees growing on a diesel-contaminated plume. Genome Announc 3:e01430
Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol Lett 251:1–7
Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica (Cairo) 2012:963401
Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169:30–39
Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B (2007) Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26:227–242
Govindasamy V, Franco CMM, Gupta VVSR (2014) Endophytic actinobacteria: diversity and ecology. In Advances in endophytic research (pp. 27–59). Springer India
Gray E, Smith D (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol Biochem 37:395–412
Gutiérrez-Mañero FJ, Ramos-Solano B, Probanza A, Mehouachi JR, Tadeo F, Talon M (2001) The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiol Plant 111:206–211
Haas D, Keel C (2003) Regulation of antibiotic production in root-colonising *Pseudomonas* spp. and relevance for biological control of plant disease. Annu Rev Phytopathol 41:117–153

Hardoim PR, van Overbeek LS, Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol 16:463–471

Hardoim PR, van Overbeek LS, Berg G, Pirttila AM, Compant S, Campisano A, Doring M, Sessitsch A (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79:293–320

Hider RC, Kong X (2010) Chemistry and biology of siderophores. Nat Prod Rep 27:637–657

Hoster F, Schmitz JE, Daniel R (2005) Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. Appl Microbiol Biotechnol 66:434–442

Ijaz A, Imran A, Anwar ul Haq M, Khan QM, Afzal M (2015) Phytoremediation: recent advances in plant-endophytic synergistic interactions. Plant Soil 405:179–195

Iniguez AL, Dong Y, Triplett EW (2004) Nitrogen fixation in wheat provided by *Klebsiella pneumoniae* 342. Mol Plant Microbe Interact 17:1078–1085

Jones MD, Crandell DW, Singleton DR, Aitken MD (2011) Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil. Environ Microbiol 13:2623–2632

Kaimi E, Mukaidani T, Tamaki M (2007) Screening of twelve plant species for phytoremediation of petroleum hydrocarbon-contaminated soil. Plant Prot Sci 10:211–218

Kamath R, Rentz J, Schnoor J, Alvarez P (2004) Phytoremediation of hydrocarbon-contaminated soils: principles and applications. Stud Surf Sci Catal 151:447–478

Kanaly R, Harayama S (2010) Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria. J Microbial Biotechnol 3:136–164

Kästner M (2008) Degradation of aromatic and polyaromatic compounds. In: Biotechnology set. Wiley-VCH Verlag GmbH, Weinheim, pp 211–239

Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, Wright SJ, Green JL (2014) Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. Proc Natl Acad Sci U S A 111:13715–13720

Khan S, Afzal M, Iqbal S, Khan QM (2013) Plant-bacteria partnerships for the remediation of hydrocarbon contaminated soils. Chemosphere 90:1317–1332

Kim BS, Moon SS, Hwang BK (1999) Isolation, identification, and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. Can J Bot 77:850–858

Kloepper JW, Leong J, Teintze M, Schroth MN (1980a) Pseudomonas siderophores: a mechanism explaining disease-suppressive soils. Curr Microbiol 4:317–320

Kloepper JW, Leong J, Teintze M, Schroth MN (1980b) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885–886

Kloepper JW, Ryu C-M, Zhang S (2004) Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259–1266

Kluepfel DA (1993) The behavior and tracking of bacteria in the rhizosphere. Annu Rev Phytopathol 31:441–472

Kogel KH, Franken P, Huckelhoven R (2006) Endophyte or parasite – what decides? Curr Opin Plant Biol 9:358–363

Kotani T, Yamamoto T, Yurimoto H, Sakai Y, Kato N (2003) Propane monooxygenase and NAD+−dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. J Bacteriol 185:7120–7128

Krechel A, Faupel A, Hallmann J, Ulrich A, Berg G (2002) Potato-associated bacteria and their antagonistic potential towards plant-pathogenic fungi and the plant-parasitic nematode Meloidogyne incognita (Kofoid & White) Chitwood. Can J Microbiol 48:772–786

Kudoyarova GR, Melentiev AI, Martynenko EV, Timergalina LN, Arkhipova TN, Shendel GV, Kuz'mina LY, Dodd IC, Veselov SY (2014) Cytokinin producing bacteria stimulate amino acid deposition by wheat roots. Plant Physiol Biochem: PPB/Soc Fr Physiol Veg 83:285–291

Kuiper I, Kravchenko LV, Bloemberg GV, Lugtenberg BJJ (2002) *Pseudomonas putida* strain PCL1444, selected for efficient root colonization and naphtalene degradation, effectively utilizes root exudate components. Mol Plant-Microbe Interact 15:734–741

Kuklinsky-Sobral J, Araújo WL, Mendes R, Geraldi IO, Pizzirani-Kleiner AA, Azevedo JL (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ Microbiol 6:1244–1251

Kulik N, Goi A, Trapido M, Tuhkanen T (2006) Degradation of polycyclic aromatic hydrocarbons by combined chemical pre-oxidation and bioremediation in creosote contaminated soil. J Environ Manage 78:382–391

Ladha JK, Barraquio WL, Watanabe I (1983) Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. Can J Microbiol 29:1301–1308

Ladha JK, Pathak H, Krupnik TJ, Six J, van Kessel C (2005) Efficiency of fertilizer nitrogen in cereal production: retrospects and prospects. In: Advances in Agronomy, vol 87. Academic, pp 85–156. Elsevier

Lagendijk EL, Validov S, Lamers GE, de Weert S, Bloemberg GV (2010) Genetic tools for tagging Gram-negative bacteria with mCherry for visualization in vitro and in natural habitats, biofilm and pathogenicity studies. FEMS Microbiol Lett 305:81–90

Leveau JH, Lindow SE (2001) Appetite of an epiphyte: quantitative monitoring of bacterial sugar consumption in the phyllosphere. Proc Natl Acad Sci U S A 98:3446–3453

Lodewyckx C, Mergeay M, Vangronsveld J, Clijsters H, Van Der Lelie D (2002a) Isolation, characterization, and identification of bacteria associated with the zinc hyperaccumulator *Thlaspi caerulescens* subsp. calaminaria. Int J Phytoremediation 4:101–115

Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M, von der Lelie D (2002b) Endophytic bacteria and their potential applications. Crit Rev Plant Sci 21:583–606

Loper JE, Hassan KA, Mavrodi DV et al (2012) Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. PLoS Genet 8:e1002784

López-Bucio J, Campos-Cuevas JC, Hernández-Calderón E, Velásquez-Becerra C, Farías-Rodríguez R, Macías-Rodríguez LI, Valencia-Cantero E (2007) *Bacillus megaterium* rhizobacteria promote growth and alter root-system architecture through an auxin- and ethylene-independent signaling mechanism in *Arabidopsis thaliana*. Mol Plant-Microbe Interact 20:207–217

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556

Lugtenberg BJ, Chin AWTF, Bloemberg GV (2002) Microbe-plant interactions: principles and mechanisms. Antonie Van Leeuwenhoek 81:373–383

Lundberg DS, Lebeis SL, Paredes SH et al (2012) Defining the core *Arabidopsis thaliana* root microbiome. Nature 488:86–90

Maestri E, Marmiroli N (2011) Transgenic plants for phytoremediation. Int J Phytoremediation 13 (Suppl 1):264–279

Malinowski DP, Alloush GA, Belesky DP (2000) Leaf endophyte *Neotyphodium coenophialum* modifies mineral uptake in tall fescue. Plant Soil 227:115–126

Martin KE, Ozsvar J, Coleman NV (2014) SmoXYB1C1Z of Mycobacterium sp. strain NBB4: a soluble methane monooxygenase (sMMO)-like enzyme, active on C2 to C4 alkanes and alkenes. Appl Environ Microbiol 80:5801–5806

Mason OU, Scott NM, Gonzalez A et al (2014) Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. ISME J 8:1464–1475

Mastretta C, Barac T, Vangronsveld J, Newman L, Taghavi S, Dvd L (2006) Endophytic bacteria and their potential application to improve the phytoremediation of contaminated environments. Biotechnol Genet Eng Rev 23:175–188

Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Gonnelli C, Barac T, Boulet J, Weyens N, Vangronsveld J (2009) Endophytic bacteria from seeds ofnicotiana tabacumcan reduce cadmium phytotoxicity. Int J Phytoremediation 11:251–267

McCully ME (2001) Niches for bacterial endophytes in crop plants: a plant biologist's view. Funct Plant Biol 28:983–990

Mehnaz S, Mirza MS, Haurat J, Bally R, Normand P, Bano A, Malik KA (2001) Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. Can J Microbiol 47:110–117

Miethke M, Marahiel MA (2007) Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev 71:413–451

Mohite B (2013) Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. J Soil Sci Plant Nutr 13:638–649

Moldes AB, Paradelo R, Rubinos D, Devesa-Rey R, Cruz JM, Barral MT (2011) Ex situ treatment of hydrocarbon-contaminated soil using biosurfactants from *Lactobacillus pentosus*. J Agric Food Chem 59:9443–9447

Murray JD (2011) Invasion by invitation: rhizobial infection in legumes. Mol Plant Microbe Interact 24:631–639

Nakayama N, Smith RS, Mandel T, Robinson S, Kimura S, Boudaoud A, Kuhlemeier C (2012) Mechanical regulation of auxin-mediated growth. Curr Biol 22:1468–1476

Nelson KE, Weinel C, Paulsen IT et al (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 4:799–808

Newman LA, Reynolds CM (2005) Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends Biotechnol 23:6–8

Nichols D, Cahoon N, Trakhtenberg EM, Pham L, Mehta A, Belanger A, Kanigan T, Lewis K, Epstein SS (2010) Use of ichip for high-throughput in situ cultivation of "uncultivable" microbial species. Appl Environ Microbiol 76:2445–2450

Nielsen TH, Sorensen J (2003) Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. Appl Environ Microbiol 69:861–868

Palleroni NJ, Pieper DH, Moore ERB (2010) Microbiology of hydrocarbon-degrading *Pseudomonas*. In: Timmis K (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin/Heidelberg, pp 1787–1798

Panagos P, Van Liedekerke M, Yigini Y, Montanarella L (2013) Contaminated sites in Europe: review of the current situation based on data collected through a European network. J Environ Public Health 2013:158764

Pandey P, Kang S, Maheshwari D (2005) Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*. Curr Sci 89:177–180

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5:308–316

Pirttilä AM, Frank AC (2011) Endophytes of forest trees, vol 80. Springer Netherlands

Pitts RJ, Cernac A, Estelle M (1998) Auxin and ethylene promote root hair elongation in Arabidopsis. Plant J 16:553–560

Poole K (2012) Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol 20:227–234

Porteous Moore F, Barac T, Borremans B, Oeyen L, Vangronsveld J, van der Lelie D, Campbell CD, Moore ER (2006) Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: the characterisation of isolates with potential to enhance phytoremediation. Syst Appl Microbiol 29:539–556

Raaijmakers JM, Mazzola M (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annu Rev Phytopathol 50:403–424

Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. Antonie Van Leeuwenhoek 81:537–547

Radzki W, Manero FG, Algar E, García JL, García-Villaraco A, Solano BR (2013) Bacterial siderophores efficiently provide iron to iron-starved tomato plants in hydroponics culture. Antonie Van Leeuwenhoek 104:321–330

Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C (2004) Biofilm formation in plant-microbe associations. Curr Opin Microbiol 7:602–609

Rashid S, Charles TC, Glick BR (2012) Isolation and characterization of new plant growth-promoting bacterial endophytes. Appl Soil Ecol 61:217–224
Reichenauer TG, Germida JJ (2008) Phytoremediation of organic contaminants in soil and groundwater. ChemSusChem 1:708–717
Reinhold-Hurek B, Hurek T (2011) Living inside plants: bacterial endophytes. Curr Opin Plant Biol 14:435–443
Remans R, Beebe S, Blair M et al (2007) Physiological and genetic analysis of root responsiveness to auxin-producing plant growth-promoting bacteria in common bean (*Phaseolus vulgaris* L.). Plant Soil 302:149–161
Roca A, Pizarro-Tobias P, Udaondo Z, Fernandez M, Matilla MA, Molina-Henares MA, Molina L, Segura A, Duque E, Ramos JL (2013) Analysis of the plant growth-promoting properties encoded by the genome of the rhizobacterium *Pseudomonas* putida BIRD-1. Environ Microbiol 15:780–794
Rodrıguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339
Rodríguez-Trigo G, Zock J-P, Pozo-Rodríguez F et al (2010) Health changes in fishermen 2 years after clean-up of the prestige oil spill. Ann Intern Med 153:489–498
Rosenblueth M, Martinez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact 19:827–837
Rylott EL (2014) Endophyte consortia for xenobiotic phytoremediation: the root to success? Plant Soil 385:389–394
Ryu C-M, Hu C-H, Reddy MS, Kloepper JW (2003a) Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. New Phytol 160:413–420
Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW (2003b) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci U S A 100:4927–4932
Ryu C-M, Hu C-H, Locy RD, Kloepper JW (2005) Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. Plant Soil 268:285–292
Sajjad Mirza M, Ahmad W, Latif F, Haurat J, Bally R, Normand P, Malik KA (2001) Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane in vitro. Plant Soil 237:47–54
Saleem M, Arshad M, Hussain S, Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J Ind Microbiol Biotechnol 34:635–648
Santaella C, Schue M, Berge O, Heulin T, Achouak W (2008) The exopolysaccharide of *Rhizobium* sp. YAS34 is not necessary for biofilm formation on *Arabidopsis thaliana* and *Brassica napus* roots but contributes to root colonization. Environ Microbiol 10:2150–2163
Scheublin TR, Deusch S, Moreno-Forero SK, Muller JA, van der Meer JR, Leveau JH (2014) Transcriptional profiling of Gram-positive Arthrobacter in the phyllosphere: induction of pollutant degradation genes by natural plant phenolic compounds. Environ Microbiol 16:2212–2225
Schlaeppi K, Dombrowski N, Oter RG, Ver Loren van Themaat E, Schulze-Lefert P (2014) Quantitative divergence of the bacterial root microbiota in *Arabidopsis thaliana* relatives. Proc Natl Acad Sci U S A 111:585–592
Schulz B, Boyle C (2006) What are endophytes. In: Schulz B, Boyle C, Sieber TN (eds) Microbial root endophytes, vol 9. Springer, Berlin/Heidelberg
Schulz BJE, Boyle CJC, Sieber TN (2006) Microbial root endophytes. Springer, Berlin/Heidelberg
Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56
Segura A, Ramos JL (2013) Plant-bacteria interactions in the removal of pollutants. Curr Opin Biotechnol 24:467–473
Segura A, Rodríguez-Conde S, Ramos C, Ramos JL (2009) Bacterial responses and interactions with plants during rhizoremediation. Microb Biotechnol 2:452–464
Sessitsch A, Hardoim P, Doring J et al (2012) Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. Mol Plant Microbe Interact 25:28–36

Siciliano SD, Fortin N, Mihoc A et al (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl Environ Microbiol 67:2469–2475

Skoog F, Armstrong DJ (1970) Cytokinins. Annu Rev Plant Physiol 21:359–384

Sloan SS, Lebeis SL (2015) Exercising influence: distinct biotic interactions shape root microbiomes. Curr Opin Plant Biol 26:32–36

Somers E, Vanderleyden J, Srinivasan M (2004) Rhizosphere bacterial signalling: a love parade beneath our feet. Crit Rev Microbiol 30:205–240

Spaepen S (2015) Plant hormones produced by microbes. In: Lugtenberg B (ed) Principles of plant-microbe interactions Microbes for sustainable agriculture. Springer International Publishing, Switzerland, pp 247–256

Spaepen S, Vanderleyden J (2011) Auxin and plant-microbe interactions. Cold Spring Harb Perspect Biol 3:a001438

Spaepen S, Vanderleyden J, Okon Y (2009) Chapter 7: plant growth-promoting actions of Rhizobacteria. In: Loon LCV (ed) Advances in botanical research, vol 51. Academic, pp 283–320

Stewart EJ (2012) Growing unculturable bacteria. J Bacteriol 194:4151–4160

Su C, Lei L, Duan Y, Zhang KQ, Yang J (2012) Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. Appl Microbiol Biotechnol 93:993–1003

Syranidou E, Christofilopoulos S, Gkavrou G, Thijs S, Weyens N, Vangronsveld J, Kalogerakis N (2016) Exploitation of endophytic bacteria to enhance the phytoremediation potential of the wetland helophyte *Juncus acutus*. Front Microbiol 07:1016

Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J, van der Lelie D (2005) Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. Appl Environ Microbiol 71:8500–8505

Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl Environ Microbiol 75:748–757

Taghavi S, van der Lelie D, Hoffman A, Zhang YB, Walla MD, Vangronsveld J, Newman L, Monchy S (2010) Genome sequence of the plant growth promoting endophytic bacterium *Enterobacter* sp. 638. PLoS Genet 6:e1000943

Tanaka H, Watanabe T (1995) Glucanases and chitinases of *Bacillus circulans* WL-12. J Ind Microbiol 14:478–483

Tang J, Wang M, Wang F, Sun Q, Zhou Q (2011) Eco-toxicity of petroleum hydrocarbon contaminated soil. J Environ Sci (China) 23:845–851

Thijs S, Weyens N, Sillen W, Gkorezis P, Carleer R, Vangronsveld J (2014a) Potential for plant growth promotion by a consortium of stress-tolerant 2,4-dinitrotoluene-degrading bacteria: isolation and characterization of a military soil. Microb Biotechnol 7:294–306

Thijs S, Van Dillewijn P, Sillen W et al (2014b) Exploring the rhizospheric and endophytic bacterial communities of *Acer pseudoplatanus* growing on a TNT-contaminated soil: towards the development of a rhizocompetent TNT-detoxifying plant growth promoting consortium. Plant Soil 385:15–36

Thijs S, Sillen W, Rineau F, Weyens N, Vangronsveld J (2016) Towards an enhanced understanding of plant-microbiome interactions to improve phytoremediation: engineering the metaorganism. Front Microbiol 7:341

Thomassin-Lacroix EJ, Eriksson M, Reimer KJ, Mohn WW (2002) Biostimulation and bioaugmentation for on-site treatment of weathered diesel fuel in Arctic soil. Appl Microbiol Biotechnol 59:551–556

Torsvik V, Øvreås L, Thingstad TF (2002) Prokaryotic diversity – magnitude, dynamics, and controlling factors. Science 296:1064–1066

Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J (2015a) The effects of the growth substrate on cultivable and total endophytic assemblages of *Arabidopsis thaliana*. Plant Soil 405(1-2):325–336

Truyens S, Weyens N, Cuypers A, Vangronsveld J (2015b) Bacterial seed endophytes: genera, vertical transmission and interaction with plants. Environ Microbiol Rep 7(1):40–50

Truyens S, Beckers B, Thijs S, Weyens N, Cuypers A, Vangronsveld J (2016) Cadmium-induced and transgenerational changes in the cultivable and total seed endophytic community of *Arabidopsis thaliana*. Plant Biol (Stuttg) 18(3):376–381

Uhlik O, Wald J, Strejcek M, Musilova L, Ridl J, Hroudova M, Vlcek C, Cardenas E, Mackova M, Macek T (2012) Identification of bacteria utilizing biphenyl, benzoate, and naphthalene in long-term contaminated soil. PLoS One 7:e40653

Van Aken B, Correa PA, Schnoor JL (2010) Phytoremediation of polychlorinated biphenyls: new trends and promises. Environ Sci Technol 44:2767–2776

van Beilen JB, Panke S, Lucchini S, Franchini AG, Rothlisberger M, Witholt B (2001) Analysis of Pseudomonas putida alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. Microbiology 147:1621–1630

Van der Lelie D, Taghavi S, Monchy S et al (2009) Poplar and its bacterial endophytes: coexistence and harmony. Crit Rev Plant Sci 28:346–358

van Elsas JD, Turner S, Bailey MJ (2003) Horizontal gene transfer in the phytosphere. New Phytol 157:525–537

Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549

Vangronsveld J, Herzig R, Weyens N et al (2009) Phytoremediation of contaminated soils and groundwater: lessons from the field. Environ Sci Pollut Res Int 16:765–794

Wang L, Wang W, Lai Q, Shao Z (2010a) Gene diversity of CYP153A and AlkB alkane hydroxylases in oil-degrading bacteria isolated from the Atlantic Ocean. Environ Microbiol 12:1230–1242

Wang W, Wang L, Shao Z (2010b) Diversity and abundance of oil-degrading bacteria and alkane hydroxylase (alkB) genes in the subtropical seawater of Xiamen Island. Microb Ecol 60:429–439

Wasmund K, Burns KA, Kurtböke DI, Bourne DG (2009) Novel alkane hydroxylase gene (alkB) diversity in sediments associated with hydrocarbon seeps in the Timor Sea, Australia. Appl Environ Microbiol 75:7391–7398

Watt M, Hugenholtz P, White R, Vinall K (2006) Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence in situ hybridization (FISH). Environ Microbiol 8:871–884

Wenzel WW (2009) Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321:385–408

Weyens N, van der Lelie D, Taghavi S, Vangronsveld J (2009a) Phytoremediation: plant-endophyte partnerships take the challenge. Curr Opin Biotechnol 20:248–254

Weyens N, van der Lelie D, Taghavi S, Newman L, Vangronsveld J (2009b) Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends Biotechnol 27:591–598

Weyens N, Taghavi S, Barac T, van der Lelie D, Boulet J, Artois T, Carleer R, Vangronsveld J (2009c) Bacteria associated with oak and ash on a TCE-contaminated site: characterization of isolates with potential to avoid evapotranspiration of TCE. Environ Sci Pollut Res Int 16:830–843

Weyens N, Croes S, Dupae J, Newman L, van der Lelie D, Carleer R, Vangronsveld J (2010) Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination. Environ Pollut 158:2422–2427

Weyens N, Schellingen K, Beckers B, Janssen J, Ceulemans R, van der Lelie D, Taghavi S, Carleer R, Vangronsveld J (2012) Potential of willow and its genetically engineered associated bacteria to remediate mixed Cd and toluene contamination. J Soil Sediment 13:176–188

Weyens N, Gielen M, Beckers B, Boulet J, Lelie D, Taghavi S, Carleer R, Vangronsveld J (2014) Bacteria associated with yellow lupine grown on a metal-contaminated soil: in vitro screening and in vivo evaluation for their potential to enhance Cd phytoextraction. Plant Biol 16:988–996

Yergeau E, Sanschagrin S, Beaumier D, Greer CW (2012) Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. PLoS One 7:e30058

Yergeau E, Sanschagrin S, Maynard C, St-Arnaud M, Greer CW (2014) Microbial expression profiles in the rhizosphere of willows depend on soil contamination. ISME J 8:344–358

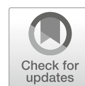
Yousaf S, Andria V, Reichenauer TG, Smalla K, Sessitsch A (2010) Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and Birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment. J Hazard Mater 184:523–532

Yousaf S, Afzal M, Reichenauer TG, Brady CL, Sessitsch A (2011) Hydrocarbon degradation, plant colonization and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains. Environ Pollut 159:2675–2683

Zamioudis C, Pieterse CM (2012) Modulation of host immunity by beneficial microbes. Mol Plant Microbe Interact 25:139–150

Zhu X, Ni X, Liu J, Gao Y (2014) Application of endophytic bacteria to reduce persistent organic pollutants contamination in plants. Clean – Soil, Air, Water 42:306–310

Zhuang X, Chen J, Shim H, Bai Z (2007) New advances in plant growth-promoting rhizobacteria for bioremediation. Environ Int 33:406–413



Removal of Hydrocarbons and Other Related Chemicals via the Rhizosphere of Plants

10

Lázaro Molina, Pieter van Dillewijn, Estrella Duque, Craig Daniels, Tino Krell, Manuel Espinosa-Urgel, María Isabel Ramos-González, Sara Rodríguez-Conde, Miguel A. Matilla, Regina Wittich, Juan Luis Ramos, and Ana Segura

Contents

1 Introduction 158
2 Phytoremediation Strategies 159
3 Rhizoremediation 160
3.1 Rhizoremediation Requirements 160
3.2 Rhizoremediation Experiments 163
3.3 Monitoring Rhizoremediation 164
4 Improving Rhizoremediation: Research Needs 165
References 166

L. Molina · P. van Dillewijn · E. Duque · T. Krell · M. Espinosa-Urgel · M. I. Ramos-González · S. Rodríguez-Conde · M. A. Matilla · R. Wittich · J. L. Ramos · A. Segura (✉)
Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain
e-mail: lazaro.molina@cnb.csic.es; pieter.vandillewijn@eez.csic.es; estrella.duque@eez.csic.es; tino.krell@eez.csic.es; t.krell@eez.csic.es; manuel.espinosa@eez.csic.es; maribel.ramos@eez.csic.es; sara.rodriguez.conde@gmail.com; miguel.matilla@eez.csic.es; regina.wittich@eez.csic.es; jlramos@eez.csic.es; juanluis.ramos@eez.csic.es; ana.segura@eez.csic.es

C. Daniels
Department of Environmental Protection, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Granada, Spain

Developmental and Stem Cell Biology Program, Brain Tumour Research Centre, The Hospital for Sick Children, Toronto, ON, Canada
e-mail: craig.daniels@sickkids.ca

© Springer Nature Switzerland AG 2019
R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_10

Abstract
Recalcitrant organic toxic chemicals have been accumulating for decades as a consequence of industrial activity. Concerns about environmental contamination have been rising in the last three decades and therefore the need for soil remediation has become a priority. Although physicochemical techniques are currently the most efficient methods being used to remove contaminants, they are very expensive and therefore impractical in many locations. The use of plants in the bioremediation of soils has been proposed as an attractive strategy; however, plants lack the extraordinary biodegradative capabilities of microorganisms. Consequently rhizoremediation, a technology which combines microorganisms that eliminate contaminants in the plant roots which provide nutrients for these microorganisms, has emerged. To design a successful rhizoremediation strategy, microorganisms need to proliferate in the root system, and the bacterial catabolic pathways have to be operative. Recent advances in these aspects, together with some techniques to improve biodegradation in the rhizosphere will be presented in this chapter.

1 Introduction

Industrialization has allowed human development and higher living standards; however, these benefits have come at a high price in environmental terms. The inappropriate use of fertilizers and pesticides to improve crop production to cope with the needs of a growing population is leading to the contamination of underground water by nitrates and to the pollution of soil and surface water. The greater energy needs of society have increased CO_2 production which is now one of the main concerns for governments. Industrial waste has resulted in the accumulation of recalcitrant organic chemicals such as DDT, lindane, triazines, and polychlorinated biphenyls (PCBs). In addition, soils are being gradually affected by the deposition of fuel particles enriched in polycyclic aromatic hydrocarbons (PAHs), as well as benzene and other chemicals derived from petrochemical activities. Recently, an increase in contaminants derived from personal care products and pharmaceuticals (PCPPs) has been detected in water. All these contaminants provoke an adverse effect on ecosystems and are a serious threat to human health. Due to their toxicity and in many cases their carcinogenic, teratogenic, endocrine disruption and mutagenic properties, a number of these chemicals have been declared pollutants and their removal is a priority for Environmental Protection Agencies. The removal of pollutants from air, water, and soil requires different technologies, and in this chapter, we will focus on the removal of contaminants from soil. Removal of pollutants from soil is a very complex and expensive procedure as it depends on numerous uncontrollable factors. Most of the successful remediation examples have included the physical removal of the contaminated soil (excavation, truck, and dump) and/or physical and/or chemical treatments; however, these techniques are quite expensive and involve the disruption of the soil structure (Gómez-Sagasti et al. 2016). In general,

biological treatments are cheaper than physicochemical ones; however, they have been used in recent years with different degrees of success (Segura and Ramos 2013). The success of biological pollutant removal depends on the particular bioremediation strategy, the soil properties, age of the contamination, initial concentration of the contaminants, and obviously on the contaminant's toxicity (dos Santos and Maranho 2018). *On-site* techniques such as land-farming or composting are promising options, but they involve the manipulation of soils and sometimes provoke the mobilization of the contaminant. *In situ* techniques, such as inoculation with microorganisms encoding the desired catalytic properties or bioaugmentation with native populations, are unreliable (Colleran 1997) and improving them by soil fertilization is expensive.

2 Phytoremediation Strategies

As an alternative to the failure of bioaugmentation, phytoremediation, the utilization of plants for the removal of pollutants, has been proposed as an attractive strategy to achieve the efficient elimination of pollutants.

The generic term, phytoremediation, is used to describe the elimination of contaminants by the plants themselves or in combination with root colonizing microorganisms, and it encompasses different strategies (Salt et al. 1998). In phytostabilization, plants either physically or by the action of the root exudates, help to sequester the contaminant to the soil particles thus making them less bioavailable (Burges et al. 2017); phytovolatilization is the term used when plants take up the contaminant from the soil and transform it into a volatile compound that is released into the atmosphere by transpiration for dispersal (Limmer and Burken 2016); phytoextraction is a technology used when plants are able to accumulate toxic compounds in the harvestable parts such as stems and leaves (Burges et al. 2017); rhizoremediation consists of the elimination of the contaminant by the microorganisms in the rhizosphere (Kuiper et al. 2004a); and phytoremediation as a strict term refers to the transformation of the contaminant by the metabolism of the plant (Macek et al. 2000).

Because plants are easy to monitor and agricultural technologies are constantly being developed, a priori the easiest way to design a phytoremediation protocol would be to use a plant as the single degradative organism. Many plants are able to transform (but in general not mineralize) some contaminants via their unique secondary metabolism, which is represented by the capacity of members of the cytochrome P450 super family of proteins to activate the contaminant that is subsequently conjugated with glutathione, malonyl, or glucosyl residues. The conjugated compound is then accumulated in vacuoles or in the plant cell wall (Burken 2004). These transformation steps are similar to those which take place in the liver of animals and led to the term “green liver” to designate the capacity of the plant to transform contaminants into less toxic compounds. Moreover, pollutants containing amino or hydroxyl groups can undergo rapid transformation by root-associated or extracellular enzymes (Shaw and Burns 2003).

Unfortunately plants do not possess the extraordinary repertoire of degradative enzymes of microorganisms, and therefore, the utilization of phytoremediation strategies based exclusively on plant activities cannot be used universally.

3 Rhizoremediation

Due to their impressive degradation capacity for xenobiotic compounds, microorganisms have long been studied and assayed in bioremediation treatments. In contrast to the successful isolation of microorganisms with specific biodegradation properties, their reintroduction into soil to remove pollutants has been frequently unsuccessful. In some cases it has been reported that reintroduced microorganisms are unable to establish themselves in the soil. Although knowledge regarding the behavior of microorganisms in the environment is incomplete, it is thought that nutrient limitation, competition with indigenous microbiota, and/or limitations in their reactions under environmental stress conditions may account for the lack of success of (re)introduced microorganisms in contaminated soils. Some of these obstacles can be overcome by using rhizoremediation, a strategy that is based on the "rhizosphere effect" that relies on the plant excreting a number of chemicals that can be used as a carbon, nitrogen, sulfur, or phosphorous source by microorganisms to proliferate and reach high cell densities in the area surrounding the roots of the plant, i.e., the rhizosphere. The advantage of rhizoremediation is that plant roots provide a large surface on which microorganisms can proliferate by creating biofilms. Roots transport microorganisms through the soil both in terms of distribution and depth, and as mentioned above, the roots provide nutrients. Root penetration into the soil also facilitates oxygen exchange and thereby the proliferation of aerobic microorganisms and oxygen availability for the initial attack on many different chemicals in reactions mediated by mono- and dioxygenases.

3.1 Rhizoremediation Requirements

One of the classic papers in the field of "natural" rhizoremediation is the report by Radwan et al. (1995) showing that plants growing in sand contaminated by oil spills after the Gulf War exhibited clean roots due to the removal of oil compounds by microorganisms. After this initial observation, many laboratories started to study rhizoremediation, and although there are not many examples detailing the removal of pollutants from soils under real conditions undertaken via the concept of "designed" rhizoremediation, there have been significant advances in the understanding of the interaction between plants and bacteria during rhizoremediation. For a successful strategy, microorganisms have to be able to proliferate in the root system, and catabolic pathways have to be operative and free of the catabolite repression effect, which is the preferential use by microorganisms of a given carbon or nitrogen source with respect to the target compound (Burken 2004), moreover the contaminant has to be bioavailable (Fig. 1).

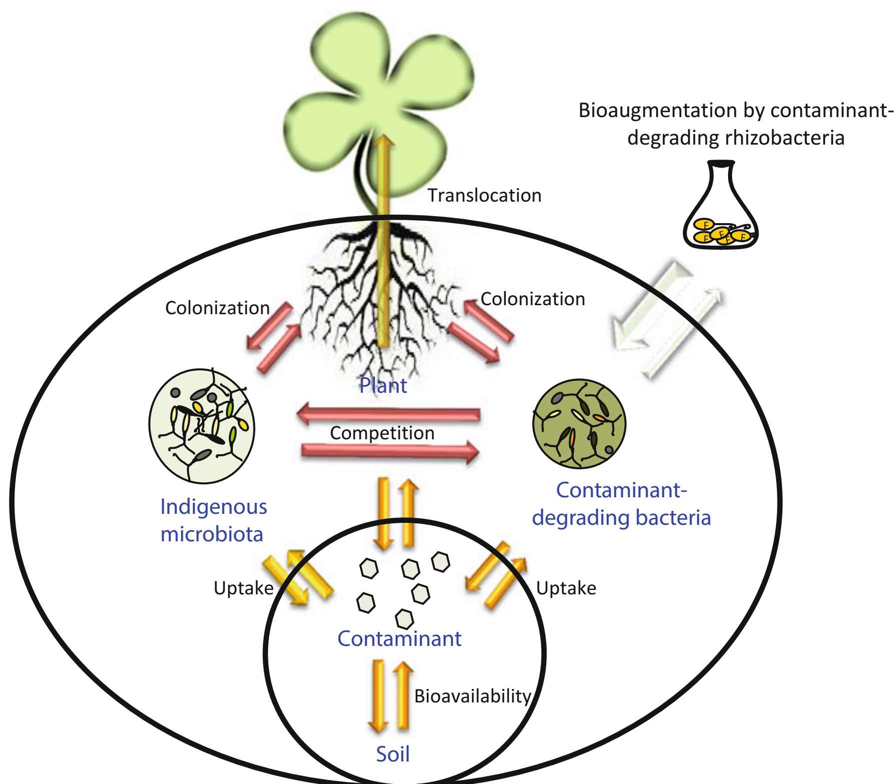


Fig. 1 Interactions influencing rhizoremediation

Rhizoremediation requires the augmentation of the desired bacteria (or consortia) in the laboratory to obtain a sufficient number of degradative bacteria to inoculate the seeds or roots of plants. After growing in the laboratory, the bacteria have to be able to proliferate on the root system under natural conditions (Fig. 1). Early experiments using *in vivo* expression technology (Rainey and Preston 2000; Ramos-González et al. 2005) and microarrays revealed the bacterial genes involved in the adaptation to the rhizosphere (Matilla et al. 2007; Attila et al. 2008). These findings indicated that after growth in the laboratory, bacteria have to reprogramme themselves to be able to live in the rhizosphere. Induction of genes related with changes in the transcriptional pattern of catabolic pathways to enable the utilization of the chemicals secreted by roots as carbon or nitrogen sources, and the induction of bacterial genes involved in oxidative stress are part of the response to the rhizosphere environment. It has also been repeatedly demonstrated that flagellar and chemotaxis genes are involved in root colonization (Lugtenberg et al. 2001; Scharf et al. 2016). These results revealed that the rhizosphere, although nutrient-rich compared with bulk soil, is a nutritionally challenging environment which is stressful to microorganisms due to the formation of reactive oxygen species produced by the respiration of root cells.

Identification of plant microbiomes (Bulgarelli et al. 2012) has confirmed that microbial populations in the rhizosphere depend on plant age, physiological status, and even the cultivar, supporting the idea that plant roots exert a certain selection toward the microorganisms living in their rhizosphere. The idea of specific plant-microbe communication is becoming more popular and, although most of the efforts in rhizoremediation have been focused on degradative bacteria, the utilization of different plants can also influence the outcome of contaminant removal. Microbial elimination of contaminants in the rhizosphere provides a positive effect for the plant as this leads to decreased pollutant concentrations in the area near the roots and thereby improved plant growth in contaminated areas (Rodriguez-Conde et al. 2016). Because of this mutual benefit, it has been proposed that plants can select for specific bacterial genotypes to be present on their roots. Experiments performed by Siciliano et al. (2002) demonstrated that the presence of the alkane monooxygenase gene was more prevalent in endophytic and rhizosphere microbial communities than in bulk soil contaminated with hydrocarbons. However, the results obtained when they studied the prevalence of xylene monooxygenase or naphthalene dioxygenase genes were just the opposite, their presence was higher in bulk soil microbial communities than those near to or on the plant. This effect was also dependent on the plant species used during the rhizoremediation strategy (Siciliano et al. 2003). Therefore, the combined influence of plants and contaminants determines the associated plant microbiota (Fig. 1). The presence of certain contaminants may affect the relative abundance of genotypes able to degrade this particular contaminant (Rodriguez-Conde et al. 2016) or the diversity of rhizosphere or endophytic bacteria (Liu et al. 2017). This has led to the hypothesis that the effectiveness of rhizoremediation strategies also depends on the selection of the best plant-bacteria combination for a specific contaminant (Thijs et al. 2016).

To complicate the general scheme, foreign bacteria also have to be able to compete with indigenous microorganisms suggesting another level of communication; they have to be able to sense their environment (Venturi and Keel 2016; Fig. 1). It is well known that bacteria are able to establish an intraspecific communication using auto-inducers that are synthesized when populations reach a critical density in a process called *quorum sensing*. The *luxI* gene encodes the auto-inducer synthase and the *luxR* codes for an auto-inducer-dependent activator responsible for coordinating the expression of several genes as well as the induction of the *luxI* gene (Stevens and Greenberg 1997; Fuqua and Greenberg 2002). *luxR* and *luxI* are generally located near to each other in the chromosome (Choudhary et al. 2013). However, as more microbial genomic information has become available in databases, it has been observed that many microorganisms encode several *luxR*-type genes that are not in the vicinity of any *luxI* and that there are more *luxR* genes than *luxI*. These genes have been named "orphan-*luxR*" or "*luxR*-solos" (Fuqua 2006). Some of these *luxR*-solos have been involved in intraspecific communication (microbe-microbe interactions) whilst others have been involved in inter-kingdom (plant-microbe) communication (Subramoni and Venturi 2009; Gonzalez and Venturi 2013).

Even if the reintroduced degradative-bacteria are: (i) able to grow with the nutrients available in the rhizosphere; (ii) able to cope with stressful environments;

and (iii) able to compete with other microorganisms and to communicate with their environment, all these properties are useless if the degradative genes are not expressed in the rhizosphere. The rationale behind rhizoremediation strategies is that the rhizosphere environment contains additional C-sources that favor microbial growth which consequently accelerates degradation rates (Kuiper et al. 2004a; Lu et al. 2017). However, several reports have indicated that biodegradative genes may be affected by catabolic repression. Catabolic repression consists of the preferential utilization of some carbon sources over others; i.e., the toluene degradation pathway of the pWW0 plasmid is repressed in the presence of glucose or succinate (Duetz et al. 1996). Rentz et al. (2004) reported that root exudates from different plants inhibited the phenanthrene-degrading activity of *Pseudomonas putida* ATCC 17484, although these exudates allowed the growth of the strain. Other reports, however, indicate the fortuitous induction of catabolic genes in the rhizosphere (Casavant et al. 2003; Segura et al. 2017), probably as a consequence of the structural similarities between certain plant secondary metabolites and some xenobiotic chemicals (Singer et al. 2003).

3.2 Rhizoremediation Experiments

Different rhizoremediation strategies have been used to demonstrate the utility of this approach for the degradation of contaminants. Initially, bacterial strains that were well characterized for their capacity to degrade a certain contaminant were chosen, but frequently these strains were not well suited for survival in the rhizosphere. This has led to the current tendency to use rhizosphere bacteria. Using a double enrichment approach in which lindane degradation and root proliferation of bacteria were prerequisites for selection, Böltner et al. (2008) isolated *Sphingomonas* strains able to adhere to corn seeds at levels two orders of magnitude higher than the reference strain *Sphingomonas* UT26 and were able to colonize the root at approximately 5×10^8 CFUs (colony forming units) per gram of root. In controlled rhizoremediation experiments, these recently isolated strains were able to degrade 30% of the γ-hexaclorohexane of contaminated soil (0.5 mg per gram of soil) in 25 days. Amongst the disadvantages of this approximation are the lack of information about the degradative abilities of the bacteria and the lack of information about the ability of these bacteria to persist in the specific plant environment. These disadvantages can be alleviated if bacteria are isolated directly from the contaminated soil that has to be cleaned up (Zafra et al. 2017), but this approach requires additional experimentation in order to find the optimal bioaugmentation conditions to ensure that most of the new inoculum contains biodegradative bacteria.

Another approach is the utilization of bacteria that are generally present in contaminated environments. For example, different metagenomic experiments have revealed that in PAHs-contaminated soils, bacteria belonging to the genera *Arthrobacter*, *Rhodobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus*, *Mycobacterium,* or to the *Sphingomonadaecea* family are frequently found to be the most abundant (Uhlik et al. 2012; Kappell et al. 2014; Rodriguez-Conde et al. 2016). Despite

possible biases in the metagenomic analysis, it has been demonstrated that members of these genera and families are good PAH degraders (Lu et al. 2011). It is therefore interesting to test members of these families in rhizoremediation experiments. Utilization of single strains as well as consortia has demonstrated the utility of this approach (Zafra et al. 2017; Rodriguez-Conde et al. 2016).

Finally, the utilization of endophytes in the biodegradation of pollutants is an interesting option that is currently being explored (Afzal et al. 2014). In order to be successful, endophytes with interesting catabolic properties have to be isolated (Taghavi et al. 2009); alternatively, degradative bacteria in the rhizosphere have to be able to transfer interesting genetic determinants to plant endophytes (Mastretta et al. 2006). Natural horizontal transfer of the pTOM plasmid from *Burkholderia cepacia* isolated from yellow lupine, to poplar endophytes that then acquired the capacity to degrade toluene have already been demonstrated (Taghavi et al. 2005). For the successful utilization of this strategy, however, contaminants have to be transported into the plant interior and the transport mechanisms of the contaminants into the root cells have to be explored.

3.3 Monitoring Rhizoremediation

In rhizoremediation there are several parameters that have to be monitored: plant survival and growth, inoculum survival and contaminant elimination. Whilst initially, the number of colony forming units (CFUs) using selective media was almost the only method to follow up microbial survival and rhizosphere colonization, the advances in the utilization of reporter genes, such as the green-fluorescent protein (Gfp) and luciferase, have allowed the simplification of methods to monitor microbial survival in the rhizosphere (Ramos et al. 2000; Buddrus-Schiemann et al. 2010). However, these methods involve the genetic modification of the organisms and therefore, monitorization under natural conditions is mainly achieved by counting cells on selective media.

Contaminant biodegradation monitoring requires the extraction of the contaminant from soil and subsequent determination by analytical methods. These methods (mainly gas chromatography coupled to mass spectrometry [GC-MS] and high-pressure liquid chromatography [HPLC]) require expensive equipment and frequently, analyses of the samples in specialized laboratories. In the last decade, several biosensors (devices based on biological parts that sense a signal and transform it into a quantifiable response) for easy *in situ* detection of contaminants have been developed (Tecon and van der Meer 2008; Segura et al. 2017). However, the toxicity of the contaminant toward the biosensor (if whole cell biosensors are used) and the possibility of synergistic effects with other compounds in the environment can alter the results making biosensors, in general, not as accurate as chemical methods (Diplock et al. 2010). Nevertheless, robust biosensors with high specificity for oil-derived compounds are available (Kohlmeier et al. 2008; Sevilla et al. 2015; Hernández-Sánchez et al. 2016). When chemical methods are used, the total amount of the

contaminant is detected, whereas the biosensors detect the bioavailable fraction; this is important as the bioavailable fraction determines the ecotoxicity of the contaminant (Tecon and van der Meer 2008).

4 Improving Rhizoremediation: Research Needs

One important problem for soil bioremediation, whatever technique is used, is the bioavailability of the pollutant. Most organic contaminants are highly hydrophobic compounds that dissolve poorly in water and many can form complexes with soil particles; this lack of bioavailability often lowers removal efficiencies. Soils with a long history of contamination are generally much less responsive to rhizoremediation than freshly spiked soils (Olson et al. 2007). It has been demonstrated that surfactants, in general, can improve the degradation of the contaminants (Ortega-Calvo et al. 2013). However, as they lead to an increase in the bioavailability of the contaminant, the addition of these compounds may produce negative effects on microbial and plant growth during rhizoremediation. Therefore, rhizoremediation techniques have to be carefully designed to improve the bioavailability of the contaminant without affecting growth. Microbial biosurfactants have been widely studied (Marchant and Banat 2012) and commercial biosurfactants are already available; however, their use in rhizoremediation has not been thoroughly investigated (Liduino et al. 2018). The selection of bacteria which are able to produce biosurfactants in the rhizosphere of plants is an interesting alternative for improving the removal efficiency. In this context, Kuiper et al. (2004b) identified bacteria growing in a PAH contaminated area that produce biosurfactants (putisolvins) that facilitate the solubilization of PAHs and hence biodegradation by microorganisms. This property is also of interest because a number of biodegradative microorganisms exhibit positive chemotaxis towards the pollutants. Therefore, the combined action of biosurfactants and chemotaxis can contribute to bacterial proliferation and to microbial spread in polluted soils, in order to clean larger zones. Besides amino acids, sugars, and other small molecular-sized compounds, plant rhizodeposition including mucilage, high molecular compounds, and other substances could improve the bioavailability of contaminants fostering biodegradation by microbial activity. To the best of our knowledge, no studies about the solubilization of hydrophobic contaminants by plant rhizodeposits have been reported.

Advances in knowledge regarding the molecular interactions between plants and microorganisms, in the expression of catabolic genes in the rhizosphere, and in the selection of the best plant-microbe combinations will have to be converted into field strategies that demonstrate the usefulness of this approach. The release of non-native and recombinant microorganisms into the soil is still a controversial subject and more studies to analyze the impact on indigenous microbial communities are needed.

It is important to know the fate of the contaminants during rhizoremediation as the translocation of contaminants to the aerial part of the plant may facilitate their spread through the trophic chain. Adsorption of the contaminants to the root cell wall

and/or transport of the toxic chemical into the plant are research topics that have received increasing attention in recent years (Dupuy et al. 2016; Cennerazzo et al. 2017). As the economic cost of bioremediation efforts is one of the main aspects that are being considered by contaminant producing industries and it has been demonstrated that the microbial biodegradation activity in soil favors the optimal growth of the plant (Van Dillewijn et al. 2007; Rodriguez-Conde et al. 2016; Vergani et al. 2017), it will be interesting to study the possibility of receiving direct economic benefits through the cultivation of crops while remediation is taking place. The economics of this new perspective and the safety-related issues regarding the possibility of the introduction of contaminants into the food-chain are areas of research that remain unexplored.

Acknowledgments The work by authors was supported by research grants from the Spanish Ministry of Science and Innovation and the Andalusian Regional Government, (Junta de Andalucía). We thank Angela Tate for improving the use of English in the manuscript.

References

Afzal M, Khan QM, Sessitsch A (2014) Endophytic bacteria: prospects and applications for the phytoremediation of organic pollutants. Chemosphere 117:232–342. https://doi.org/10.1016/j.chemosphere.2014.06.078

Attila C, Ueda A, Cirillo SLG, Chen W, Wood TK (2008) Pseudomonas aeruginosa PAO1 virulence factors and poplar tree response in the rhizosphere. Microb Biotechnol 1:17–29

Böltner D, Godoy P, Muñoz-Rojas J, Duque E, Moreno-Morillas S, Sánchez L, Ramos JL (2008) Rhizoremediation of lindane by root-colonizing *Sphingomonas*. Microbial Biotech 1:87–93

Buddrus-Schiemann K, Schmid M, Schreiner K, Welzl G, Hartmann A (2010) Root colonization by *Pseudomonas* sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of barley. Microb Ecol 60:381–393. https://doi.org/10.1007/s00248-010-9720-8

Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, Rauf P, Huettel B, Reinhardt R, Schmelzer E et al (2012) Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. Nature 488:91–95. https://doi.org/10.1038/nature11336

Burges A, Alkorta I, Epelde L, Garbisu C (2017) From phytoremediation of soil contaminants to phytomanagement of ecosystem services in metal contaminated sites. Int J Phytoremediation. https://doi.org/10.1080/15226514.2017.1365340

Burken JG (2004) Uptake and metabolism of organic compounds: green-liver model. In: McCutcheon SD, Schnoor JL (eds) On phytoremediation. Wiley, New York, pp 59–84

Casavant NC, Thompson D, Beattie GA, Phillips GJ, Halverson LJ (2003) Use of a site-specific recombination-based biosensor for detecting bioavailable toluene and related compounds on roots. Environ Microbiol 5:238–249

Cennerazzo J, de Junet A, Audinot J-N, Leyval C (2017) Dynamics of PAHs and derived organic compounds in a soil-plant mesocosm spiked with 13C-phenanthrene. Chemosphere 168:1619–1627

Choudhary KS, Hudaiberdiev S, Gelencser Z, Goncalves Coutinho B, Venturi V, Pongor S (2013) The organization of the quorum sensing luxI/R family genes in *Burkholderia*. Int J Mol Sci 14:13727–13747. https://doi.org/10.3390/ijms140713727

Colleran E (1997) Uses of bacteria in bioremediation. In: Sheehan D (ed) Methods in biotechnology, vol 2: bioremediation protocols. Humana Press Inc., Totowa

Diplock EE, Alhadrami HA, Paton GI (2010) Application of microbial bioreporters in environmental microbiology and bioremediation. Adv Biochem Eng Biotechnol 118:189–209

Dos Santos JJ, Maranho LT (2018) Rhizospheric microorganisms as a solution for the recovery of soils contaminated by petroleum: a review. J Environ Manage 210:104–113. https://doi.org/10.1016/j.jenvman.2018.01.015

Duetz WA, Marqués S, Wind B, Ramos JL, van Andel JG (1996) Catabolite repression of the toluene degradation pathway in *Pseudomonas putida* harboring pWW0 under various conditions of nutrient limitation in chemostat culture. Appl Environ Microbiol 62:601–606

Dupuy J, Leglize P, Vincent Q, Zelko I, Mustin C, Ouvrard S, Sterckeman T (2016) Effect and localization of phenanthrene in maize roots. Chemosphere 149:130–136

Fuqua C (2006) The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. J Bacteriol 188:3169–3171. https://doi.org/10.1128/JB.188.9.3169-3171.2006

Fuqua C, Greenberg EP (2002) Listening in on bacteria: acyl-homoserine lactone signalling. Nat Rev Mol Cell Biol 3:685–695. https://doi.org/10.1038/nrm907

Gómez-Sagasti MT, Epelde L, Alkorta I, Garbisu C (2016) Reflections on soil contamination research from a biologist's point of view. Appl Soil Ecol 105:207–210. https://doi.org/10.1016/j.apsoil.2016.04.004

Gonzalez JF, Venturi V (2013) A novel widespread interkingdom signaling circuit. Trends Plant Sci 18:167–174. https://doi.org/10.1016/j.tplants.2012.09.007

Hernández-Sánchez V, Molina L, Ramos JL, Segura A (2016) New family of biosensors for monitoring BTX in aquatic and edaphic environments. Microb Biotechnol 9:858–867. https://doi.org/10.1111/1751-7915.12394

Kappell AD, Wei Y, Newton RJ, Van Nostrand JD, Zhou J, McLellan SL, Hristova KR (2014) The polycyclic aromatic hydrocarbon degradation potential of Gulf of Mexico native coastal microbial communities after the Deepwater Horizon oil spill. Front Microbiol 5:205. https://doi.org/10.3389/fmicb.2014.00205

Kohlmeier S, Mancuso M, Deepthike U, Tecon R, van der Meer JR, Harms H, Wells M (2008) Comparison of naphthalene bioavailability determined by whole-cell biosensing and availability determined by extraction with Tenax. Environ Pollut 156:803–808. https://doi.org/10.1016/j.envpol.2008.06.001

Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004a) Rhizoremediation: a beneficial plant–microbe interaction. Mol Plant Microbe Interact 17:6–15

Kuiper I, Lagendijk EL, Pickford R, Derrick JP, Lamers GEM, Thomas-Oates JE, Lugtenberg BJJ, Bloember GV (2004b) Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and breakdown existing biofilms. Mol Microbiol 51:97–113

Liduino VS, Servulo EFC, Oliveira FJS (2018) Biosurfactant-assisted phytoremediation of multi-contaminated industrial soil using sunflower (*Helianthus annuus* L.). J Environ Sci Health A Tox Hazard Subst Environ Eng 1:1–8. https://doi.org/10.1080/10934529.2018.1429726

Limmer M, Burken J (2016) Phytovolatilization of organic contaminants. Environ Sci Technol 50:6632–6643. https://doi.org/10.1021/acs.est.5b04113

Liu J, Xiang Y, Zhang Z, Ling W, Gao Y (2017) Inoculation of a phenanthrene-degrading endophytic bacterium reduces the phenanthrene level and alters the bacterial community structure in wheat. Appl Microbiol Biotechnol 101:5199–5212. https://doi.org/10.1007/s00253-017-8247-z

Lu XY, Zhang T, Fang HH (2011) Bacteria-mediated PAH degradation in soil and sediment. Appl Microbiol Biotechnol 89:1357–1371. https://doi.org/10.1007/s00253-010-3072-7

Lu H, Sun J, Zhu L (2017) The role of artificial root exudate components in facilitating the degradation of pyrene in soil. Sci Rep 7:7130. https://doi.org/10.1038/s41598-017-07413-3

Lugtenberg BJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461–490

Macek T, Mackova M, Kas J (2000) Exploitation of plants for the removal of organics in environmental remediation. Biotechnol Adv 18:23–34

Marchant R, Banat IM (2012) Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnol Lett 34:1597–1605. https://doi.org/10.1007/s10529-012-0956-x
Mastretta C, Barac T, Vangronsveld J, Newman L, Taghavi S, Van der Lelie D (2006) Endophytic bacteria and their potential application to improve the phytoremediation of contaminated environments. Biotechnol Genet Eng Rev 23:175–207
Matilla MA, Espinosa-Urgel M, Rodríguez-Hervá JJ, Ramos JL, Ramos-González MI (2007) Genomic analysis reveals the major driving forces of bacterial life in the rhizosphere. Genome Biol 8:R179
Olson PE, Castro A, Joern M, DuTeau NM, Pilon SEAH, Reardon KF (2007) Comparison of plant families in a greenhouse phytoremediation study on an aged polycyclic aromatic hydrocarbon contaminated soil. J Environ Qual 36:1461–1469
Ortega-Calvo JJ, Tejeda-Agredano MC, Jimenez-Sanchez C, Congiu E, Sungthong R, Niqui-Arroyo JL, Cantos M (2013) Is it possible to increase bioavailability but not environmental risk of PAHs in bioremediation? J Hazard Mater 261:733–745. https://doi.org/10.1016/j.jhazmat.2013.03.042
Radwan S, Sorkhoh N, El-Nemr I (1995) Oil biodegradation around roots. Nature 376:302
Rainey PB, Preston GM (2000) In vivo expression technology strategies: valuable tools for biotechnology. Curr Opin Biotechnol 11:440–444
Ramos C, Molina L, Mølbak L, Ramos JL, Molin S (2000) A bioluminescent derivative of *Pseudomonas putida* KT2440 for deliberate release into the environment. FEMS Microbiol Ecol 34:91–102
Ramos-González MI, Campos MJ, Ramos JL (2005) Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: in vivo expression technology capture and identification of root activated promoters. J Bacteriol 187:4033–4041
Rentz JA, Alvarez PJJ, Schnoor JL (2004) Repression of *Pseudomonas putida* phenanthrene-degrading activity by plant root extracts and exudates. Environ Microbiol 6:574–583
Rodriguez-Conde S, Molina L, González P, García-Puente A, Segura A (2016) Degradation of phenanthrene by *Novosphingobium* sp. HS2a improved plant growth in PAHs-contaminated environments. Appl Microbiol Biotechnol 100:10627–10636
Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Plant Physiol Plant Mol Biol 49:643–668
Scharf BE, Hynes MF, Alexandre GM (2016) Chemotaxis signaling systems in model beneficial plant-bacteria associations. Plant Mol Biol 90:549–559. https://doi.org/10.1007/s11103-016-0432-4
Segura A, Ramos JL (2013) Plant–bacteria interactions in the removal of pollutants. Curr Opin Biotechnol 24:467–473. https://doi.org/10.1016/j.copbio.2012.09.011
Segura A, Hernández-Sánchez V, Marqués S, Molina L (2017) Insights in the regulation of the degradation of PAHs in *Novosphingobium* sp. HR1a and utilization of this regulatory system as a tool for the detection of PAHs. Sci Total Environ 590–591:381–393. https://doi.org/10.1016/j.scitotenv.2017.02.180
Sevilla E, Yuste L, Rojo F (2015) Marine hydrocarbonoclastic bacteria as whole-cell biosensors for n-alkanes. Microb Biotechnol 8:693–706. https://doi.org/10.1111/1751-7915.12286
Shaw LJ, Burns RG (2003) Biodegradation of organic pollutants in the rhizosphere. Adv Appl Microbiol 53:1–60
Siciliano SD, Fortin N, Mihoc A, Wisse G, Labelle X, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K, Greer CW (2002) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl Environ Microbiol 67:2469–2475
Siciliano SD, Germida JJ, Banks K, Greer CW (2003) Changes in microbial community composition and function during a polyaromatic hydrocarbon phytoremediation field trial. Appl Environ Microbiol 69:483–489
Singer AC, Crowley DE, Thompson IP (2003) Secondary plant metabolites in phytoremediation and biotransformation. Trends Biotechnol 21:123–130

Stevens AM, Greenberg EP (1997) Quorum sensing in *Vibrio fischeri*: essential elements for activation of the luminescence genes. J Bacteriol 179:557–562
Subramoni S, Venturi V (2009) LuxR-family 'solos': bachelor sensors/regulators of signaling molecules. Microbiology 155:1377–1385. https://doi.org/10.1099/mic.0.026849-0
Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J, van der Lelie D (2005) Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. Appl Environ Microbiol 71:8500–8505
Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J, van der Lelie D (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Appl Environ Microbiol 75: 748–757. https://doi.org/10.1128/AEM.02239-08
Tecon R, Van der Meer JR (2008) Bacterial biosensors for measuring availability of environmental pollutants. Sensors 8:4062–4080
Thijs S, Sillen W, Rineau F, Weyens N, Vangronsveld J (2016) Towards an enhanced understanding of plant–microbiome interactions to improve phytoremediation: engineering the metaorganism. Front Microbiol 7:341. https://doi.org/10.3389/fmicb.2016.00341
Uhlik O, Wald J, Strejcek M, Musilova L, Ridl J, Hroudova M, Vlcek C, Cardenas E, Mackova M, Macek T (2012) Identification of bacteria utilizing biphenyl, benzoate, and naphthalene in long-term contaminated soil. PLoS One 7(7):e40653. https://doi.org/10.1371/journal.pone.0040653
Van Dillewijn P, Caballero A, Paz JA, González-Pérez MM, Oliva JM, Ramos JL (2007) Bioremediation of 2,4,6-trinitrotoluene under field conditions. Environ Sci Technol 41:1378–1383
Venturi V, Keel C (2016) Signaling in the rhizosphere. Trends Plant Sci 21:187–198. https://doi.org/10.1016/j.tplants.2016.01.005
Vergani L, Mapelli F, Zanardini E, Terzaghi E, Di Guardo A, Morosini C, Raspa G, Borin S (2017) Phyto-rhizoremediation of polychlorinated biphenyl contaminated soils: an outlook on plant-microbe beneficial interactions. Sci Total Environ 575:1395–1406. https://doi.org/10.1016/j.scitotenv.2016.09.218
Zafra G, Absalón ÁE, Anducho-Reyes MÁ, Fernandez FJ, Cortés-Espinosa DV (2017) Construction of PAH-degrading mixed microbial consortia by induced selection in soil. Chemosphere 172:120–126. https://doi.org/10.1016/j.chemosphere.2016.12.038

Natural Attenuation of Hydrocarbon Compounds in Groundwater

11

Steven F. Thornton

Contents

1 Introduction 172
2 Conceptual Model for Natural Attenuation of Hydrocarbons in Groundwater 173
3 Sorption of Hydrocarbon Compounds in Groundwater 175
4 Biodegradation of Hydrocarbon Compounds in Groundwater 176
5 Performance Assessment of Natural Attenuation for Hydrocarbon Compounds 180
5.1 Mass Transport Heterogeneity 182
5.2 Process Heterogeneity 185
5.3 Stable Isotopes 188
6 Research Needs 189
6.1 Mechanistic Understanding of Microbial Community Interactions at Plume Interfaces 189
6.2 Quantitative Description of Microbial Interactions at Plume Interfaces 190
References 190

Abstract

Natural attenuation (NA) is a widely used strategy for the risk-based management of groundwater contaminated with hydrocarbons. New process-based conceptual models highlight the spatial distribution of biodegradation processes in plumes, controlled by microbial activity, electron acceptor bioavailability, and aquifer properties, as a key control on NA performance. The plume fringe is identified as a critical interface for enhanced microbial activity and biodegradation of hydrocarbons in plumes. Mass transport and process heterogeneity in aquifers must be adequately resolved to ensure reliable estimates of hydrocarbon attenuation. The adoption of mass discharge as a performance measure and use of high-

S. F. Thornton (✉)
Groundwater Protection and Restoration Group, Department of Civil and Structural Engineering, University of Sheffield, Sheffield, UK
e-mail: s.f.thornton@sheffield.ac.uk

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_3

resolution multilevel monitoring devices is recommended to address these issues. These concepts are illustrated with a case study example. Stable isotope methods are important tools in NA assessments and, when combined with hydrochemical analyses, offer new possibilities to characterize contaminant sources, biodegradation pathways, redox processes, element cycling, the relative contribution of different attenuation processes, and quantify biodegradation of hydrocarbons in groundwater.

1 Introduction

Natural attenuation (NA) of polluted subsurface environments refers to naturally occurring physical, chemical, and biological processes which occur in situ and act, in isolation or combination, without human intervention to reduce the mass, toxicity, mobility, flux, volume, or concentration of contaminants in soil and groundwater (Wiedemeier et al. 1995; ASTM 1998; U.S. EPA 1998a, b; Environment Agency 2000; McLaughlan et al. 2006). These in situ processes include biodegradation, abiotic degradation, dispersion, dilution, sorption, volatilization, and chemical or biological stabilization, transformation, or destruction (U.S. EPA 1999; Wiedemeier et al. 1999; McLaughlan et al. 2006; Rivett and Thornton 2008). The technical application of NA processes for the management of soil and contaminated groundwater is termed *monitored natural attenuation* (MNA). This emphasizes rigorous performance assessment of NA using appropriate monitoring strategies and analytical tools to demonstrate an acceptable reduction in environmental risk at a specific compliance point or receptor within a reasonable timeframe (U.S. EPA 1998a; Environment Agency 2000; Rügner et al. 2006). Example receptors include humans (by direct exposure), potable water supply wells, and surface water bodies, whereas the legal boundary of a site is a typical compliance point, although many other examples may be identified in specific circumstances. Different receptors may be identified at various stages in the management of a contaminated site, during future site development or use and for different contaminants, according to their properties. While NA can be implemented to manage inorganic contaminants in both soil and groundwater, this chapter describes the theoretical concepts and practical application for hydrocarbon compounds in groundwater. These are illustrated by reference to petroleum hydrocarbons, a common pollutant in groundwater, but also underpin the implementation of NA for other similar organic compounds (e.g., polyaromatic hydrocarbons, diesel, phenols, coal tar compounds). The reader is referred to other texts for the assessment of NA in soils (Mulligan and Yong 2004; Khan et al. 2011), for chlorinated organic compounds (RTDF 1997; U.S. EPA 1998b; Environment Agency 1999; Wiedemeier et al. 1999) and for inorganic compounds (U.S. EPA 2007a, b).

Scientifically and operationally NA is distinct from bioremediation in that only biological processes are considered in bioremediation, whereas these contribute with many other nonbiological processes to NA (Alvarez and Illman 2006; U.S. EPA

2006; Rivett and Thornton 2008). The key technical components underpinning the implementation of NA for groundwater are (i) a site-specific risk assessment, (ii) development of a conceptual model which describes NA processes and translation of this understanding into a mathematical model for prediction, and (iii) demonstration and performance assessment of NA against stated site management and remediation objectives (Wiedemeier et al. 1999; Environment Agency 2000; Alvarez and Illman 2006; McLaughlan et al. 2006). The risk assessment seeks to identify the relevant contaminant linkage(s) in terms of the source-pathway-receptor (or target) framework (Environment Agency 2006). Characterizing the source (e.g., type, form, distribution, quantity of contaminants) and quantifying NA processes along the pathway (e.g., aquifer) are critical to the robust prediction of potential impacts on receptors, risk reduction, and effectiveness of NA in groundwater. If this analysis indicates remediation is necessary at a site, then the identified risks can be mitigated to an acceptable level by reducing the source mass or concentration, and/or reducing the flux of contaminants or preventing migration along the pathway, and/or removing or isolating the receptor. In most cases source reduction measures will be required to support the implementation of NA for contaminated groundwater, to ensure the achievement of remediation objectives in reasonable timescales (Environment Agency 2000; Chapelle et al. 2003).

2 Conceptual Model for Natural Attenuation of Hydrocarbons in Groundwater

Groundwater contamination by hydrocarbons originates from sources (e.g., leaking underground storage tanks, pipeline distribution systems, refining facilities, coal-gasification and chemical manufacturing plants, fuel transportation, and waste disposal practices) that release pure or dissolved phase hydrocarbon compounds to soil or the unsaturated zone. The dissolved phase compounds migrate to the water table in recharge water, which infiltrates the unsaturated zone. Pure liquids move under gravity to the water table, where they either form an immiscible phase in the capillary fringe if less dense (light nonaqueous phase liquid, LNAPL) than water (e.g., petroleum fuel) (CL:AIRE 2014), or migrate below the water table if more dense (e.g., coal tar) (Environment Agency 2003). The constituent compounds also dissolve into groundwater, limited thermodynamically by their effective solubility in the mixture (Thornton et al. 2013).

A general conceptual model of a contaminant plume formed in groundwater from a release of hydrocarbons is shown in Fig. 1. The plume is shown within the Source-Pathway-Target risk assessment framework. It is created by the continuous dissolution of organic compounds from the hydrocarbon source and transport of these to the water table, where groundwater advection and dispersion distribute the mass down hydraulic gradient of the release point. The plume source geometry and composition, ambient flow field, physical heterogeneity of the aquifer, and availability of electron acceptors for biodegradation exert an important control on the spatial and temporal distribution of hydrocarbon compounds in groundwater. Volatilization may reduce

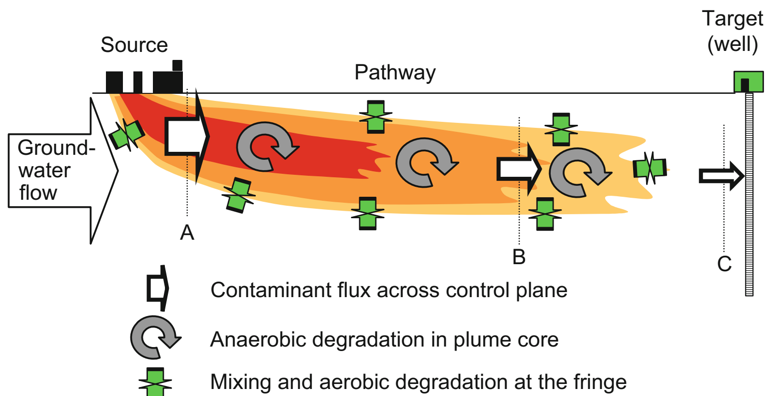


Fig. 1 Conceptual model for natural attenuation of hydrocarbon compounds in groundwater, in which contaminant fluxes are reduced during flow from a source along the aquifer pathway to a receptor by a combination of spatially distributed aerobic and anaerobic biodegradation processes, as shown

the concentration of dissolved contaminants in groundwater, where the plume is located at the water table (CL:AIRE 2014). Other abiotic processes such as sorption contribute to the attenuation of hydrocarbon compounds by slowing (retarding) their migration in the plume, according to the contaminant-specific properties (e.g., relative hydrophobicity) and the aquifer geochemistry (Schwarzenbach et al. 2003). When originating from a single source area, the plume boundary is usually sharply defined by transverse dispersion in the aquifer, a process which induces mixing of the contaminated and uncontaminated groundwater. Dispersion is controlled by the aquifer physical properties and is important in diluting the contaminant concentrations and supplying dissolved electron acceptors for biodegradation in the plume (Chapelle et al. 2003; McLaughlan et al. 2006). In most cases, biodegradation of hydrocarbons at the plume fringe will largely be limited by the small magnitude of transverse dispersion in aquifers (Lerner et al. 2000; Thornton et al. 2001a, b; Rahman et al. 2005; Watson et al. 2005; Gutierrez-Neri et al. 2009). However, in general biodegradation is usually more important than either physical or chemical processes for the natural attenuation of hydrocarbon compounds in groundwater.

Contaminated groundwater is transported from the source at A, through the plume B, to the receptor at C. If the flux and concentration of pollutants measured across the *control planes* is reduced sufficiently to make the risks to the receptor acceptably low, then NA is considered to be successful. The control planes are virtual points of reference, used at different locations along the plume flowpath to estimate the change in contaminant concentration or flux over the distance between them. This measure is used in more sophisticated monitoring strategies to assess the performance of NA for site management (see Sect. 5). Biodegradation of the organic contaminants by aerobic respiration, nitrate reduction, and sulfate reduction using dissolved electron acceptors (O_2, NO_3, SO_4) will occur at the periphery or *fringe* of the plume, driven by mixing between the plume and background groundwater (Lerner et al. 2000; Thornton et al. 2001a; Bauer et al.

2008; Gutierrez-Neri et al. 2009; Eckert et al. 2015). Slower anaerobic biodegradation by manganese reduction, iron reduction, and methanogenesis will occur inside the plume core, using mineral Mn and Fe oxide fractions on the aquifer sediment and fermentation processes. Generally, microbial activity and biodegradation processes at the plume fringe are more important for attenuation than processes occurring in the plume core (Thornton et al. 2001b; McLaughlan et al. 2006; Tuxen et al. 2006; Bauer et al. 2008; Tischer et al. 2013; Eckert et al. 2015). The plume will increase in length until a steady state is reached, in which the contaminant flux from the source is balanced by the rate of attenuation from the combined fringe mixing-controlled and the slower internal reactions (Chapelle et al. 2003; Gutierrez-Neri et al. 2009). Source management measures are typically implemented with NA to both reduce the duration and steady-state length of the plume (McLaughlan et al. 2006). If the source is depleted (e.g., by natural weathering) or removed through source management measures (e.g., remediation), the plume may become detached from the source and migrate down hydraulic gradient as a separate entity (Wiedemeier et al. 1999).

3 Sorption of Hydrocarbon Compounds in Groundwater

Sorption of dissolved hydrophobic hydrocarbons in groundwater is generally well described by the partitioning of the organic compound within the particulate organic matter fraction of the aquifer sediment (Schwarzenbach et al. 2003; Chapelle et al. 2003). At the low dissolved concentrations of organic chemicals typically found in most plumes, this process can be described by a linear isotherm and estimated from laboratory batch sorption tests or using empirical relationships (McLaughlan et al. 2006):

$$K_d = K_{oc} . f_{oc}$$

where K_d is the distribution coefficient of the organic chemical between the groundwater and aquifer at equilibrium (L^3/M), K_{oc} is the distribution coefficient of the organic chemical normalized to the total organic carbon content (L^3/M), and f_{oc} is the fraction of organic carbon present in the aquifer material (fraction). Other relationships have been developed to estimate K_d according to the fundamental properties of the organic chemicals, such as their octanol-water partition coefficient, K_{ow} (Fetter 2001), and also extended to predict sorption at higher concentrations using Freundlich and Langmuir isotherms (Schwarzenbach et al. 2003). The transport of organic chemicals in a plume can then be estimated according to the following relationship:

$$R_f = 1 + \frac{\rho_b K_d}{\theta}$$

where R_f is the retardation factor for the organic chemical, relative to the groundwater velocity [dimensionless], ρ_b is the bulk density of aquifer material (M/L^3), and θ is the porosity of the aquifer material (fraction).

Table 1 Relative mobility of common organic chemicals in groundwater

Compound	Solubility (ppm)	K_{oc}	Mobility class
Acetone	Miscible	1	Very high
2-Methylphenol (*o*-cresol)	31,000	15	Very high
4-Methylphenol (*p*-cresol)	24,000	17	Very high
2,4-Dimethylphenol (2,4-xylenol)	17,000	21	Very high
Phenol	82,000	27	Very high
2-Chlorophenol	11,087	27	Very high
Benzene	1,780	97	High
Toluene	500	242	Moderate
o-Xylene	170	363	Moderate
p-Xylene	156	552	Low
m-Xylene	146	588	Low
Ethylbenzene	150	622	Low
3,5-Dimethylphenol (3,5-xylenol)		1,038	Low
2,6-Dimethylphenol (2,6-xylenol)		1,060	Low
Naphthalene	32	1,300	Low
Phenanthrene	1.3	23,000	Immobile

Data source: Fetter (2001)

Sorption results in retardation of contaminants relative to the groundwater velocity and limits plume migration until it reaches steady state (Chapelle et al. 2003). It can be important for the NA of more hydrophobic hydrocarbon compounds in aquifers with relatively higher particulate organic carbon (e.g., alluvial and carbonaceous materials). Table 1 compares the relative mobility of organic chemicals in groundwater, according to their solubility and respective K_{oc} value. More soluble organic chemicals (e.g., phenol and cresols) are highly mobile in groundwater and typically migrate further from the point of release, whereas polyaromatic hydrocarbons (e.g., naphthalene and phenanthrene) have relatively limited mobility and may be restricted to the plume source area. This knowledge helps inform monitoring programs for NA, by identifying the expected type and spatial distribution of organic chemicals which may be present in groundwater, according to the known source composition (e.g., a release of coal tar vs. gasoline).

4 Biodegradation of Hydrocarbon Compounds in Groundwater

Biodegradation of hydrocarbon compounds in groundwater occurs by aerobic and anaerobic pathways, coupled to the reduction of dissolved electron acceptors (oxygen, nitrate, sulfate, and carbon dioxide) and mineral oxidants (manganese and iron oxides) on the aquifer sediment (Chapelle 1993; Borden 1994). In most cases, biodegradation of these compounds occurs through primary metabolism, in which the microorganisms use the hydrocarbon compounds as carbon and energy sources

(Wiedemeier et al. 1999), although co-metabolism is also important (Borden 1994; Alvarez and Illman 2006). The various pathways by which hydrocarbon compounds can be biodegraded in groundwater to organic intermediates or mineralized to carbon dioxide and the microorganisms responsible have been extensively studied. A detailed analysis of these is outside the scope of this chapter, but can be found in many excellent reviews (Smith 1991; Cerniglia 1992; Harayama and Timmis 1992; Haritash and Kaushik 2009). This section describes the biogeochemical processes which develop in hydrocarbon-contaminated groundwater as a basis to interpret and predict the natural attenuation of these compounds.

Molecular oxygen is used by aerobic bacteria as the terminal electron acceptor for respiration, whereas both facultative and obligate anaerobic microorganisms can facilitate anaerobic oxidation of hydrocarbons (Borden 1994). For a given oxidizable substrate (i.e., hydrocarbon compound), these electron acceptors are typically consumed in the order oxygen, nitrate, Mn-oxide, Fe-oxide, sulfate, and carbon dioxide, according to the decreasing energy yield to microorganisms mediating the reactions (Chapelle 1993; Reinhard 1994). The microorganisms release this energy by coupling the oxidation of an electron donor (the hydrocarbon compound) with the reduction of an electron acceptor in a redox reaction. The specific energy yield of a redox reaction can be quantified using the Gibbs free energy of the reaction (ΔG_r^o). Calculated values of ΔG_r^o for selected electron acceptor and electron donor (hydrocarbon compound) half-cell reactions are shown in Table 2 and expressed per mole e- transferred. Coupled redox reactions are expected to occur in the order of their thermodynamic energy yield, provided microorganisms facilitating each reaction are present and there is adequate supply of electron acceptors for biodegradation. For a given redox condition and availability of electron acceptors, this implies a preferred order of hydrocarbon biodegradation in mixtures. For example, aerobic respiration of BTEX would theoretically occur in the order $B > T = E > X$, although other factors (e.g., presence of competing substrates and viable microorganisms supporting biodegradation) may affect this sequence (Environment Agency 1999; Wiedemeier et al. 1999; Spence et al. 2005).

In hydrocarbon-contaminated aerobic groundwater, these microbially mediated reactions create a characteristic sequence of spatially and temporally dynamic redox processes, comprising successive zones of predominantly aerobic respiration, denitrification, Mn/Fe-reduction, sulfate reduction, and methanogenesis along the flowpath from the plume source (Chapelle 1993; Borden 1994; Williams et al. 2001). Characteristically this creates steep biogeochemical gradients across the plume fringe, driven by microbial activity and consumption of electron acceptors, where hydrocarbon biodegradation is enhanced (Thornton et al. 2001a; Thornton et al. 2014; Tuxen et al. 2006; Prommer et al. 2009; Tischer et al. 2013). The development of these redox zones (Fig. 2) is sustained by the supply of hydrocarbon (and other oxidizable) compounds from the plume source and the availability of both dissolved and mineral-based electron acceptors in the aquifer. This is critical for the natural attenuation of hydrocarbons in groundwater, given that biodegradation of specific compounds may only occur under specific redox conditions (Borden et al. 1997; Wiedemeier et al. 1999; Banwart and Thornton 2003, 2010; Wilson et al. 2004).

Table 2 Gibbs free energy of reaction for electron donor and electron acceptor half-cell reactions for selected hydrocarbon compounds

Species	Electron donor half-cell reaction[a]	ΔGro (kJ/mol e⁻)	Species	Electron acceptor half-cell reaction[b]	ΔGro (kJ/mol e⁻)
Benzene	$C_6H_6 + 12H_2O > 6CO_2 + 30H^+ + 30e^-$	−29.44	Hexachlorobenzene[c]	$2e^- + H^+ + C_6Cl_6 > C_6HCl_5 + Cl^-$	−39.33
Toluene	$C_7H_8 + 14H_2O > 7CO_2 + 36H^+ + 36e^-$	−28.91	Tetrachlorobenzene[c]	$2e^- + H^+ + C_6H_2Cl_4 > C_6H_3Cl_3 + Cl^-$	−36.40
Ethylbenzene	$C_8H_{10} + 16H_2O > 8CO_2 + 42H^+ + 42e^-$	−28.91	Trichlorobenzene[c]	$2e^- + H^+ + C_6H_3Cl_3 > C_6H_4Cl_2 + Cl^-$	−34.73
m-Xylene	$C_6H_4(CH_3)_2 + 16H_2O > 8CO_2 + 42H^+ + 42e^-$	−28.62	**Redox process[d]**		
1,2,4-Trimethylbenzene	$C_6H_3(CH_3)_3 + 18H_2O > 9CO_2 + 48H^+ + 48e^-$	−28.43	Aerobic respiration	$4e^- + 4H^+ + O_2 > 2H_2O$	−77.40
Phenol	$C_6H_6O + 11H_2O > 6CO_2 + 28H^+ + 28e^-$	−30.81	Denitrification	$5e^- + 6H^+ + NO_3^- > N_2 + 3H_2O$	−70.71
Naphthalene	$C_{10}H_8 + 20H_2O > 10CO_2 + 48H^+ + 48e^-$	−28.82	Mn(IV) reduction	$2e^- + 4H^+ + MnO2(s) > Mn^{2+} + 2H_2O$	−36.03
Tetrachlorobenzene[c]	$C_6H_2Cl_4 + 12H_2O > 6CO_2 + 26H^+ + 4Cl^- + 22e^-$	−51.86	Fe(III) reduction	$e^- + 3H^+ + FeOOH > Fe^{2+} + 2H_2O$	−64.62
Trichlorobenzene[c]	$C_6H_3Cl_3 + 12H_2O > 6CO_2 + 27H^+ + 3Cl^- + 24e^-$	−44.49	Sulfate reduction	$8e^- + 8H^+ + SO_4^{2-} > S^{2-} + 4H_2O$	22.45
Dichlorobenzene[c]	$C_6H_4Cl_2 + 12H_2O > 6CO_2 + 28H^+ + 2Cl^- + 26e-$	−38.41	Methanogenesis	$8e^- + 8H^+ + CO_{2(g)} > CH_{4(g)} + 2H_2O$	24.95
Chlorobenzene[c]	$C_6H_5Cl + 12H_2O > 6CO_2 + 29H^+ + Cl^- + 28e^-$	−33.51			
Carbohydrate	$CH_2O + H_2O > CO_2 + 4H^+ + 4e^-$	−41.84			

[a]Electron donor half-cell reactions define the relevant species which will be oxidized in a redox reaction involving an electron acceptor
[b]Electron acceptor half-cell reactions define the relevant species which will be reduced in a redox reaction involving an electron donor
[c]Chlorobenzene compounds are chlorinated aromatic hydrocarbons which can be biodegraded and biotransformed in redox reactions as an electron donor or electron acceptor, according to the relative oxidation status of the compound
[d]Common redox processes in hydrocarbon-contaminated aquifers, presented as generic electron acceptors which participate in the reduction of organic and inorganic electron donors

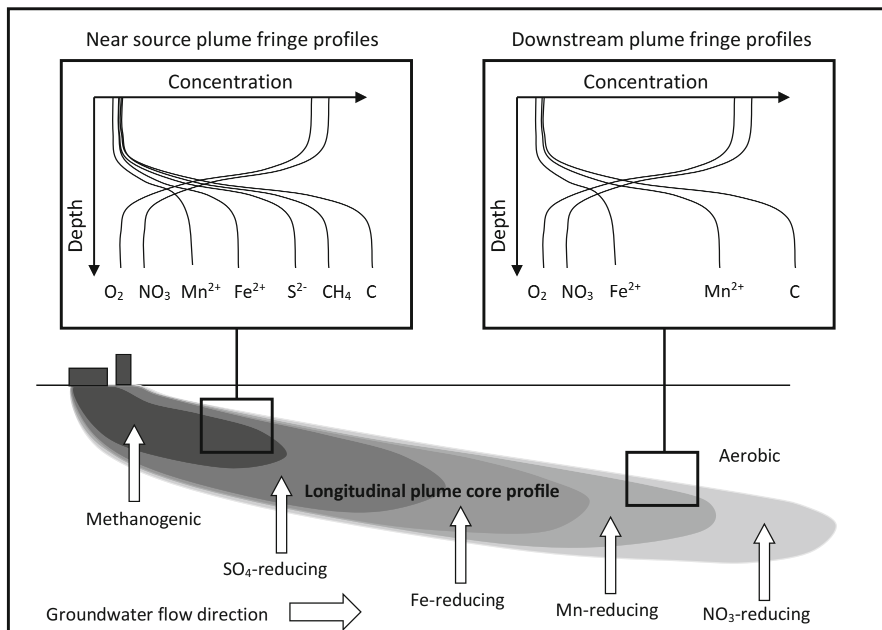


Fig. 2 Idealized conceptual model in longitudinal section and vertical profile of redox zone distribution in groundwater for a typical plume of oxidizable hydrocarbon compounds

The aquifer oxidation capacity (OXC) provides a convenient measure of the total reservoir of electron acceptors from all sources that can support hydrocarbon biodegradation in a per unit volume of aquifer (Heron et al. 1994). This is given by:

$$\begin{aligned} \mathrm{OXC} = {} & 4[\mathrm{O_2(aq)}] + 5[\mathrm{NO_3(aq)}] + 2[\mathrm{MnO_2(s)}] + [\mathrm{FeOOH(s)}] + 8[\mathrm{SO_4(aq)}] \\ & + 8[\mathrm{CO_2(aq)}] \end{aligned}$$

where [ ] denotes concentration or mass in moles, (aq) denotes aqueous concentration, and (s) denotes solid mineral species. A key assumption of this analysis is that all electron acceptors are available for biodegradation. However, biodegradation using dissolved electron acceptors is potentially limited by the relatively low solubility of oxygen or rate of mass transfer of oxygen, nitrate, and sulfate into the plume by dispersion (Thornton et al. 2001a, b; 2014). Moreover, while aquifer sediments typically have a significant reservoir of mineral-bound electron acceptors as Mn- and Fe-oxides, only the easily reducible amorphous forms will contribute oxidation capacity for hydrocarbon biodegradation in groundwater (Borden 1994; Wiedemeier et al. 1999; Banwart and Thornton 2003; Thornton et al. 2011). Estimates of OXC therefore represent an upper limit on the maximum concentration of hydrocarbons that can be biodegraded according to the theoretical consumption of the total mass of electron acceptors in the aquifer.

5 Performance Assessment of Natural Attenuation for Hydrocarbon Compounds

The performance assessment of natural attenuation for hydrocarbons in groundwater aims to (i) characterize the nature and spatial extent of in situ biodegradation processes for the hydrocarbons, (ii) estimate hydrocarbon biodegradation rates, and (iii) confirm NA occurs at a rate which will reduce environmental risk, protect identified receptors, and achieve site management/remediation objectives. It also identifies the need for additional measures if NA will not achieve remediation requirements in isolation. These aims are fulfilled through an intensive long-term monitoring program of groundwater quality and source composition, supported by a technically rigorous evaluation of the site investigation and groundwater quality data, as documented in relevant technical protocols and guidance (AFCEE 1995; Buscheck and O'Reilly (1995); ASTM 1998; Environment Agency 1999, 2000; U.S. EPA 1999; Wiedemeier et al. 1999; CGER 2000). Current best practice for the performance assessment of NA may involve the integrated analysis of hydrochemical, isotopic, microbiological, and other data using a wide range of qualitative and quantitative techniques (reviewed in Bombach et al. (2010) and Hunkeler and Aravena (2010)) at different scales (Table 3) to document the occurrence and extent of hydrocarbon attenuation by in situ processes.

This analysis is formalized within a "lines of evidence" framework, which includes the collection of different types of information (AFCEE 1995; ASTM 1998; U.S. EPA 1998a, b; Wiedemeier et al. 1999; Environment Agency 1999, 2000; National Academy of Sciences 2000; Alvarez and Illman 2006; McLaughlan et al. 2006):

- **Primary line of evidence**: Field time-series data showing a consistent reduction in the contaminant concentration or flux over time at one or more points along the source-pathway-receptor linkage, typically used to deduce the status of the plume (i.e., expanding, stable or shrinking), relative to predictions of groundwater flow
- **Secondary line of evidence**: Field data demonstrating in situ biodegradation of hydrocarbons in the plume, typically based on the consumption of dissolved (e.g., O_2, NO_3, SO_4) and mineral phase (e.g., MnO_4, FeOOH) electron acceptors in the aquifer and corresponding increase in organic metabolites, inorganic reaction products (e.g., Mn^{2+}, Fe^{2+}, HS^-), and other chemical species (e.g., dissolved CO_2 and CH_4) that verify different biodegradation processes
- **Tertiary line of evidence**: Supporting evidence related to the verification of natural attenuation processes, such as laboratory microcosm studies to quantify biodegradation rates, confirmation of biodegradation mechanisms and pathways using stable isotope analysis, or the use of molecular biological techniques to demonstrate the activity of appropriate hydrocarbon-degrading microorganisms

Not all lines of evidence are required in every case, but rather an appropriate level of data collection and interpretation will apply. However, this analysis will typically include mathematical modeling studies to verify the site conceptual model

Table 3 Data collection and analysis typically used for the performance assessment of hydrocarbon natural attenuation in groundwater

Indicator or measurement	Basis for performance assessment
Hydrochemical-based	
Decreased electron acceptor concentration	Utilization by microorganisms during biodegradation of specific hydrocarbons
Increased inorganic carbon concentration	Production from biodegradation and mineralization of hydrocarbon compounds and organic metabolites
Stoichiometry and mass balance between reactants and products	Balanced consumption of electron acceptors and organic compounds (hydrocarbons and relevant organic metabolites) during biodegradation under specific redox conditions
Increased concentrations of intermediate-stage and final products	Identification of specific biodegradation pathways and transformation for hydrocarbons
Increased ratio of transformation products to parent compounds	Progressive biodegradation of parent compound to metabolite over space and time
Decreased ratio of reactant to inert tracer	Preferential biodegradation of hydrocarbon species relative to a nonreactive species in the same mixture, accounting natural attenuation due to abiotic processes
Relative rates of transformation of different hydrocarbons consistent with laboratory data	Independent confirmation of biodegradation potential at field-scale based on controlled laboratory studies
Stable isotope-based	
Changes in carbon, chloride, and hydrogen isotope ratios of specific organic contaminants (depending on respective chemical group), and isotope ratios of electron acceptors and inorganic carbon in CO_2 and CH_4	Biodegradation of specific organic compounds (if based on compound-specific isotope analysis, CSIA), biodegradation of nonspecific compounds (if based on total dissolved carbon isotope ratios), and specific redox processes (if based on isotope ratios of electron acceptors)
Microbiological-based	
Increased number of (live/active) bacteria in hydrocarbon plume	Increase in size of viable microbial community for in situ biodegradation
Increase in relative abundance of known degraders in hydrocarbon plume	Increased contribution of specific microorganisms within community with capability for hydrocarbon biodegradation
Increase in abundance of genes associated with biodegradation in hydrocarbon plume	Development of metabolic pathways in microorganisms which enable biodegradation of specific hydrocarbons

underpinning the interpretation and to predict the long-term behavior of the plume for site management. Primary and secondary lines of evidence are obtained from the distribution of dissolved reactants along the plume flowpath, using groundwater samples collected from monitoring wells in the plume source area, uncontaminated and contaminated sections of the aquifer. The plume is presumed to have a centerline, characterized by the monitoring wells, for this analysis. Visual, graphical, and quantitative methods, including regression techniques, statistical analyses, and mass

balances (e.g., Table 3), are available to interpret these data using concentration versus time and concentration versus distance plots (see Wiedemeier et al. 1999; Environment Agency 2000; Alvarez and Illman 2006 for summary). An important objective is the estimation of plume-scale contaminant biodegradation rates from this analysis, often assumed to follow first-order kinetics for mathematical simplicity and use in numerical reactive transport codes (Buscheck and Alcantar 1995; Suarez and Rifai 1999; Beyer et al. 2007). Similarly, the maximum steady-state plume length and time to plume stabilization must also be predicted, usually to evaluate receptor impacts and remediation timescales. Various mathematical approaches and modeling tools have been developed to undertake this for hydrocarbons plumes, based on different conceptual models of plume development, substrate mixtures, and biodegradation processes (Chapelle et al. 2003; Atteia and Guillot 2007; Gutierrez-Neri et al. 2009).

A relatively sophisticated site assessment and monitoring program is required to verify the effectiveness of NA for hydrocarbons in groundwater (Wiedemeier et al. 2006). This is because the technical appraisal relies primarily on the evaluation of subsurface transport and biodegradation processes using the aquifer characteristics and groundwater quality data. However, data quality can be strongly influenced by heterogeneity in the spatial and temporal distribution of contaminants, geological and hydrogeological properties, and biodegradation processes, which must be resolved for correct performance assessment of NA. These issues are incorporated in the concepts of *mass transport heterogeneity* and *process heterogeneity*, which must be resolved in NA assessments.

5.1 Mass Transport Heterogeneity

Mass transport heterogeneity results from the spatial and temporal variability in contaminant distribution and plume development due to the combined effects of variation in plume source term (composition and location) aquifer properties (geological structure, stratigraphy and geochemistry) and hydrogeology (recharge, groundwater flow direction). The effect of these factors on plume development is illustrated by considering a series of generic plume scenarios (not exhaustive of all possible scenarios), shown in Fig. 3. If the plume has a unique or representative centerline that can be identified (Fig. 3a), then centerline monitoring may be possible and a pseudo-first-order contaminant biodegradation rate estimated from the contaminant concentration versus distance profile (Buscheck et al. 1995; Wiedemeier et al. 1999). However, where a plume has a single dominant flowpath (i.e., a centerline), it may be missed by monitoring wells installed using an assumed, but incorrect, groundwater flow direction or if there is periodic lateral migration of the plume due to seasonal (or induced) variations in groundwater flow direction (Fig. 3b). Furthermore, macroscale physical heterogeneity (e.g., buried stream channels) may result in a "centerline" that deviates according to the subsurface geological structure (Fig. 3c) (Mackay et al. 2001). Rather than follow a unique centerline, contaminants may be transported along a "flowpath" within a series of relatively discrete plumes of

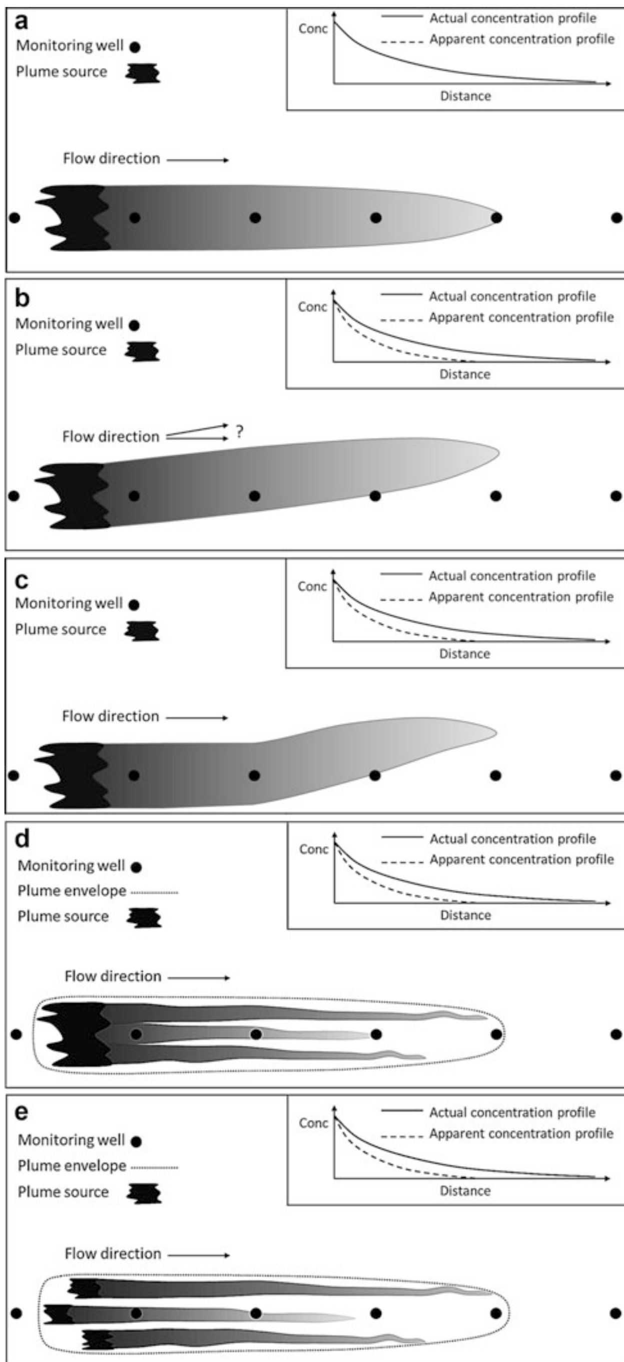


Fig. 3 Conceptual diagrams illustrating problem in establishing and instrumenting representative centerline monitoring in different scenarios: (**a**) uniformly distributed plume in homogeneous,

varying strength and/or migration rate (Jankowski et al. 1997; Lee et al. 2001). This can reflect preferential pathways due to heterogeneity in the aquifer hydrogeology, for example, layers with different permeability, cross-bedding or discontinuities (Fig. 3d) or distributed sources (Fig. 3f).

Hydrocarbon releases from source zones may also vary temporally as the source term naturally weathers or is depleted in more soluble components (Thornton et al. 2013), or due to water table fluctuations, recharge variations, and changes in groundwater flow direction (McMahon and Bruce 1997; Davis et al. 1999; McGuire et al. 2000; Mackay et al. 2001; Wilson et al. 2002). This variability may occur on a scale of weeks to months (water table, recharge and flow direction changes) or years (source term aging and compositional changes). Therefore, centerline monitoring typically requires more detailed site characterization to accurately define the spatial-temporal variation in plume flowpath.

The effect of mass transport heterogeneity on NA assessments is potentially significant. The assumption of a fixed centerline for a plume can mean that the primary pathway contributing most to contaminant migration can be missed. When the monitoring wells which intersect the plume are sampled, this can result in an apparent longitudinal contaminant concentration profile, which is less than the actual profile (Fig. 3a–c). A misleading interpretation of a much shorter plume length and overestimation of hydrocarbon attenuation along the flowpath would then arise.

An alternative approach is to use changes in contaminant *mass discharge* and *mass flux* through a series of monitoring wells set transverse to the plume flowpath (i.e., functioning as control planes) and capturing the full depth and width of the plume for performance assessment of NA (Kao and Wang 2001; Wilson et al. 2004; Alvarez and Illman 2006; ITRC 2010). Mass discharge and flux estimates quantify source or plume strength at a given time and location (ITRC 2010). *Mass flux* is a rate measurement specific to a defined area, which is usually a subset of a plume cross section, and expressed as mass/time/area. *Mass discharge* is an integrated mass flux estimate, representing the sum of all mass flux measures across an entire plume. It therefore gives the total mass of any solute transported in groundwater through a defined plane. If the total mass discharge of contaminants migrating through successive control planes decreases over time, NA is evident (Alvarez and Illman 2006).

The mass discharge of hydrocarbons (or any other dissolved chemical species) can be estimated from (API 2003; ITRC 2010):

Fig. 3 (continued) isotropic media, (**b**) uniformly distributed plume in homogeneous, isotropic media where hydraulic gradient is inaccurately defined or is temporally variable, (**c**) uniformly distributed plume in media with undetected macroscale heterogeneity (e.g., buried stream channel), (**d**) nonuniformly distributed plume migrating along preferred pathways in heterogeneous media within plume envelope, (**e**) nonuniformly distributed plume generated from heterogeneously distributed sources within plume envelope. Insert diagrams show resulting apparent and actual contaminant concentration-distance profiles

$$M_d = \sum_{i=1}^{i=n} C_i q_i A_i$$

where M_d is the total hydrocarbon mass discharge from the plume source that passes a given control plane (M/T), C_i is the concentration of a specific hydrocarbon measured at the flow area within the given control plane (M/L^3), q_i is the specific discharge (L/T), and A_i is the flow area associated with specific hydrocarbon measurement within the control plan (L^2). The value of q_i can be calculated from:

$$q_i = K.i$$

where K is the hydraulic conductivity of the flow area (L/T) and i is the aquifer hydraulic gradient (L/L).

Flux planes can capture spatially and temporally variable contaminant distribution, as well as estimate biodegradation rates using different methods, such as the sampling of a dense monitoring array (Borden et al. 1997; Kao and Wang 2001; Béland-Pelletier et al. 2011), control plane pumping of a more sparse array (Bockelmann et al. 2003; ITRC 2010; Béland-Pelletier et al. 2011), or a passive flux meter approach (Hatfield et al. 2004; ITRC 2010). In addition to defining the source strength and plume attenuation rate, mass flux estimates can identify areas of a plane through which most contaminant mass is moving. This helps target monitoring and remediation efforts (e.g., amendments to enhance NA) to optimize treatment. As such, flux-based methods provide a superior analysis of NA performance compared with methods based on assumed centerlines of plumes, by integrating results from many monitoring wells and accommodating plume heterogeneity in estimates of mass loss (API 2003; Wilson et al. 2004; Alvarez and Illman 2006; Beyer et al. 2007). However, this approach requires a higher density of groundwater monitoring wells or sampling stations, ideally using higher resolution MLS, to characterize the contaminant and hydraulic conductivity distribution in sufficient detail (API 2003; Alvarez and Illman 2006; ITRC 2010).

5.2 Process Heterogeneity

Process heterogeneity refers to the spatial and temporal distribution of biodegradation processes that occurs in plumes. As biodegradation is the most important attenuation mechanism for hydrocarbon compounds in groundwater, monitoring should focus on deducing the type and location of biodegradation processes in plumes from the groundwater chemistry and other data. This information is necessary to estimate biodegradation rates (Thornton et al. 2001b), and experience shows that contaminant biodegradation is strongly influenced by the evolution in redox conditions (Lyngkilde and Christensen 1992; Lerner et al. 2000; Cozzarelli et al. 2001; Thornton et al. 2001a), contaminant concentrations (Pickup et al. 2001; Wu et al. 2006; Baker et al. 2012) and microbial communities (Elliott et al. 2010;

Rizoulis et al. 2013) in plumes. While the characteristic longitudinal development of redox zones resulting from biodegradation of hydrocarbon compounds in groundwater (Fig. 2) has long been known (Champs et al. 1979; Barcelona et al. 1989; Chapelle et al. 2002), the importance of biodegradation at the plume fringe is now increasingly recognized as a major contribution to overall biodegradation in organic contaminant plumes (Pickup et al. 2001; Anneser et al. 2008; Prommer et al. 2009; Tischer et al. 2013; Thornton et al. 2014). However, weak vertical dispersive mixing means that the plume fringe zone may be less than 1 m thick (Davis et al. 1999; Schreiber and Bahr 1999; Cozzarelli et al. 1999; Cozzarelli et al. 2001; McGuire et al. 2000), with steep gradients in chemical species, such as electron acceptors, electron donors, and metabolites (see profiles in Fig. 2), and microorganisms supporting biodegradation (Tuxen et al. 2006).

The challenge of monitoring process heterogeneity in hydrocarbon plumes is therefore to adequately resolve the spatial and temporal distribution of biodegradation processes. This objective can only satisfactorily be achieved using high-resolution multilevel sampling (MLS) devices (Thornton et al. 2001a; Einarson and Cherry 2002; Spence et al. 2005; Anneser et al. 2008). Figure 4 shows vertical profiles of phenol and selected redox-sensitive chemical species across the plume fringe at two locations in an aromatic hydrocarbon plume, obtained from a MLS (1 m sampling interval), with black bars indicating the lengths of single screen monitoring wells typically used at the site. (Thornton et al. 2001b). It is evident that neither the vertical distribution of chemical species in the plume nor the steep gradients in these across the plume fringe can be adequately discriminated with the single screen monitoring wells, which straddle multiple redox zones and induce dilution, mixing, or averaging of dissolved chemical concentrations within the borehole, destroying any gradients. This artifact can result in erroneous interpretation of dominant biodegradation processes in hydrocarbon plumes and underestimation of biodegradation capacity (Martin-Hayden and Robbins 1997; Schreiber and Bahr 1999).

The MLS devices provide superior understanding of the chemical framework needed to quantify contaminant biodegradation and predict the bioattenuation potential of the plume as a whole. At the study site, they suggest that simultaneous aerobic respiration and NO_3^- and Fe^{3+} reduction occurs over a narrow 1 m mixing zone at the plume fringe. Further illustration is provided in Table 4, which shows a depth-integrated electron acceptor and carbon mass balance for the plume, estimated from the MLS at locations A and B using the methodology developed by Thornton et al. (2001b). The mass balance compares total electron acceptor consumption with contaminant consumption and production of organic and inorganic metabolites. It shows that aerobic respiration and denitrification at the plume fringe account for significantly greater biodegradation and carbon turnover than anaerobic biodegradation processes in the plume core, apart from fermentation (Table 4). This mass balance was also used to estimate. An overall plume-scale pseudo-first-order biodegradation rate of 0.005 $year^{-1}$ was estimated from this mass balance, two orders of magnitude lower than that obtained using data from the single-screen monitoring wells.

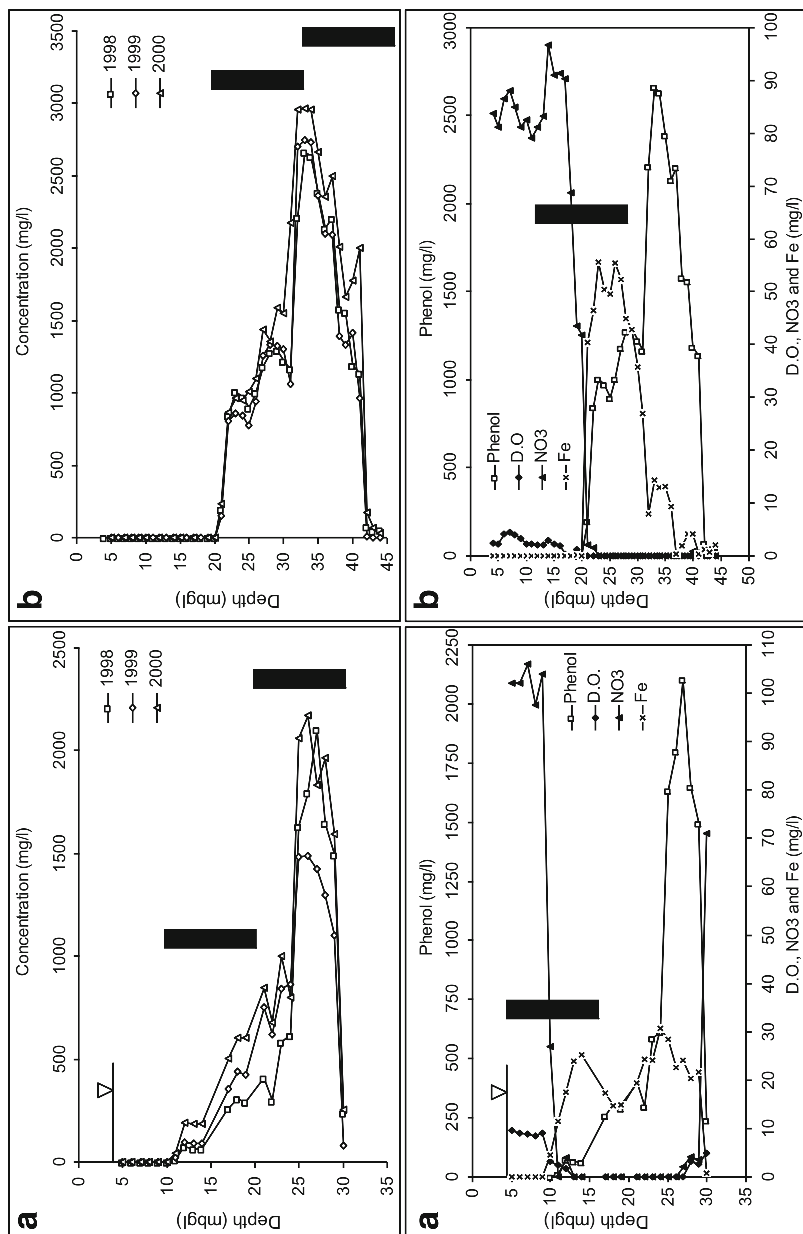


Fig. 4 Vertical profiles of phenol (successive annual surveys) and selected redox-sensitive chemical species (1998 survey) at near source MLS (**a**) and downstream MLS (**b**) in an aromatic hydrocarbon plume. *Black* bars are typical screen lengths for single screen monitoring wells at the site

Table 4 Contaminant carbon and aquifer oxidant consumption estimated from MLS vertical profiles at locations A and B

	Oxidant consumption ($kg \cdot m^{-2}$)[a]		Contaminant consumption (kg phenol-C$\cdot m^{-2}$)[a, b]			
Species	Borehole A	B	A	Residual phenol-C	Borehole B	Residual phenol-C
O_2	0.42	0.21	0.14		0.066	
NO_3	4.67	4.2	0.97		0.87	
SO_4	0.0016	0.001	0.00034		0.00022	
MnO_2	0.041	0.24	0.0032		0.018	
FeOOH	0.15	0.24	0.0056		0.009	
Acetate[c]			0.31		0.19	
Total	5.28	4.89	1.43	7.18	1.15	15.5

[a]Values are integrated over the depth of the MLS profile
[b]Consumption is presented in terms of equivalent phenol carbon and by assuming that all contaminants are present as phenol
[c]Acetate is assumed to be produced by fermentation of the hydrocarbon compounds

5.3 Stable Isotopes

Measurements of hydrochemical species in groundwater will not always provide conclusive proof of hydrocarbon biodegradation for NA assessments (Borden 1994; Hunkeler and Aravena 2010). For example, biodegradation of specific hydrocarbon compounds in mixtures can be difficult to deduce if common pathways exist, when organic metabolites are similar to potential parent compounds, or if analytes are present at trace levels that are difficult to quantify (Alvarez and Illman 2006; Spence et al. 2005). Also changes in redox conditions which are attributed to hydrocarbon biodegradation in groundwater can also arise from metabolism of other co-contaminants, including natural substances, confounding quantitative interpretation, and mass balances at the plume scale.

Many of these problems can be addressed using natural abundance and compound-specific stable isotope analysis (CSIA) of H, C, N, O, S, and Cl in hydrocarbon compounds, their biodegradation metabolites, and the respective elements in NO_3^-, SO_4^{2-}, CO_2, CH_4. The fundamental principles and environmental applications of stable isotope analysis have been extensively reviewed (Meckenstock et al. 2004; Hunkeler and Elsner 2010; Elsner and Imfeld 2016). With respect to the natural attenuation of hydrocarbons in groundwater, stable isotope analysis can be used to (i) identify the source(s) and origin (e.g., via biological or abiotic transformation) of a compound (both organic and inorganic) released to or formed in a given environment; (ii) deduce specific pathways and redox processes (e.g., aerobic vs. anaerobic) for biodegradation; (iii) assess the contribution of biotic and abiotic transformation processes affecting contaminants during subsurface transport; (iv) quantify the extent of biodegradation; (v) verify reactive transport models for prediction of environmental impacts, remediation system design, and treatment

timescales; and (vi) understand element cycling in natural and contaminated systems using isotope-labeled compounds (Höhener and Aelion 2010).

More recent development of stable isotope methods include the use of dual isotope analysis to interpret in situ biodegradation processes for aromatic hydrocarbons and other organic contaminants. Attention has mainly focused on understanding the reaction mechanisms and enzymes involved (Morasch et al. 2001), relevant metabolic pathways (Mancini et al. 2003; Fischer et al. 2008), characterizing the physiological groups of microorganisms facilitating biodegradation (Kümmel et al. 2015), deducing the dominant redox processes supporting biodegradation (Spence et al. 2001, 2005; Feisthauer et al. 2012; Thornton et al. 2014), and quantifying biodegradation at field scale (Hunkeler et al. 2001; Mancini et al. 2002, 2003; Richnow et al. 2003; Griebler et al. 2004; Meckenstock et al. 2004; Spence et al. 2005; Fischer et al. 2007; Hunkeler and Aravena 2010; Thornton et al. 2011; Lesser-Carillo 2014).

6 Research Needs

The process-based conceptual model which describes the spatial distribution of microbiological activity and biodegradation potential in hydrocarbon plumes is increasingly recognized as a fundamental biogeochemical framework to understand and predict the natural attenuation of a wide range of aromatic hydrocarbon contaminants in groundwater. The steep gradients in biogeochemical processes which characterize the plume fringe interface provide a unique environment for the biodegradation of oxidizable organic chemicals. However, further research is needed in two areas to understand the complex relationships that exist within the microbial communities which develop at this interface and to interpret their interactions in response to changing environmental conditions.

6.1 Mechanistic Understanding of Microbial Community Interactions at Plume Interfaces

It is likely that the composition, function, and activity of specific populations within the microbial community at the plume fringe will be sensitive to selective pressures created by the availability of organic substrates and electron acceptors, which in turn are controlled by the aquifer properties and groundwater flow field. Both competitive and synergistic interactions between microbial populations may characterize the biodegradation of hydrocarbons under these conditions, but this is poorly understood. Moreover, little is known about the characteristic response times of the microbial community to temporal variations in plume conditions, such as those which occur by natural evolution in the source term composition and by engineered interventions (e.g., injection of amendments to increase microbial activity and contaminant turnover). The work of Thornton et al. (2014) highlighted the potential to increase rates of NA in such circumstances, but the response, in particular the

recovery timescales, of planktonic and attached populations in a hydrocarbon plume to such intervention must be qualified and formerly linked to the biogeochemical drivers controlling this behavior.

6.2 Quantitative Description of Microbial Interactions at Plume Interfaces

Datasets to quantitatively measure and interpret the microbial interactions which develop at the plume fringe, supported by a theoretical framework which enables these observations to be translated into predictions of NA performance at field scale. This necessarily requires the improved mechanistic understanding outlined above and new modeling tools which can represent such dynamic relationships across different scales (microscale to plume scale). The work completed in Watson et al. (2005) highlighted the importance of such adaptive modeling frameworks to scale biogeochemical processes across the plume fringe but also emphasized the conceptual and theoretical limitations of existing modeling approaches.

References

AFCEE (Air Force Centre for Environmental Excellence) (1995) Technical protocol for implementing intrinsic remediation with long-term monitoring for natural attenuation of fuel contamination dissolved in groundwater, vol 1–2. AFCEE, Transfer Division, Brooks Air Force Base, San Antonio

Alvarez PJJ, Illman WA (2006) Bioremediation and natural attenuation. Process fundamentals and mathematical models. Wiley, Hoboken, p 614

Anneser B, Einsiedl F, Meckenstock RU, Richters L, Frank Wisotzky F, Griebler C (2008) High-resolution monitoring of biogeochemical gradients in a tar oil-contaminated aquifer. Appl Geochem 23:1715–1730

API (American Petroleum Institute) (2003). Groundwater remediation strategies tool, Publication no. 4730, p 80

ASTM (American Society for Testing and Materials) (1998) Standard guide for remediation of ground water by natural attenuation at petroleum release sites. ASTM Standard Guide, West Conshohocken. E1943-98

Atteia O, Guillot C (2007) Factors controlling BTEX and chlorinated solvents plume length under natural attenuation conditions. J Contam Hydrol 90:81–104

Baker K, Bottrell SH, Thornton SF, Peel K, Spence MJ (2012) Effect of contaminant concentration on in-situ bacterial sulphate reduction and methanogenesis in a phenol-contaminated aquifer. Appl Geochem 27:2010–2018

Banwart SA, Thornton SF (2003) The geochemistry and hydrology of groundwater bioremediation by natural attenuation. In: Head I, Singleton I (eds) Bioremediation: a critical review. Horizon Scientific Press, Wymondham, pp 93–138

Banwart SA, Thornton SF (2010) Natural attenuation of hydrocarbons in groundwater. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer Verlag, Berlin/Heidelberg, pp 2474–2486

Barcelona MJ, Holm TR, Schock MR, George GK (1989) Spatial and temporal gradients in aquifer oxidation-reduction conditions. Water Resour Res 25:991–1003

Bauer RD, Maloszewski P, Zhang Y, Meckenstock RU, Griebler C (2008) Mixing-controlled biodegradation in a toluene plume – results from two-dimensional laboratory experiments. J Contam Hydrol 96:150–168

Béland-Pelletier C, Fraser M, Barker J, Ptak T (2011) Estimating contaminant mass discharge: a field comparison of the multilevel point measurement and the integral pumping investigation approaches and their uncertainties. J Contam Hydrol 122:63–75

Beyer C, Chen C, Gronewold J, Kolditz O, Bauer S (2007) Determination of first-order degradation rate constants from monitoring networks. Groundwater 45:774–785

Bockelmann A, Zamfirescu D, Ptak T, Grathwohl P, Teutsch G (2003) Quantification of mass fluxes and natural attenuation rates at an industrial site with a limited monitoring network: a case study. J Contam Hydrol 60:97–121

Bombach P, Richnow HH, Kastner M, Fischer A (2010) Current approaches for the assessment of *in situ* biodegradation. Appl Microbiol Biotechnol 86:839–852

Borden RC (1994) Natural bioremediation of hydrocarbon-contaminated ground water. In: Handbook of bioremediation. Lewis Publishers, Boca Raton, pp 177–199

Borden RC, Daniel RA, LeBrun LE IV, Davis CW (1997) Intrinsic biodegradation of MTBE and BTEX in a gasoline-contaminated aquifer. Water Resour Res 33:1105–1115

Buscheck TE Alcantar CM (1995) Regression techniques and analytical solution to demonstrate intrinsic bioremediation. Intrinsic bioremediation. RE Hinchee, JT Wilson D Downey. Columbus, Battelle Press, 109–116

Buscheck T, O'Reilly K (1995) Protocol for monitoring intrinsic bioremediation in groundwater. Chevron Research and Technology Company, Health, Environment and Safety Group, Richmond,CA, p 20

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

CGER (Commission on Geosciences, Environment and Resources) (2000) Natural attenuation for groundwater remediation, chapter: 5 protocols for documenting natural attenuation. The National Academies Press, Washington, DC, pp 212–254

Champs DR, Gulens J, Jackson RE (1979) Oxidation-reduction sequences in groundwater flow systems. Can J Earth Sci 16:319–337

Chapelle FH (1993) Ground-water microbiology and geochemistry. Wiley, New York, pp 424

Chapelle FH, Bradley PM, Lovley DR, O'Neill K, Landmeyer JE (2002) Rapid evolution of redox processes in a petroleum hydrocarbon-contaminated aquifer. Groundwater 40: 353–360

Chapelle FH, Widdowson MA, Brauner JS, Mendez E, Casey CC (2003) Methodology for estimating times of remediation associated with monitored natural attenuation, U.S. Geological survey water-resources investigations report 03–4057, p 58

CL:AIRE (2014) An illustrated handbook of LNAPL transport and fate in the subsurface. CL: AIRE, London. ISBN: 978-1-905046-24-9. Download www.claire.co.uk/LNAPL

Cozzarelli IB, Herman JS, Baedecker MJ, Fischer JM (1999) Geochemical heterogeneity of a gasoline-contaminated aquifer. J Contam Hydrol 40:261–284

Cozzarelli IB, Bekins BA, Baedecker MJ, Aiken GR, Eganhouse RP, Tuccillo ME (2001) Progression of natural attenuation processes at a crude-oil spill site: I. Geochemical evolution of the plume. J Contam Hydrol 53:369–385

Davis GB, Barber C, Power TR, Thierrin J, Patterson BM, Rayner JL, Wu Q (1999) The variability and intrinsic remediation of a BTEX plume in anaerobic sulphate-rich groundwater. J Contam Hydrol 36:265–290

Eckert D, Kürzinger P, Bauer R, Griebler C, Cirpka OA (2015) Fringe-controlled biodegradation under dynamic conditions: quasi 2-D flow-through experiments and reactive-transport modeling. J Contam Hydrol 172:100–111

Einarson MD, Cherry JA (2002) A new multilevel ground water monitoring system using multichannel tubing. Ground Water Monit Remediat 22:52–65

Elliott DR, Scholes JD, Thornton SF, Rizoulis A, Banwart SA, Rolfe SA (2010) Dynamic changes in microbial community structure and function in phenol-degrading microcosms from a contaminated aquifer. FEMS Microbiol Ecol 71:247–259

Elsner M, Imfeld G (2016) Compound-specific isotope analysis (CSIA) of micropollutants in the environment – current developments and future challenges. Curr Opin Biotechnol 41:60–72

Environment Agency (1999). Natural attenuation of petroleum hydrocarbons and chlorinated solvents in groundwater, R&D technical report P305. p 135. ISBN: 1857051394

Environment Agency (2000) Guidance on the assessment and monitoring of monitored natural attenuation of contaminants in groundwater, R&D publication, vol 95. Environment Agency, Bristol

Environment Agency (2003) An illustrated handbook of DNAPL transport and fate in the subsurface. p 67. ISBN: 1844320669

Environment Agency (2006) Remedial targets methodology: hydrogeological risk assessment for land contamination. GEHO0706BLEQ-E-E, p 129

Feisthauer S, Seidel M, Bombach P, Traube S, Knöller K, Wange M, Fachmann S, Richnow HH (2012) Characterization of the relationship between microbial degradation processes at a hydrocarbon contaminated site using isotopic methods. J Contam Hydrol 133:17–29

Fetter CW (2001) Applied hydrogeology, 4th edn. Prentice-Hall, Upper Saddle River, p 615. ISBN: 0-13-088239-9

Fischer A, Theuerkorn K, Stelzer N, Gehre M, Thullner M, Richnow HH (2007) Applicability of stable isotope fractionation analysis for the characterization of benzene biodegradation in a BTEX-contaminated aquifer. Environ Sci Technol 41:3689–3696

Fischer A, Herklotz I, Herrmann S, Thullner M, Weelink SAB, Stams AJM, Scholmann M, Richnow H-H, Vogt C (2008) Combined carbon and hydrogen isotope fractionation investigations for elucidating benzene biodegradation pathways. Environ Sci Technol 42:4356–4363

Griebler C, Safinowski M, Vieth A, Richnow HH, Meckenstock RU (2004) Combined application of stable carbon isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. Environ Sci Technol 38:617–631

Gutierrez-Neri M, Ham PAS, Schotting RJ, Lerner DN (2009) Analytical modelling of fringe and core biodegradation in groundwater plumes. J Contam Hydrol 107:1–9

Harayama S, Timmis KN (1992) Aerobic biodegradation of aromatic hydrocarbons. In: Sigel H, Sigel A (eds) Metal ions in biological systems. Vol. 28: degradation of environmental pollutants by microorganisms and their metalloenzymes. Marcel Dekker, New York, pp 99–155

Haritash, A.K. and C.P. Kaushik, C.P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J Hazard Mater, 169, 1–15.

Hatfield K, Annable M, Chob J, Rao PSC, Klammler H (2004) A direct passive method for measuring water and contaminant fluxes in porous media. J Contam Hydrol 75:155–181

Heron G, Christensen TH, Tjell JC (1994) Oxidation capacity of aquifer sediments. Environ Sci Technol 28:153–158

Höhener P, Aelion CM (2010) Fundamentals of environmental isotopes and their use in biodegradation. In: Aelion CM et al (eds) Environmental isotopes in biodegradation and bioremediation. CRC Press, Boca Raton, pp 3–22

Hunkeler D, Aravena R (2010) Investigating the origin and fate of organic contaminants in groundwater using stable isotope analysis. In: Aelion CM et al (eds) Environmental isotopes in biodegradation and bioremediation. CRC Press, Boca Raton, pp 249–292

Hunkeler D, Elsner M (2010) Principles and mechanisms of isotope fractionation. In: Aelion CM et al (eds) Environmental isotopes in biodegradation and bioremediation. CRC Press, Boca Raton, pp 43–78

Hunkeler D, Anderson N, Aravena R, Bernasconi SM, Butler BJ (2001) Hydrogen and carbon isotope fractionation during aerobic biodegradation of benzene. Environ Sci Technol 35:3462–3467

ITRC (2010) Use and measurement of mass flux and mass discharge. Interstate Technology & Regulatory Council, Integrated DNAPL Site Strategy Team, Washington, DC, p 154. www.itrcweb.org

Jankowski J, Beck P, Acworth RI (1997) Vertical heterogeneity in the Botany Sands aquifer, Sydney, Australia: implications for chemical variations and contaminant delineation. Groundwater in the urban environment: problems, processes and management. Balkema, Rotterdam, pp 445–450

Kao CM, Wang YS (2001) Field investigation of natural attenuation and intrinsic biodegradation rates at an underground storage tank site. Environ Geol 40:622–631

Khan M, Zaidi A, Goel R, Musarrat J, (2011) Biomanagement of metal-contaminated soils, Environmental pollution, vol 20. Springer, Dordrecht. ISBN: 978-94-007-1913-2

Kümmel S, Herbst FA, Bahr A, Duarte M, Pieper DH, Jehmlich N, Seifert J, von Bergen M, Bombach P, Richnow HH, Vogt C (2015) Anaerobic naphthalene degradation by sulfate-reducing *Desulfobacteraceae* from various anoxic aquifers. FEMS Microbiol Ecol 91. https://doi.org/10.1093/femsec/fiv006

Lee JY, Cheon JY, Lee KK, Lee SK, Lee MH (2001) Factors affecting the distribution of hydrocarbon contaminants and hydrogeochemical parameters in a shallow sand aquifer. J Contam Hydrol 50:139–158

Lerner DN, Thornton SF, Spence MJ, Banwart SA, Bottrell SH, Higgo JJ, Mallinson HEH, Pickup RW, Williams GM (2000) Ineffective natural attenuation of degradable organic compounds in a phenol-contaminated aquifer. Ground Water 38:922–928

Lesser-Carillo LE (2014) $^{13}C/^{12}C$ isotope fractionation during aerobic and anaerobic biodegradation of naphthalene. Int J Geosci 5:206–213

Lyngkilde J, Christensen TH (1992) Fate of organic contaminants in the redox zones of a landfill leachate pollution plume (Vejen, Denmark). J Contam Hydrol 10:291–307

Mackay DM, Wilson RD, Scow KS, Einarson MD, Fowler B, Wood IA (2001) *In situ* remediation of MTBE at Vandenberg Air Force Base, California. Contam Soil Sediment Water Spring pp 43–46

Mancini SA, Lacrampe-Couloume G, Jonker H, van Breukelen BM, Groen J, Volkering F, Sherwood Lollar B (2002) Hydrogen isotopic enrichment: an indicator of biodegradation at a petroleum hydrocarbon contaminated field site. Environ Sci Technol 36:2464–2470

Mancini SA, Ulrich AC, Lacrampe-Couloume G, Sleep B, Edwards EA, Sherwood Lollar B (2003) Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. Appl Environ Microbiol 69:191–198

Martin-Hayden JM, Robbins GA (1997) Plume distortion and apparent attenuation due to concentration averaging in monitoring wells. Ground Water 35:339–346

McGuire JT, Smith EW, Long DT, Hyndman DW, Haack SK, Klug MJ, Velbel MA (2000) Temporal variations in parameters reflecting terminal-electron accepting processes in an aquifer contaminated with waste fuel and chlorinated solvents. Chem Geol 169:471–485

McLaughlan RG, Merrick NP, Davis GB (2006) Natural attenuation: a scoping review. CRC for Contamination Assessment and Remediation of the Environment technical report no. 3. p 73

McMahon PB, Bruce BW (1997) Distribution of terminal electron-accepting processes in an aquifer having multiple contaminant sources. Appl Geochem 12:507–516

Meckenstock RU, Morasch B, Griebler C, Richnow H-H (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated aquifers. J Contam Hydrol 75:215–255

Morasch B, Richnow HH, Schink B, Meckenstock R (2001) Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl Environ Microbiol 67:4842–4849

Mulligan CN, Yong RN (2004) Natural attenuation of contaminated soils. Environ Int 30:587–601

National Academy of Sciences (2000) Natural attenuation for groundwater remediation. Committee on ground water clean-up alternatives. National Research Council, National Academy Press, Washington, DC

Pickup RW, Rhodes G, Alamillo ML, Mallinson HEH, Thornton SF, Lerner DN (2001) Microbiological analysis of multilevel borehole samples from a contaminated groundwater system. J Contam Hydrol 53:269–284

Prommer H, Anneser B, Rolle M, Insiedl F, Griebler C (2009) Biogeochemical and isotopic gradients in a BTEX/PAH contaminant plume: model-based interpretation of a high-resolution field data set. Environ Sci Technol 43:8206–8282

Rahman MA, Jose SC, Nowak W, Cirpka OA (2005) Experiments on vertical transverse mixing in a large-scale heterogeneous model aquifer. J Contam Hydrol 80:130–148

Reinhard M (1994) In-situ bioremediation technologies for petroleum-derived hydrocarbons based on alternate electron acceptors (other than molecular oxygen). Handbook of bioremediation. Lewis Publishers, Boca Raton, Florida, pp 131–147
Richnow HH, Annweiler E, Michaelis W, Meckenstock RU (2003) Microbial degradation of aromatic hydrocarbons in a contaminated aquifer monitored by $^{13}C/^{12}C$ isotope fractionation. J Contam Hydrol 64:59–72
Rivett MO, Thornton SF (2008) Monitored natural attenuation of organic contaminants in groundwater: principles and application. Water Manag J 161:381–392
Rizoulis A, Elliott DR, Rolfe SA, Thornton SF, Banwart SA, Pickup RW, Scholes JS (2013) Diversity of planktonic and attached bacterial communities in a phenol-contaminated sandstone aquifer. Microb Ecol 66:84–95
RTDF (1997) Natural attenuation of chlorinated solvents in groundwater: principles and practices. Version 3.0. August. Remediation Technologies Development Forum
Rügner H, Finkel M, Kaschl A, Bittens M (2006) Application of monitored natural attenuation in contaminated land management – a review and recommended approach for Europe. Environ Sci Pol 9:568–576
Schreiber ME, Bahr JM (1999) Spatial electron acceptor variability: implications for assessing bioremediation potential. Biorem J 3:363–378
Schwarzenbach RP, Gschwend PM, Imboden DM (2003) Environmental organic chemistry, 2nd edn. Wiley-Interscience, New York, p 1327. ISBN: 0-47 1-35750-2
Smith MR (1991) The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation 1:191–206
Spence MJ, Bottrell S, Thornton SF, Lerner DN (2001) Isotopic modelling of the significance of sulphate reduction for phenol attenuation in a polluted aquifer. J Contam Hydrol 53:285–304
Spence MJ, Bottrell SH, Thornton SF, Richnow H, Spence KH (2005) Hydrochemical and isotopic effects associated with fuel biodegradation pathways in a chalk aquifer. J Contam Hydrol 79:67–88
Suarez MP, Rifai HS (1999) Biodegradation rates for fuel hydrocarbons and chlorinated solvents in groundwater. Biorem J 3:337–362
Thornton SF, Quigley S, Spence M, Banwart SA, Bottrell S, Lerner DN (2001a) Processes controlling the distribution and natural attenuation of phenolic compounds in a deep sandstone aquifer. J Contam Hydrol 53:233–267
Thornton SF, Lerner DN, Banwart SA (2001b) Assessing the natural attenuation of organic contaminants in aquifers using plume-scale electron and carbon balances: model development with analysis of uncertainty and parameter sensitivity. J Contam Hydrol 53:199–232
Thornton SF, Bottrell SH, Spence KS, Pickup R, Spence MJ, Shah N, Mallinson HEHM, Richnow HH (2011) Assessment of MTBE biodegradation in contaminated groundwater using ^{13}C and ^{14}C analysis: field and laboratory microcosm studies. Appl Geochem 26:828–837
Thornton SF, Tobin K, Smith JWH (2013) Comparison of constant and transient-source zones on simulated contaminant plume evolution in groundwater: implications for hydrogeological risk assessment. Ground Water Monit Remediat 33:78–91
Thornton SF, Baker KM, Bottrell SH, Rolfe SA, McNamee P, Forrest F, Duffield P, Wilson RD, Fairburn AW, Cieslak L (2014) Enhancement of in situ biodegradation of organic compounds in groundwater by targeted pump and treat intervention. Appl Geochem 48:28–40
Tischer K, Kleinsteuber S, Schleinitz KM, Fetzer I, Spott O, Stange F, Lohse U, Franz J, Neumann F, Gerling S, Schmidt C, Hasselwander E, Harms H, Wendeberg A (2013) Microbial communities along biogeochemical gradients in a hydrocarbon-contaminated aquifer. Environ Microbiol 15:2603–2615
Tuxen N, Albrechtsen HJ, Bjerg PL (2006) Identification of a reactive degradation zone at a landfill leachate plume fringe using high resolution sampling and incubation techniques. J Contam Hydrol 85:179–194
U.S. EPA (1998a) EPA policy on use of monitored natural attenuation for site remediation. Seminars on monitored natural attenuation for ground water, 1–3 to 1–6. U.S. EPA/625/K-98/001

U.S. EPA (1998b) Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water. Office of Research and Development, Washington, DC, p 248
U.S. EPA (1999) Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. U.S. EPA Office of Solid Waste and Emergency Response Directive 9200.4-17P, p 32
U.S. EPA (2006) In situ and ex situ biodegradation technologies for remediation of contaminated sites. Engineering issue, solid waste and emergency response, EPA 625/R-06/015, p 22
U.S. EPA (2007a) Monitored natural attenuation of inorganic contaminants in ground water, vol 1. Technical basis for assessment. EPA/600/R-07/139
U.S. EPA (2007b) Monitored natural attenuation of inorganic contaminants in ground water, vol 2. Assessment for non-radionuclides including arsenic, cadmium, chromium, copper, lead, nickel, nitrate, perchlorate, and selenium. EPA/600/R-07/140, p 124
Watson IA, Oswald SE, Banwart SA, Crouch RS, Thornton SF (2005) Modeling the dynamics of fermentation and respiratory processes in a groundwater plume of phenolic contaminants interpreted from laboratory-to field-scale. Environ Sci Technol 39:8829–8839
Wiedemeier T, Wilson JT, Kampbell DH, Miller RN, Hansen JE (1995) Technical protocol for implementing intrinsic remediation with long-term monitoring for natural attenuation of fuel contamination dissolved in groundwater. Air Force Center for Environmental Excellence – Technology Transfer Division, San Antonio
Wiedemeier TH, Rifai HS, Newell CJ, Wilson JT (1999) Natural attenuation of fuels and chlorinated solvents in the subsurface. Wiley, New York. ISBN: 0 471 19749 1
Wiedemeier TH, Barden MJ, Haas PE, Dickson WZ (2006) Designing monitoring programs to effectively evaluate the performance of natural attenuation. In: Nielsen DM (ed) Practical handbook of environmental site characterization and groundwater monitoring, 2nd edn. CRC Taylor and Francis, Boca Raton, pp 574–634
Williams GM, Pickup RW, Thornton SF, Lerner DN, Mallinson HEH, Moore Y, White C (2001) Biogeochemical characterisation of a coal-tar distillate plume. J Contam Hydrol 53:175–198
Wilson RD, Mackay DM, Scow KM (2002) *In situ* MTBE biodegradation supported by diffusive oxygen release. Environ Sci Technol 36:190–199
Wilson RD, Thornton SF, Mackay DM (2004) Challenges in monitoring the natural attenuation of spatially variable plumes. Biodegradation 15:359–369
Wu Y, Lerner DN, Banwart SA, Thornton SF, Pickup RW (2006) Persistence of fermentative process to phenolic toxicity in ground water. J Environ Qual 35:2021–2025

In Situ Groundwater Bioremediation

12

Terry C. Hazen

Contents

1 Introduction 198
2 Terminology 198
3 Characterization and Monitoring Feasibility 199
4 Biostimulation and Bioaugmentation of Groundwater 205
5 Intrinsic Bioremediation and Modeling 209
6 Research Needs 210
References 211

Abstract

In situ groundwater bioremediation of hydrocarbons has been used for more than 40 years. Most strategies involve biostimulation; however, recently bioaugmentation have been used for dehalorespiration. Aquifer and contaminant profiles are critical to determining the feasibility and strategy for in situ groundwater bioremediation. Hydraulic conductivity and redox conditions, including concentrations of terminal electron acceptors, are critical to determine the feasibility and strategy for potential bioremediation applications. Conceptual models followed by characterization and subsequent numerical models are critical for efficient and cost-effective bioremediation. Critical research needs in this area include better modeling and integration of remediation strategies with natural attenuation.

T. C. Hazen (✉)
Department of Civil and Environmental Engineering, Department of Earth and Planetary Sciences, Department of Microbiology, Institute for Secure and Sustainable Environment, University of Tennessee, Knoxville, TN, USA

Oak Ridge National Laboratory, Oak Ridge, TN, USA
e-mail: tchazen@utk.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_11

1 Introduction

A patent for in situ bioremediation of groundwater contaminated with gasoline by stimulating indigenous bacteria via nutrient injection into the terrestrial subsurface was issued to Dick Raymond in 1974 (U.S. Patent 3,846,290). He successfully demonstrated this technology and began commercial applications in 1972 (Raymond et al. 1977). Clearly in situ groundwater bioremediation has been used successfully for more than 50 years, and much is understood about where it is applicable, especially for petroleum contaminants. The really new bioremediation applications that have been done in the last 20 years are in the area of chlorinated solvent, PAH, PCB, dioxin, MTBE, metals, and radionuclides. Bioremediation has been around for a long time, only its application breadth in terms of types of contaminants and environments has increased in the last 20 years. This explosive proliferation of new applications and environments in the last 20 years, especially by companies trying to establish themselves with a proprietary edge, has led to a large number of terms, many of which are highly redundant, in what they try to uniquely describe. Also, the bioremediation field applications that have been reported frequently lack comprehensive field data, especially in the terrestrial subsurface. Though bioremediation has been used at a large number of sites, these applications were nearly all done by companies trying to do the study for (1) clients, who usually wanted to remain confidential, (2) the least possible cost to the client and the vendor, and (3) protecting the vendors proprietary edge for their product. This has led to a paucity of peer-reviewed data, miss application of terminology, and confusion as to what some terms mean. More importantly it has also led to many “failures” of in situ groundwater bioremediation due to a lack of fundamental understanding of requirements, and limitations, in terms of hydrology, geology, and biogeochemistry at various scales.

2 Terminology

Biological Treatment – Any treatment process that involves organisms or their products, e.g., enzymes.

Biotransformation – A biological treatment process that involves changing the contaminant, e.g., valence states of metals, chemical structure, etc.

Intrinsic Bioremediation – Unmanipulated, unstimulated, unenhanced biological remediation of an environment; i.e., biological natural attenuation of contaminants in the environment, also known as monitored natural attenuation.

Engineered Bioremediation – Any type of manipulated or stimulated or enhanced biological remediation of an environment.

Biostimulation – The addition of organic or inorganic compounds to cause indigenous organisms to effect remediation of the environment, e.g., fertilizer.

Bioaugmentation – The addition of organisms to effect remediation of the environment, e.g., contaminant-degrading bacteria injection into an aquifer.

Biosparging – Injection of air or specific gases below ground, usually into saturated sediments (aquifer material) to increase biological rates of remediation.

Bioslurping – This treatment combines soil vapor extraction with removal of light nonaqueous phase liquid contaminants from the surface of the groundwater table, thereby enhancing biological treatment of the unsaturated zone and the groundwater, especially the capillary fringe zone where hydrocarbons tend to smear.

Biofilters – Normally used to refer to treatment of gases by passing through a support material containing organisms, e.g., soil, compost, trickle filter. Sometimes used to refer to treatment of groundwater via passage through a biologically active area in the subsurface.

Biocurtain – The process of creating a subsurface area of high biological activity to contain or remediate, usually in aquifer material.

Bioremoval – A biological treatment involving uptake of the contaminant from the environment by an organism or its agent.

Bioimmobilization – A biological treatment process that involves sequestering the contaminant in the environment. No biodegradation of the contaminant, e.g., metal bioreduction.

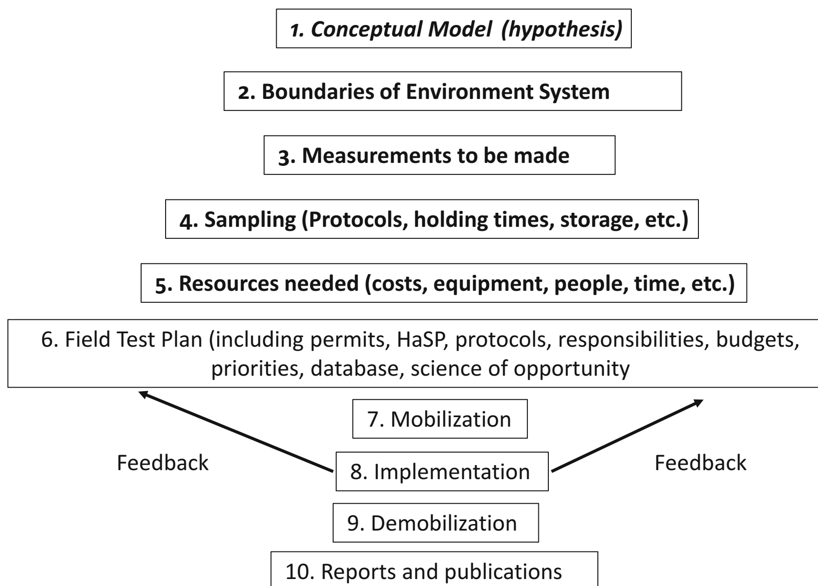
Biomobilization – A biological treatment process that involves making the contaminant more mobile in the environment. No biodegradation of the contaminant but usually requires removal of the contaminant.

Permeable Reactive Barriers (PRBs) – Are often referred to as iron filing walls, reactive barriers, funnel, and gate systems, or passive treatment walls. They are constructed underground to intercept groundwater flows and to provide preferential flow paths through bioreactive materials, e.g., as groundwater moves through the bioreactive materials, contaminants are treated and transformed into harmless by-products.

3 Characterization and Monitoring Feasibility

The success of any bioremediation application will be highly dependent on the careful planning of the overall project including the characterization and monitoring that is done before and during the field deployment. The overall planning of the remediation needs to take into account a number of steps from conceptual model to demobilization and report writing (Table 1, Hazen and Sayler 2016). For any field remediation, the first step is to form a conceptual model of the contaminant plume in the environment and how that environment effects that plume. The uncertainties in this conceptual model provide the drivers for the characterization and monitoring needs (EPA 2013). For example, characteristics of the aquifer will have a profound impact on the remediation strategy (Table 2). The largest part of the expense of any remediation project is the characterization and monitoring. Hydraulic conductivities can have a severe effect on your ability to deliver nutrients to the subsurface (Fig. 1) and can be the most limiting part of the environment. Fortunately, new advances in geophysics and hydraulic push technology (Geoprobe) have enabled us to

Table 1 In situ groundwater field plan



characterize sites in a fraction of the time and cost. Once we have established the hydrology and basic geochemistry at the site and used that data to refine our conceptual model, a baseline characterization of the microbiology is essential to establish that the right microorganisms are present, that they can be stimulated, and that no undesirable reactions with the stimulants or daughter products from the stimulation will occur. This usually requires some treatability and soil compatibility studies and monitoring of microbial community structure and function to establish the base conditions prior to stimulation (Plaza et al. 2001). For example, some metals like arsenic actually increase solubility under the same redox potentials that precipitate Cr and U. Table 3 provides an example list of the types of measurements that should be performed from either treatability slurries, soil columns, or in situ sampling (Hazen 1997). The in situ sampling including push/pull studies are usually the best sort of information for rates of biodegradation, effective porosity, and colloidal borescope measurements of groundwater flow rates and vectors (Paradis et al. 2016, 2018). This data and the refined conceptual model provide the functional design criteria for the remediation and can be used to develop a numerical model to predict the remediation rates, stability, and legacy management needs, e.g., monitoring, especially if the remediation is an immobilization strategy.

Bioremediation strategies will be limited most by our ability to deliver the stimulus to the environment. The permeability of the formation must be sufficient

Table 2 Aquifer and contaminant characteristics

Aquifer Profile	
Site Characteristics	Impact on Remediation Program
a) soil type	a) level of difficulty
- homogeniety	
- permeability	
- chemistry	
b) aquifer type and use	b) remediation goals, urgency, level of difficulty, treatment strategy
- confined, perched	
- drinking water, agriculture, etc.	c) urgency
c) groundwater flow	d) duration
d) sustainable pumping rate	e) level of difficulty
e) water table location	
- current depth to water	
- depth to water	
- water table fluctuation (seasonal and extreme)	f) level of difficulty, treatment strategy
f) recharge	
- location	
- seasonal rainfall	
Contamination Profile	
a) number and types (classes or specific compounds)	a) treatment strategy, level of difficulty
b) quantity	b) difficulty
c) solubility	c) treatment strategy, level of difficulty
d) volatility	d) treatment strategy
e) biodegradability	e) treatment strategy
f) toxicity	f) urgency, remediation goal

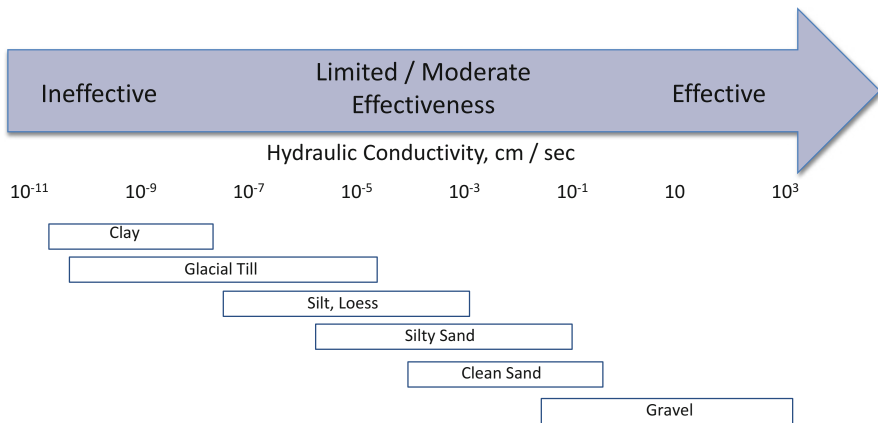


Fig. 1 Hydraulic conductivity

Table 3 Bioremediation characterization and monitoring parameters

Measurements	Parameter
Biomass	
Viable Counts	plate counts, Most Probable Number (MPN), enrichments, BIOLOG™
Direct Counts	Acridine Orange Direct Count (AODC), Fluorescien Isothiocyanate (FITC), Direct Fluorescent Antibody (DFA)
Signature Compounds	Phospholipid Fatty Acid (PLFA), DNA, RNA, qPCR, phylochips, functional gene arrays
Bioactivity and Bioremediation	
Daughter Products	Cl, CO_2, CH_4, stable isotopic C, reduced contaminants, stable isotopic fractionation of contaminants
Intermediary Metabolites	epoxides, reduced contaminants
Signature Compounds	PLFA, ribosome probes, BIOLOG™, phosphatase, dehydrogenase, Iodophenyl-Nitrophenyl, Tetrazolium Chloride (INT), acetylene reduction, recalcitrant contaminants
Electron Acceptors	O_2, NO_3,SO_4, Fe(III), CO_2
Conservative Tracers	He, CH_4, Cl, Br
Radiolabeled Mineralization	^{14}C, ^{3}H – labeled contaminants, acetate, thymidine
Sediment	
Nutrients	PO_4, NO_3, NH_4, O_2, total organics, SO_4
Physical/Chemical	porosity, lithology, cationic exchange, redox potential, pH, temperature, moisture, heavy metals
Toxicity	Microtox™, Mutatox™

to allow perfusion of the nutrients and/or microorganisms through the formation. The minimum average hydraulic conductivity for a formation is generally considered to be 10^{-4} cm/sec (Thomas and Ward 1989). Additionally, the stimulants required must be compatible with the environment. For example, hydrogen peroxide is an excellent source of oxygen, but it can cause precipitation of metals in soils, and such dense microbial growth around the injection site that all soil pores is plugged. It is also toxic to bacteria at high concentrations, >100 ppm (Thomas and Ward 1989). Ammonia also can be problematic, because it adsorbs rapidly to clays, causes pH changes in poorly buffered environments, and can cause clays to swell, decreasing permeability around the injection point. It is generally accepted that soil bacteria need a C:N:P ratio of 30:5:1 for unrestricted growth (Paul and Clark 1989). The actual injection ratio used is usually slightly higher (a ratio of 100:10:2) (Litchfield 1993), since these nutrients must be bioavailable, a condition that is much more difficult to measure and control in the terrestrial subsurface. It may also be necessary to remove light nonaqueous phase liquid (LNAPL) contaminants that are floating on the water table or smearing the capillary fringe zone, hence bioslurping (Keet 1995). This strategy greatly increases the biostimulation response time by lowering the highest concentration of contaminant the organisms are forced to transform.

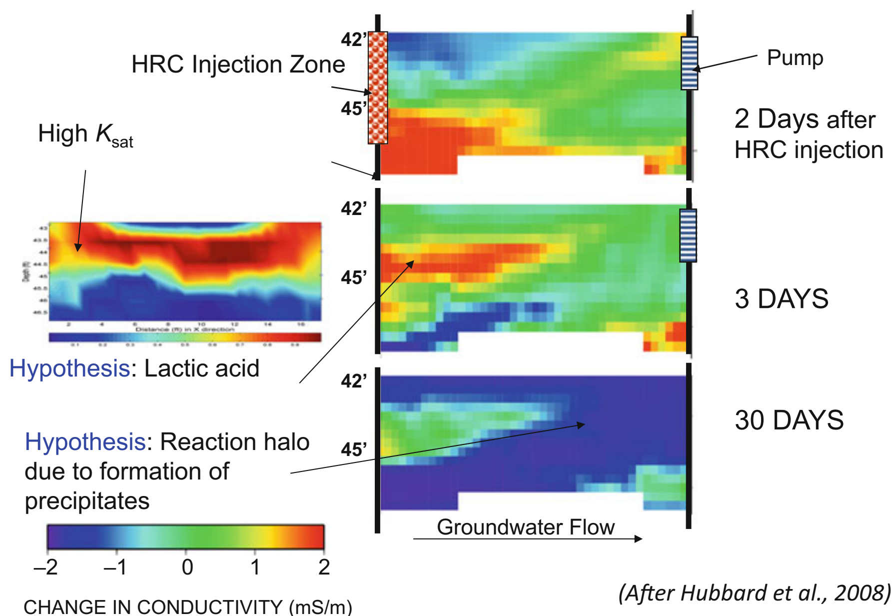


Fig. 2 Geophysical measurements of polylactate injection for groundwater bioremediation

Recent advances in geophysics are now enabling us to determine aquifer heterogeneity, hydraulic conductivity, amendment movement in the subsurface, changes in biogeochemistry, and real-time monitoring of changes (Fig. 2). These measurements can potentially save time, expense, and increase our resolution of biogeochemical changes, hydrology, contaminant inventory, and amendment injection pathway (Hubbard et al. 2008; Faybishenko et al. 2008).

The type of sample used for monitoring and characterization of groundwater can have a significant impact on a bioremediation project. Hazen et al. (1991) demonstrated that deep oligotrophic aquifers have dense attached communities of bacteria that are not reflected in the groundwater from that aquifer. This has serious implications for the in situ bioremediation of deep contaminated aquifers, since monitoring of groundwater is the principal method used to characterize and control biodegradation by indigenous bacteria stimulated by nutrient infiltration. Groundwater monitoring may not indicate community or population numbers, or physiological activity of the sediment attached microbes, the principal biologically active component of these aquifers. Harvey et al. (1984) and Harvey and George (1987) have shown that shallow, eutrophic, rapidly moving aquifers behave quite differently, in that there are no significant differences between groundwater and attached sediment communities. This is reasonable because attachment in such an environment would have no significant advantage, unlike the oligotrophic deep aquifers. Enzien et al. (1994) further underscored the need for careful sampling when they showed significant anaerobic reductive dechlorination processes occurring in an aquifer whose bulk groundwater was aerobic (>2 mg/L O_2).

The state and fate of contaminants in all environments is highly dependent on the redox or valence state of the environment. The redox potential of the environment will control the direction of chemical equilibria and whether the contaminant is reduced or oxidized. This in turn controls the possible compounds that the contaminant can form and the relative solubility of these metals in the environment. To stimulate microbes to produce conditions that are appropriate for remediation of specific contaminants requires a thorough knowledge of the geochemistry of that environment. Since electron acceptors vary greatly as to the energy that can be derived from their use in respiration, the most common terminal electron acceptors (TEA) will be utilized in a set order, according to the energy that can be derived (Fig. 3). Thus, oxygen is the preferred TEA and first TEA to be utilized, followed by nitrate, iron (III), sulfate, and carbon dioxide. Since dehalorespiration is not favored until the redox potential is in methanogenic conditions, O_2, NO_3, Fe(III), and SO_4 would have to be depleted first. Indeed, for sites that also have PCE/TCE, the iron (III) and the sulfate would have to be depleted before sustained methanogenesis, and subsequently dehalorespiration can occur. Failure to deplete these electron donors for chlorinated solvents will result in a dichloroethylene or vinyl chloride stall (frequently a fatal flaw in the conceptual model and remediation plan). For field applications, this means that enough electron donor would have to be added to deplete all the oxygen and nitrate present, at a minimum. By monitoring the TEA and their daughter products, it

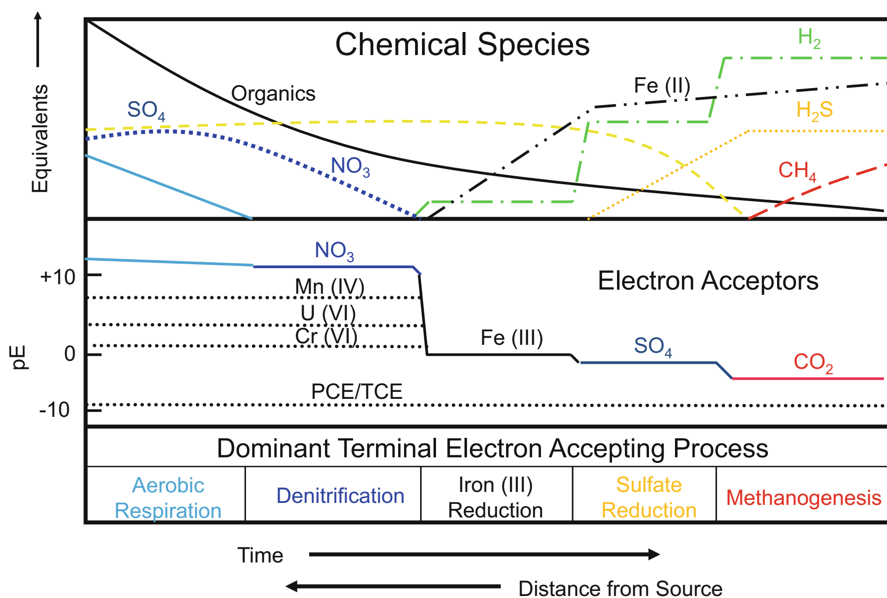


Fig. 3 Critical biogeochemistry involving terminal electron acceptors and their hierarchical redox potential relationships

provides an excellent measure of the redox conditions at the site and the potential for degradation of the contaminants of concern (Hazen and Tabak 2005; Nelson et al. 1994).

4 Biostimulation and Bioaugmentation of Groundwater

All engineered bioremediation can be characterized as either biostimulation, i.e., the addition of nutrients, or bioaugmentation, i.e., the addition of organisms, or processes that use both. The problems with adding chemical nutrients to sediment and groundwater are fundamentally different from those of adding organisms. Simple infiltration of soil and subsequently groundwater is physically quite different in the two processes (Alfoldi 1988). Even the smallest bacterium has different adsorption properties from chemicals. For example, clayey soils have very low porosity and may not physically allow bacteria to penetrate. These clays may also bind the microbes that are added, e.g., cationic bridges involving divalent metals and the net negative charge on the surface of the bacteria and the surface of the clay. In some soils, inorganic chemicals that are injected may precipitate metals, swell clays, change redox potentials, and conductivity, thus having a profound effect on groundwater flow and biogeochemistry of the environment. Indeed, bacterial plugging of subsurface formations has been successfully used for enhanced oil recovery in oil reservoirs (Cusack et al. 1992).

Biostimulation is dependent on the indigenous organisms and thus requires that they be present and that the environment be capable of being altered in a way that will have the desired bioremediation effect (Table 4). In most terrestrial subsurface

Table 4 Biostimulation versus bioaugmentation strategy requirements

Biostimulation requirements
1. correct microbes must be present
2. ability to stimulate target microbes
3. ability to deliver nutrients
4. C:N:P – 30:5:1 for balanced growth (Paul and Clark 1989) 100:10:2 in field practice (Litchfield 1993)
Gases: air, oxygen, nitrous oxide, propane, methane, triethyl phosphate, etc.
Liquids: lactic acid, molasses, vegetable oil, acetate, Chitin, hydrogen release compound (HRC®), MRC®, etc.
Solids: bulking agents (saw dust, agricultural byproducts), oxygen release compound (ORC®), etc.
Bioaugmentation advantages
1. “new” spills where microflora has not had time to adapt or grow (vector)
2. recalcitrant contaminants (GMO)
3. biomass can not establish or maintain itself (GMO)
4. biobarrier (ultramicrobacteria, GMO)
5. controlled environment (GMO)
Pseudomonads (oil spills) – several commercial products
Dehalococcoides ethenogenes (chlorinated solvents) new products from Regenesis, GeoSyntec, and others

environments, the indigenous organisms have been exposed to the contaminant for extended periods of time and have adapted or even naturally selected (USEPA 1988). Recent studies have demonstrated that microbial community structures at any given sampling can be used to predict ambient geochemistry and suggest bacteria that are stimulated by the amendment or hampered by the amendments (Smith et al. 2015). Using the Structured Learning in Microbial Ecology (SLiME) model (Smillie et al. 2011) and analyzing over 100 wells for 58 biogeochemical parameters in a contaminated water shed, 36 geochemical parameters were accurately predicted from the 16S rDNA community structure (Fig. 4). In addition, two OTUs (*Brevundimonas* and *Sediminibacterium*) were found to have geochemical drivers that would be important for in situ groundwater bioremediation (Fig. 5) (Smith et al. 2015). Many contaminants, especially organic compounds, are naturally occurring or have natural analogs in the environment. Rarely can a terrestrial subsurface environment be found that does not have a number of organisms already present that can degrade or transform any contaminant present. Indeed, even pristine environments have bacteria with an increasing number of plasmids with sediment depth in response to increasing recalcitrance of the organics present (Fredrickson et al. 1988).

Oxygen is quite often limiting since the contaminant can be used as a carbon and energy source by the organisms, and the contaminant concentration greatly exceeds the oxygen input needed by the organisms. Introduction of air, oxygen, or hydrogen peroxide via infiltration galleries, tilling, sparging, or venting has proven to be extremely effective in bioremediating petroleum contaminants and a variety of

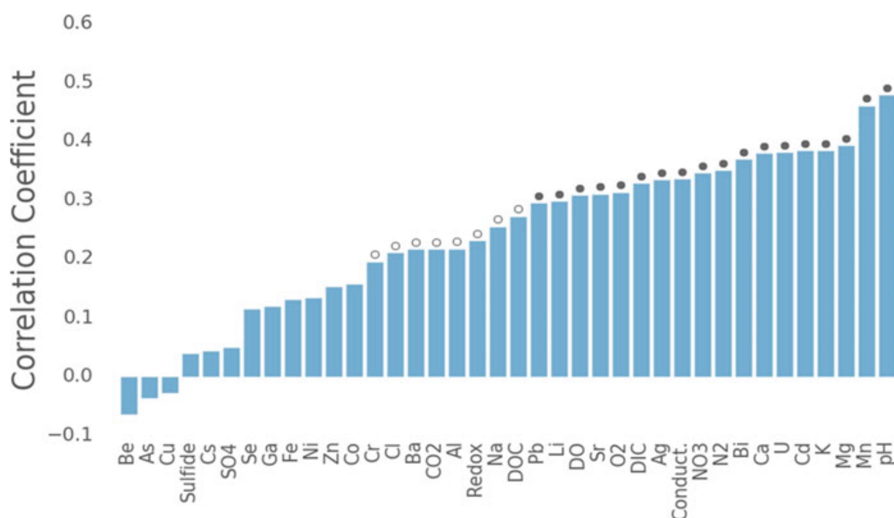


Fig. 4 Bacterial 16S sequence data can be used to predict quantitative values for a variety of geochemical measurements. Correlation coefficient (Kendall's tau) between true and predicted values. Eighteen of these correlations are highly significant ($p < 0.0001$, indicated by ●), 8 are significant ($p < 0.01$, indicated by o), and 12 of these correlations are not significant (Smith et al. 2015)

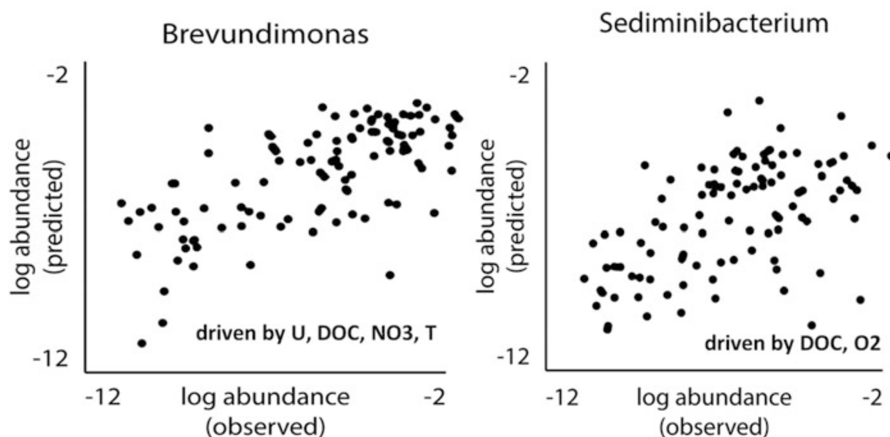


Fig. 5 Geochemical drivers for OTUs can be predicted using SLiME

other organic compounds that are not particularly recalcitrant (Thomas and Ward 1992). However, if the environment has been anaerobic for extended periods of time and the contaminant has a high carbon content, it is likely that denitrification has reduced the overall nitrogen content of the environment making this nutrient limiting. Nitrogen has been successfully introduced into the terrestrial subsurface for biostimulation using ammonia, nitrate, urea, and nitrous oxide (EPA 1989). Phosphorus is naturally quite low in most environments and, in terrestrial subsurface environments, even if phosphorus concentrations are high, it may be in a mineral form that is biologically unavailable, e.g., apatite. Several inorganic and organic forms of phosphate have been successfully used to biostimulate contaminated environments (EPA 1989). In environments where the contaminant is not a good carbon or energy source and other sources of carbon or energy are absent or unavailable, it will be necessary to add an additional source of carbon (Horvath 1972). An additional source of organic carbon will also be required if the total organic carbon concentration in the environment falls below 1 ppm and the contaminant clean-up levels have still not been met. Methane, methanol, acetate, molasses, sugars, agricultural compost, phenol, and toluene have all been added as secondary carbon supplements to the terrestrial subsurface to stimulate bioremediation (National Research Council 1993).

Bioaugmentation may provide significant advantages over biostimulation for (1) environments where the indigenous bacteria have not had time to adapt to the contaminant; (2) particularly recalcitrant contaminants that only a very limited number of organisms are capable of transforming or degrading; (3) environments that don't allow a critical biomass to establish and maintain itself; (4) applications where the desired goal is to plug the formation for contaminant containment, e.g., biocurtain; and (5) controlled environments where specific inocula of high rate degraders will greatly enhance the process, e.g., permeable reactive barriers. Like biostimulation, a major factor effecting the use of bioaugmentation in the terrestrial

subsurface is hydraulic conductivity. The 10^{-4} cm/sec limit for biostimulation will need to be an order of magnitude higher for bioaugmentation and may need to be higher yet, depending on the size and adherence properties of the organism being applied (Baker and Herson 1990; Ginn et al. 2002). Studies have shown the less adherent strains of some contaminant degraders can be produced, allowing better formation penetration (DeFlaun et al. 1994; Johnson et al. 2001). However, the ability to rapidly clog a formation is a significant advantage of bioaugmentation in applications where containment is a primary goal. The oil industry has been using this strategy to plug fluid loss zones and enhance oil recovery for a number of years (Cusack et al. 1992).

A number of novel organisms have been successfully injected into the subsurface for in situ bioremediation of PCBs, chlorinated solvents, PAHs, and creosote (National Research Council 1993). Bioaugmentation suffers the dilemma of being indistinguishable from biostimulation in many environments, since nutrients are often injected with the organisms and since dead organisms are an excellent source of nutrients for most indigenous organisms. For many applications it is difficult, if not impossible, to determine if the added organisms provided a significant advantage over nutrient stimulation alone. Given the problems and high cost of producing the organisms for inoculation and delivery problems, bioaugmentation applications will probably remain limited. For example, if dehalorespiration was the strategy and the site had a hydraulic conductivity of only 10^{-8} cm/sec with very high nitrate and sulfate levels and high pH, it may not be cost-effective to use dehalorespiration at this site. These issues also suggest why bioaugmentation has not lived up to its hope. Though bioaugmentation promises “designer biodegraders,” it has not proven to be better than biostimulation in repeated field trials over the last two decades. Indeed, there is only one bacterium that has demonstrated that it can perform better than biostimulation in situ on most occasions, *Dehalococcoides ethenogenes* for dehalorespiration of chlorinated solvents. Several products are commercially available and have been widely used that are proprietary strains of this organism (e.g., Regenesis and Geosyntec). We suspect the reason that this microbe has been successful is that it is a strict anaerobe, chlorinated solvent dehalorespiration requires established methanogenic redox potentials, and the organism is very small irregular coccus (0.5 μm) so it can penetrate the subsurface more easily (Loffler et al. 2000). Patchy distributions of this organism in nature are also common, so bioaugmentation may provide a couple of advantages.

Bioaugmentation may also have a very significant advantage when genetically engineered microorganisms (GEMs) are used. It is possible that a GEM could be constructed with unique combinations of enzymes to facilitate a sequential biotransformation or biodegradation of a contaminant. This would be particularly helpful for contaminants that are extremely recalcitrant, e.g., PCBs, or under limited conditions, e.g., tetrachloroethylene and carbon tetrachloride, which can only be biodegraded anaerobically. In addition, this GEM could be modified with unique survival or adherence properties that would make it better suited to the environment where it was to be applied.

5 Intrinsic Bioremediation and Modeling

Intrinsic bioremediation is developing rapidly as an important alternative for many contaminated environments. This strategy of monitored natural attenuation (MNA) by thorough characterization, treatability studies, risk assessment, modeling, and verification monitoring of contaminated environments was first proposed by John Wilson of EPA's Kerr Lab in the early 1990s. Wilson organized the first symposium on MNA in August 1994, and development and regulatory acceptance has been exponential ever since. Certainly, much of this rapid deployment of intrinsic bioremediation has been due to the crushing financial burden that environmental clean-up represents and our need to use more risk-based clean-up goals for the thousands of new contaminated sites identified every year. MNA as a strategy carries with it a burden of proof of (1) risk to health and the environment and (2) a model that will accurately predict the unengineered bioremediation of the environment (EPA 2017). Thus, applications of intrinsic bioremediation have been confined to environments with few risk receptors, containing contaminants with relatively low toxicity, e.g., petroleum in fairly homogeneous, confined, and predictable subsurface environments. The EPA reported that in 1995, intrinsic bioremediation was already in use at 29,038 leaking underground petroleum storage tank (LUST) sites in 33 states (Tremblay et al. 1995). This represents 28% of the 103,479 LUST sites being remediated in 1995 and an increase of more than 100% since 1993. Intrinsic bioremediation has also been implemented at a creosote-contaminated methanogenic aquifer in Florida (Bekins et al. 1993) and in three TCE-contaminated, reducing aquifers (Martin and Imbrigotta 1994; Wilson et al. 1994; Major et al. 1994).

The coupling of intrinsic bioremediation or MNA to engineered bioremediation could be the best overall solution. Nearly all engineered bioremediation projects could substantially reduce costs by stopping the biostimulation or bioaugmentation process early and allowing intrinsic bioremediation to finish the clean-up process. The only projects that would not benefit from such a strategy would be those where immediate risk to health and the environment demanded an emergency response. Intrinsic bioremediation has the same requirements for treatability, modeling, characterization, and modeling as engineered bioremediation discussed above. The only difference is that a greater emphasis is put on risk assessment, predictive modeling, and verification monitoring. Once an intrinsic bioremediation project has been started, verification monitoring of the predictive model is initially quite rigorous. Afterward, if the model holds true, monitoring frequency and numbers of parameters gradually decline until the site is cleaned up.

Modeling of the bioremediation process has become increasingly important in determining the fate and effect of contaminants and predicting the outcome of different amendment scenarios. The models will only be as good as the data they receive from the characterization studies and the treatability studies. However, models can also be used to suggest treatability studies that should be performed from a minimum of characterization data. The simple kinetic models using Monod or Michaelis-Menten functions of 15 years ago are completely inadequate for current

bioremediation applications in the terrestrial subsurface. One- and two-dimensional models of aerobic biodegradation of organic contaminants in groundwater did not appear until quite recently (Molz et al. 1986; Widdowson et al. 1987). These models used advective and dispersive transport coupled with an assumption of micro-colonies. Widdowson et al. (1988) later added nitrate respiration as an option to their model. Perhaps the best documented and most widely used model for bioremediation has been the BIOPLUME model (Borden and Bedient 1986). This model, now in its forth version, uses a series of simultaneous equations to simulate growth, decay, and transport of microorganisms, oxygen, and hydrocarbons. Rifai et al. (1987) later modified this model (BIOPLUME II) to incorporate the USGS two-dimensional method of characteristic model (Konikow and Bredehoeft 1978). The original model was used to simulate PAH biodegradation at a Texas Superfund site (Borden and Bedient 1986). BIOPLUME II has been used to model biodegradation of aviation fuel at the US Coast Guard Station at Traverse City, Michigan (Rifai et al. 1988) and to characterize benzene biodegradation over 3 years in another shallow aquifer (Chiang et al. 1989; Choi et al. 2009). Travis and Rosenberg (1997) used a numerical simulation model to successfully predict aerobic bioremediation of chlorinated solvents in the groundwater and vadose zone using methane biostimulation at the US DOE's Savannah River Site near Aiken, South Carolina. Their model also used a series of simultaneous equations for microbial growth, nutrient limitations, and contaminant, microbe, and nutrient transport. The model predicted the amount of TCE that was biodegraded during a 14-month, full-scale demonstration and was validated by five other methods (Hazen et al. 1994). Other models that are in use these days are BIOSCREEN (http://www.epa.gov/water-research/bioscreen-natural-attenuation-decision-support-system), BIOCHLOR (https://www.epa.gov/water-research/biochlor-natural-attenuation-decision-support-system), REMChlor (http://www.epa.gov/water-research/remediation-evaluation-model-chlorinated-solvents-remchlor), REMFuel (http://www.epa.gov/water-research/remediation-evaluation-model-fuel-hydrocarbons-remfuel), and Matrix Diffusion Toolkit (http://www.gsi-net.com/en/software/free-software/matrix-diffusion-toolkit.html). Models like these are becoming increasingly important as our need to understand the terrestrial subsurface "black box" of bioremediation increases in response to increased emphasis on intrinsic bioremediation as a solution. These types of models, along with rigorous treatability studies, are required for intrinsic bioremediation to be acceptable, particularly as a solution for bioremediation of terrestrial subsurface environments.

6 Research Needs

There are a large number of ex situ and in situ bioremediation methods currently available. Ex situ methods have been around longer and are better understood, and they are easier to contain, monitor, and control. However, in situ bioremediation has several advantages over ex situ techniques. In situ treatment is useful for contaminants that are widely dispersed in the environment, present in dilute concentrations, or otherwise inaccessible (e.g., due to the presence of buildings or structures). This

approach can be less costly and less disruptive than ex situ treatments because no pumping or excavation is required. Moreover, exposure of site workers to hazardous contaminants during in situ treatment is minimal. Broadly, bioremediation strategies can be further divided into natural attenuation, biostimulation, and bioaugmentation strategies. Bioaugmentation being the most aggressive, since organisms are added to the contaminated environment. Biostimulation can be aggressive or passive, in that electron donors, electron acceptors, and trace nutrients can be injected into the environment to stimulate indigenous organisms to increase biomass or activity to affect the contaminant. Passive biostimulation techniques include simple infiltration galleries. Monitored natural attenuation relies on the intrinsic bioremediation capabilities of that environment. Environments high in organic carbon and energy sources, low contaminant concentrations, and without significant nutrient deficiencies may be able to degrade or transform the contaminants of concern without any intervention. Ideally, the most cost-effective and efficient approach to treat most large contaminant plumes is to use more aggressive approaches, e.g., bioaugmentation or even excavation and removal, at the source, grading into natural attenuation at the leading edge, or over time as the contaminant concentration declines. There are only a few bioaugmentation candidates for in situ groundwater bioremediation (*Dehalococcoides ethenogenes*); however, it is technically possible to use bacteriophage as vectors to provide indigenous bacteria with increases or new degradation capacity. The size of bacteriophages and their specificity overcomes the inherent problem particle injection in the subsurface and the minimizing nontarget effects. Much more research is needed in this area. Rarely is a single remediation approach completely effective or cost-efficient. Indeed, combining aggressive physical and chemical treatment techniques like chemical oxidation/reduction and thermal desorption with bioremediation can provide advantages to some types of contaminants and allows bioremediation to be an effective polishing or sentinel strategy for the clean-up. Much more modeling at all scales (Lee and Swartz 2007) using a systems biology approach is needed to find the fastest, most efficient, and lowest life cycle cost solution for contaminated groundwater.

References

Alfoldi L (1988) Groundwater microbiology, and problems and biological treatment-state-of-the-art report. Water Sci Technol 20:1–31

Baker KH, Herson DS (1990) In situ bioremediation of contaminated aquifers and subsurface soils. Geomicrobiol J 8:133–146

Bekins BA, Godsy EM, Goerlitz DF (1993) Modeling steady-state methanogenic degradation of phenols in groundwater. J Contam Hydrol 14:279–294

Borden RC, Bedient PB (1986) Transport of dissolved hydrocarbons influenced by reaeration and oxygen limited biodegradation 1. Theoretical development. Water Resour Res 22:1973–1982

Chiang CY, Salanitro JP, Chai EY, Colthart JD, Klein CL (1989) Aerobic biodegradation o benzene, toluene, and xylene in sandy aquifer, and data analysis and computer modeling. Ground Water 27:823–834

Choi NC, Choi JW, Kim SB, Park SJ, Kim DJ (2009) Two-dimensional modelling of benzene transport and biodegradation in a laboratory-scale aquifer. Environ Technol 30:53–62

Cusack F, Singh S, McCarthy C, Grieco J, Derocco M, Nguyen D, Lappinscott H, Costerton JW (1992) Enhanced oil-recovery – 3-dimensional sandpack simulation of ultramicrobacteria resuscitation in reservoir formation. J Gen Microbiol 138:647–655

Deflaun MF, Marshall BM, Kulle EP, Levy SB (1994) Tn5 insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl Environ Microbiol 60:2637–2642

Enzien MV, Picardal F, Hazen TC, Arnold RG, Fliermans CB (1994) Reductive Dechlorination of trichloroethylene and tetrachloroethylene under aerobic conditions in a sediment column. Appl Environ Microbiol 60:2200–2204

Faybishenko B, Hazen TC, Long PE, Brodie EL, Conrad ME, Hubbard SS, Christensen JN, Joyner D, Borglin SE, Chakraborty R, Williams KH, Peterson JE, Chen JS, Brown ST, Tokunaga TK, Wan JM, Firestone M, Newcomer DR, Resch CT, Cantrell KJ, Willett A, Koenigsberg S (2008) In situ long-term reductive bioimmobilization of Cr(VI) in groundwater using hydrogen release compound. Environ Sci Technol 42:8478–8485

Fredrickson JK, Hicks RJ, Li SW, Brockman FJ (1988) Plasmid incidence in bacteria from deep subsurface sediments. Appl Environ Microbiol 54:2916–2923

Ginn TR, Wood BD, Nelson KE, Scheibe TD, Murphy EM, Clement TP (2002) Processes in microbial transport in the natural subsurface. Adv. Water Resour 25:1017–1042

Harvey RW, George LH (1987) Growth determination for unattached bacteria in a contaminated aquifer. Appl Environ Microbiol 53:2992–2996

Harvey RW, Smith RL, George LH (1984) Effect of organic contamination upon microbial distributions and heterotrophic uptake in a cape cod, Mass. Aquifer. Appl Environ Microbiol 48:1197–1202

Hazen TC, Lombard KH, Looney BB, Enzien MV, Dougherty JM, Fliermans CB, Wear J, Eddy-Dilek CA (1994) Summary of in situ bioremediation demonstration (methane biostimulation) via horizontal wells at the Savannah River site integrated demonstration project. In: Gee GW, Wing NR (eds) Proceedings thirty-third Hanford symposium on health and the environment in-situ remediation: scientific basis for current and future technologies. Battelle Press, Columbus, pp 135–150

Hazen TC, Jimenez L, De Victoria GL, Fliermans CB (1991) Comparison of bacteria from deep subsurface sediment and adjacent groundwater. Microb Ecol 22:293–304

Hazen TC, Sayler GS (2016) Environmental systems microbiology of contaminated environments. In: Yates M, Nakatsu C, Miller R (eds) Manual of environmental microbiology, 4th edn. ASM Press, Washington, DC

Hazen TC, Tabak HH (2005) Developments in bioremediation of soils and sediments polluted with metals and radionuclides: 2. Field research on bioremediation of metals and radionuclides. Rev Environ Sci Biotechnol 4:157–183

Hazen TC (1997) Bioremediation. In: Haldeman PA (ed) Microbiology of the terrestrial subsurface. CRC Press, Boca Raton, pp 247–266

Horvath RS (1972) Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriol Rev 36:146–155

Hubbard SS, Williams K, Conrad ME, Faybishenko B, Peterson J, Chen JS, Long P, Hazen TC (2008) Geophysical monitoring of hydrological and biogeochemical transformations associated with Cr(VI) bioremediation. Environ Sci Technol 42:3757–3765

Johnson WP, Zhang P, Fuller ME, Scheibe TD, Mailloux BJ, Onstott TC, DeFlaun MF, Hubbard SS, Radtke J, Kovacik WP, Holben W (2001) Ferrographic tracking of bacterial transport in the field at the Narrow Channel focus area, Oyster, VA. Environ Sci Technol 35:182–191

Keet BA (1995) Bioslurping state of the art. In: Hinchee RE, Kittel JA, Reisinger HJ (eds) Applied bioremediation of petroleum hydrocarbons. Battelle Press, Columbus, pp 329–334

Konikow LF, Bredeheoft JD (1978) Computer model of two dimensional solute transport and dispersion in ground water. Automated data processing and computations. Techniques of Water Resources Investigations of the U.S. Geological Survey, Washington, DC

Lee ES, Schwartz FW (2007) Characterization and optimization of long-term controlled release system for groundwater remediation: a generalized modeling approach. Chemosphere 69:247–253

Litchfield CD (1993) In situ bioremediation: basis and practices. In: Levin MA, Gealt MA (eds) Biotreatment of industrial and hazardous waste. McGraw-Hill, New York, pp 167–196

Loffler FE, Sun Q, Li J, Tiedje JM (2000) 16S rRNA gene-based detection of Tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Appl Environ Microbiol 66:1369–3445

Major D, Cox E, Edwards E, Hare PW (1994) The complete dechlorination of trichloroethene to ethene under natural conditions in a shallow bedrock aquifer located in New York state. In: Proceedings of the EPA Symposium on Intrinsic Bioremediation of Ground Water, August 30–September 1, Denver, CO. U.S. Environmental Protection Agency, EPA/540/R-94/515

Martin M, Imbrigiotta TE (1994) Contamination of ground water with trichloroethylene at the building 24 site at Picatinny arsenal, New Jersey. In: Proceedings of the EPA symposium on intrinsic bioremediation of ground water, august 30-September 1, Denver, CO. U.S. Environmental Protection Agency, EPA/540/R-94/515

Molz FJ, Widdowson MA, Benefield LD (1986) Simulation of microbial growth dynamics coupled to nutrient and oxygen transport in porous media. Water Resour Res 22:1207–1216

National Research Council (U.S.). Water Science and Technology Board (1993) In situ bioremediation: when does it work? National Academy Press, Washington, DC

Nelson MJK, Compeau G, Maziarz T, Mahaffey WR (1994) Laboratory treatability testing for assessment of field applicability. In: Flathman PE, Jerger DE, Exner JH (eds) Bioremediation field experience. Lewis Publishers, Boca Raton, pp 59–80

Paradis CJ, Jagadamma S, Watson DB, McKay LD, Hazen TC, Park M, Istok JD (2016) In situ mobility of uranium in the presence of nitrate following sulfate-reducing conditions. J Contam Hydrol 187:55–64

Paradis CJ, McKay LD, Perfect E, Istok JD, Hazen TC (2018) Push-pull tests for estimating effective porosity: expanded analytical solution and in situ application. Hydrogeol J 26:381–393

Paul EA, Clark FG (1989) Soil microbiology and biochemistry. Academic, San Diego

Plaza G, Ulfig K, Hazen TC, Brigmon RL (2001) Use of molecular techniques in bioremediation. Acta Microbiol Pol 50:205–218

Raymond RL, Jamison VW, Hudson JO (1977) Beneficial stimulation of bacterial activity in groundwater containing petroleum hydrocarbons. Am Inst Chem Eng Symp Ser 73:390–404

Rifai HS, Bedient PB, Borden RC, Haasbeek JF (1987) BIOPLUME II computer model of two-dimensional contaminant transport under the influence of oxygen limited biodegradation in groundwater User's manual version 1.0 Houston. Rice University, National Center for ground water research

Rifai HS, Bedient PB, Wilson JT, Miller KM, Armstrong JM (1988) Biodegradation modeling at an aviation fuel spill. ASCE J Environ Eng 114:1007–1029

Smillie CS, Smith MB, Friedman J, Cordero OX, David LA, Alm EJ (2011) Ecology drives a global network of gene exchange connecting the human microbiome. Nature 480:241–244

Smith MB, Rocha AM, Smillie CS, Olesen SW, Paradis C, Wu L, Campbell JH, Fortney JL, Mehlhorn TL, Lowe KA, Earles JE, Phillips J, Techtmann SM, Joyner DC, Elias DA, Bailey KL, Hurt RA Jr, Preheim SP, Sanders MC, Yang J, Mueller MA, Brooks S, Watson DB, Zhang P, He Z, Dubinsky EA, Adams PD, Arkin AP, Fields MW, Zhou J, Alm EJ, Hazen TC (2015) Natural bacterial communities serve as quantitative geochemical biosensors. MBio 6: e00326–e00315

Thomas JM, Ward CH (1989) In situ biorestoration of organic contaminants in the subsurface. Environ Sci Technol 23:760–766

Thomas JM, Ward CH (1992) Subsurface microbial ecology and bioremediation. J Hazard Mater 32:179–194

Travis BJ, Rosenberg ND (1997) Modeling in situ bioremediation of TCE at Savannah River: effects of product toxicity and microbial interactions on TCE degradation. Environ Sci Technol 31:3093–3102

Tremblay D, Tulis D, Kostecki P, Ewald K (1995) Innovation skyrockets at 50,000 LUST sites, EPA study reveal technology use at LUST sites. Soil Groundwater Cleanup 1995:6–13

USEPA (1988) Clean-up of releases from petroleum USTs. Vol. Report-530-UST-88-001 USEPA, Washington, DC

USEPA (1989) Bioremediation of hazardous waste sites workshop. CERI-89-11. Washington, DC

USEPA (2013) Introduction to in situ bioremediation of groundwater. EPA 542-R-13-018

USEPA (2017) How to evaluate alternative cleanup technologies for underground storage tank sites. EPA 510-B-17-003

Widdowson MA, Molz FA, Benefield LD (1987) Development and application of a model for simulating microbial growth dynamics coupled to nutrient and oxygen transport in porous media. In: Proceedings of the Association of Groundwater Scientists and Engineers/international ground water model center, pp 28–51, Holcomb research center institute conference on solving ground water problems with models. Dublin: National Ground Water Association

Widdowson MA, Molz FJ, Benefield LD (1988) A numerical transport model for oxygen- and nitrate-based respiration linked to substrate and nutrient availability in porous media. Water Resour Res 24:1553–1565

Wilson JT, Weaver JW, Kampbel DH (1994) Intrinsic bioremediation of TCE in ground water at an NPL site in St. Joseph, Michigan. Vol. proceedings of the EPA symposium on intrinsic bioremediation of ground water, august 30-September 1, Denver, CO. U.S. Environmental Protection Agency, EPA/540/R-94/515

Microbiology of Oil- and Natural Gas-Producing Shale Formations: An Overview

13

Christopher G. Struchtemeyer

Contents

1 Introduction 216
2 The Microbiology of Oil- and Natural Gas-Producing Shale Formations 217
3 The Influence of Drilling Mud on the Microbiology of Oil- and Natural Gas-Producing Shale Formations 220
4 The Influence of Hydraulic Fracturing on the Microbiology of Oil- and Natural Gas-Producing Shale Formations 224
5 Research Needs 228
References 230

Abstract

Shales are the most abundant type of sedimentary rock on Earth. Many shale formations contain high concentrations of organic matter and can serve as both sources and reservoirs of oil and natural gas. Oil- and natural gas-producing shale formations are characterized by very low permeability and extremely small pore throat sizes, which have traditionally made it very difficult to extract economic volumes of hydrocarbons. Recent advances associated with horizontal drilling and hydraulic fracturing have led to increased exploration and extraction of oil and natural gas in shale formations throughout the world. This increased activity in shale formations has been accompanied by a variety of microbial-related issues including reservoir plugging, reservoir souring, sulfidogenesis, and corrosion. Even though it is clear that microorganisms cause a wide variety of deleterious processes in shales, very little is known about their origins in these formations. There are several plausible sources of microorganisms in shale, including the

C. G. Struchtemeyer (✉)
Department of Biology, McNeese State University, Lake Charles, LA, USA
e-mail: cstruchtemeyer@mcneese.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_18

formation itself and the fluids that are used during the drilling and hydraulic fracturing processes. This chapter contains an overview of what is currently known about the microbiological properties of shale formations and the fluids that are used during the drilling and hydraulic fracturing processes. The chapter also contains a brief description of the research issues that need to be addressed in future studies.

1 Introduction

Since the early 2000s, rising oil and natural gas prices, along with increased confidence that oil and natural gas could be profitably produced from shales, led to the exploration of these formations as possible alternative sources of fuels (Kerr 2010; Wang et al. 2014). Technological advances associated with horizontal drilling and hydraulic fracturing have made it profitable to recover oil and natural gas from shale formations (Hu and Xu 2013; Vengosh et al. 2013, 2014). These technological advances have also led to sharp increases in oil and natural gas production in the United States (USA). The quantity of oil produced from fractured wells in the USA has increased from 102,000 barrels per day (equivalent to less than 2% of total US oil production) in 2000 to more than 4.3 million barrels per day (equivalent to approximately 50% of total US oil production) in 2015 (Cook and Perrin 2016). The majority of the new oil that has been produced over this 15-year period has originated from the Eagle Ford and Permian shale formations in Texas and the Bakken and Three Forks shale formations, which are located in Montana and North Dakota (Cook and Perrin 2016). Similar trends have been observed in US shale gas production rates, which have increased from 0.32 trillion cubic feet (tcf) in 2000 to 6.84 tcf in 2011 (Hu and Xu 2013). Current projections suggest that shale gas will account for approximately 50% of the total US natural gas supply in 2035 (Vengosh et al. 2013). It is also expected that the USA will become a net natural gas exporter by this time (Hu and Xu 2013). The increased production of oil and natural gas from US shale formations has led to global exploration of shale formations (Hu and Xu 2013; Vengosh et al. 2013, 2014). Efforts are currently underway to assess the feasibility of oil and gas production from shale formations in Canada, South America, Africa, Europe, China, New Zealand, and Australia (Hu and Xu 2013; Vengosh et al. 2013, 2014).

The increase in oil and natural gas production from US shale formations has been accompanied by a number of microbial-related issues. Microorganisms that are present in oil and natural gas reservoirs have been shown to cause corrosion of metal-containing production and transport equipment, reservoir plugging, reservoir souring due to sulfide production, and reduced product quality as a result of the degradation of reservoir hydrocarbons (Fichter et al. 2009; Kermani and Harrop 1996; Youssef et al. 2009). To date, very little is known about the origin of microorganisms in oil and natural gas reservoirs (Magot 2005). The majority of studies that have examined populations of microorganisms in these reservoirs have

relied on the use of production water samples (Magot 2005). In these studies, microorganisms were generally assumed to be indigenous to a given oil or natural reservoir if their optimum growth temperatures and physiological capabilities were consistent with the in situ conditions (Magot 2005). More recent work involving the study of conventional oil reservoirs that contain biodegraded hydrocarbons have provided insight on a number of factors, including temperature, salt concentrations, and nutrient availability, which likely control microbial growth in shale formations (Foght 2010; Head et al. 2010; Jones et al. 2008). However, these studies have not provided definitive proof for the existence of indigenous populations of microorganisms in shale formations. Analysis of the microbial properties of drilling and fracturing fluids has provided a better understanding of how the drilling and fracturing processes influence the microbiology of shale formations (Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer et al. 2011; Struchtemeyer and Elshahed 2012). This work has clearly shown that drilling and fracturing stimulate microorganisms that are capable of survival and contributing to detrimental processes (e.g., sulfidogenesis, microbiologically influenced corrosion, and biofilm formation) in shale formations (Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer et al. 2011; Struchtemeyer and Elshahed 2012). The recent insight gained from microbiological studies of shale, drilling muds, and fracturing fluids has led to enhanced efforts to control microbial growth and eliminate deleterious microbial processes in oil- and gas-producing shale formations.

2 The Microbiology of Oil- and Natural Gas-Producing Shale Formations

Shales are the most abundant type of sedimentary rock on earth and contain a variety of minerals including clays (primarily illite), calcite, dolomite, siderite, feldspars, pyrite, gypsum, and mica (Boyer et al. 2011; Bruner and Smosna 2011; Gaspar et al. 2014). The concentrations of these minerals tend to vary both locally and regionally in shale formations (Bruner and Smosna 2011; Gaspar et al. 2014). Shales are composed primarily of clay- and silt-sized particles, which tend to accumulate as muds in low-energy depositional environments (tidal ponds and deepwater basins) (Arthur et al. 2008). Several different types of organic matter including algae, plant stems, and leaves infiltrate these muds during the deposition process (Alexander et al. 2011; Arthur et al. 2008). Over time (millions of years), the muds are buried under other layers of rock and become highly compacted in layers (Alexander et al. 2011; Arthur et al. 2008; Bowker 2007; Riebeek 2011). These compacted sediments are then exposed to elevated temperatures and pressures, which results in the formation of oil- and gas-bearing shales (Alexander et al. 2011). These oil- and gas-bearing shale formations are typically characterized by low permeability, low porosity, extremely small pore throat sizes, and high concentrations of organic matter (Alexander et al. 2011; Arthur et al. 2008; Bowker 2007; Riebeek 2011).

Isotope fractionation has commonly been used to assess whether microbially induced processes are occurring in oil and natural gas reservoirs. This technique

has been used to determine whether the natural gas in shale formations is generated through biogenic, thermogenic, or a combination of biogenic and thermogenic processes (Curtis 2002; Martini et al. 1998, 2008; Schoell 1980). Isotopic fractionation has also been used frequently to evaluate the origins of a variety of gaseous waste products (H_2, CO_2, H_2S, and CH_4) that are generated as a result of the degradation of hydrocarbons in oil and natural gas reservoirs (Jones et al. 2008; Kleikemper et al. 2002; Meckenstock et al. 1999, 2004). The results of these isotopic studies have shown that microorganisms, abiotic processes, or a combination of these two factors are often responsible for hydrocarbon degradation in oil and natural gas reservoirs (Jones et al. 2008; Kleikemper et al. 2002; Meckenstock et al. 1999, 2004). Even though isotopic studies have clearly demonstrated that microbial processes are often responsible for hydrocarbon production and degradation in oil- and natural gas-producing reservoirs, they cannot be used to provide definitive proof for the existence of indigenous (native) microorganisms in these formations.

Some of the strongest evidence for the presence of indigenous microorganisms in shale formations comes from a large number of studies that have observed microbially degraded hydrocarbons in conventional oil reservoirs (Foght 2010; Head et al. 2010; Jones et al. 2008; Magot 2005; Youssef et al. 2009). The results of this work have provided significant insight on the factors that dictate whether or not a given shale formation possesses an indigenous population of microorganisms. Temperature is one of the most important factors that govern whether or not biodegraded hydrocarbons are present in conventional oil reservoirs. Biodegraded hydrocarbons have not been observed in conventional oil reservoirs when temperatures exceed 80 °C (Head et al. 2010; Magot 2005). Thus, it is generally assumed that microorganisms will not be present in shale formations that contain temperatures above 80 °C. In other cases biodegraded oils have not been observed in deeply buried reservoirs which experienced temperatures above 80 °C, but cooled to temperatures that are more suitable for microbial growth (Foght 2010). In these cases, it is generally hypothesized that the formations were sterilized by elevated temperatures, cooled by uplifting, but not repopulated by microorganisms (Röling et al. 2003; Wilhelms et al. 2001). This hypothesis is likely highly applicable to thermogenic shale formations, such as the Barnett shale, which have undergone similar heating and cooling events. Even though many thermogenic shales have cooled to temperatures which are suitable for microbial growth, the extremely low permeabilities and small pore throat sizes within these formations are thought to have prevented their repopulation by microorganisms (Bowker 2003). The high concentrations of organic matter in many thermogenic shales also suggest that these formations may lack an active population of microorganisms (Loucks and Ruppel 2007). Salinity has also been shown to be an important factor that often limits the biodegradation of hydrocarbons in conventional reservoirs (Foght 2010; Head et al. 2010). Reservoirs with highly saline waters often exhibit limited hydrocarbon biodegradation, and studies have shown that it was not possible to cultivate hydrocarbon degraders from oil reservoirs when salt concentrations exceeded 100 g/L (Foght 2010; Grassia et al. 1996; Head et al. 2010). The availability of nutrients (e.g., electron donors, electron

acceptors, phosphorous, etc.) also plays a critical role in determining whether or not hydrocarbons are degraded by microorganisms in conventional reservoirs (Foght 2010; Head et al. 2010).

Only a handful of studies have collected core samples to test for the presence of indigenous microorganisms in shale formations (Fredrickson et al. 1997; Krumholz et al. 1997; Onstott et al. 1998). Shallow core samples (collected less than 250 m below ground) from the Mancos shale formation in New Mexico generally lacked detectable levels of microbial activity (Fredrickson et al. 1997; Krumholz et al. 1997). However, low levels of $^{35}SO_4^{2-}$ reduction were detected in some shale cores and sulfate-reducing bacteria were also enriched from one shale sample that was collected (Fredrickson et al. 1997; Krumholz et al. 1997). The authors of this study noted that $^{35}SO_4^{2-}$ reduction and sulfate-reducing bacteria were only observed after extended (14 days or longer) incubation periods in laboratory experiments (Fredrickson et al. 1997; Krumholz et al. 1997). Therefore, the authors concluded that sulfate-reducing microorganisms were likely dormant in the shale and stimulated by nutrient amendments during laboratory experiments (Fredrickson et al. 1997; Krumholz et al. 1997). This conclusion was supported by diglyceride fatty acid (DGFA)/phospholipid-derived fatty acid (PLFA) ratios, which also indicated that significant levels of nonviable biomass were present in shale and in many cases exceeded the amount of living biomass (Fredrickson et al. 1997). The authors found that the levels of microbial activity were highly dependent on pore throat sizes in this formation (Fredrickson et al. 1997; Krumholz et al. 1997). The shale layers that were examined in this formation contained pore throat sizes that were less than 0.2 μm in diameter (Fredrickson et al. 1997; Krumholz et al. 1997). The authors concluded that the small pore throat sizes of the shale resulted in nutrient limitations and reduced microbial activity (Fredrickson et al. 1997; Krumholz et al. 1997).

Studies by Onstott et al. collected cores from a low-permeability zone within the Taylorsville Basin of Virginia and tested for the presence of indigenous microorganisms (Onstott et al. 1998). This low-permeability zone was located approximately 2,800 m below ground and was composed of organic-rich shale and siltstone, which was interbedded with more porous sandstone and high natural gas concentrations (Onstott et al. 1998). This particular area was chosen for sampling because it was hydrologically isolated, it contained relatively few boreholes, and it was thought that the low permeability of the formation would reduce the possibility that the core samples would become contaminated with drilling muds (Onstott et al. 1998). Saline-tolerant, thermophilic-fermenting, iron (III)-reducing, and sulfate-reducing microorganisms were recovered from these samples (Onstott et al. 1998). These microorganisms possessed physiological traits that were consistent with the temperatures (76 °C), pressures (32 MPa), and salt concentrations (≈0.8% wt. % NaCl equivalent) in the sampled portion of the reservoir (Onstott et al. 1998). Thus, it was assumed that these microorganisms were indigenous to the sampled portion of the formation (Onstott et al. 1998). However, subsequent studies revealed that this particular formation had been exposed to paleotemperatures ranging from 160 to 200 °C, which geologically sterilized the formation (Tseng and Onstott 1997).

Modeling data suggests that the microorganisms in the sampled portion of the formation were deposited from overlying strata by meteoric water during the basin's last major tectonic event (Tseng and Onstott 1997).

To date, our knowledge of the microbiology in shales remains limited, due to the small number of core samples that have been collected. Isotope work and studies of biodegraded oil reservoirs have provided indirect evidence for the presence of indigenous microorganisms in shale formations. This work has also provided insight on the factors that control the growth of microbial populations in shale formations. Even though a handful of studies have directly examined the microbiological properties of shale cores, they have not provided absolute proof for the existence of active indigenous microbial communities in shale formations. While it generally seems to be accepted that thermogenic shale formations lack indigenous populations of microorganisms, the situation is less clear in shale formations that have not been exposed to temperatures above 80 °C (Bowker 2003; Loucks and Ruppel 2007). The work that was done with shale cores from the Mancos formation in New Mexico, which never exceeded 65 °C, may provide the strongest evidence for the presence of indigenous microorganisms in non-thermogenic shale formations (Fredrickson et al. 1997; Krumholz et al. 1997). Previous work has clearly shown that this formation contains populations of microorganisms (Fredrickson et al. 1997; Krumholz et al. 1997). The fact that these microorganisms appear to be dormant in the shale but are stimulated upon the addition of exogenous nutrients seems to support the notion that they are indigenous to the formation (Fredrickson et al. 1997; Krumholz et al. 1997). It appears highly unlikely that these microorganisms are surface contaminants (Fredrickson et al. 1997; Krumholz et al. 1997). The extremely small pore throat sizes (less than 0.2 μm) that created nutrient-limiting conditions in this formation would also have likely prevented the migration of microorganisms from the surface into the shale (Fredrickson et al. 1997; Krumholz et al. 1997). The fact that these dormant microorganisms were stimulated by exogenous nutrients also has important implications from a well construction standpoint in oil- and gas-producing shale formations. During the drilling and fracturing processes, large volumes of nutrient-laden drilling and fracturing fluids are pumped into the formation (Struchtemeyer et al. 2011; Struchtemeyer and Elshahed 2012). Previous work has shown that significant volumes of these fluids are lost to the formation (Darley and Gray 1988; Veil 2010). Thus, it is likely that these lost fluids could stimulate the growth of any indigenous microorganisms that are present in oil- and gas-producing shale formations.

3 The Influence of Drilling Mud on the Microbiology of Oil- and Natural Gas-Producing Shale Formations

Drilling muds serve a number of important functions during the construction of oil and natural gas wells in shale formations. These muds transport shale cuttings to the surface, balance surface and formation pressures in order to prevent blowouts, cool and lubricate the drilling bit, and help preserve and protect the hole during the

drilling process (Darley and Gray 1988). Water-based muds are most commonly used to construct oil and natural gas wells in shale formations due to their low cost, readily disposable nature, and low ecological footprint (Burke and Veil 1995; Murali Mohan et al. 2013). However, in some settings such as reactive shale formations, deep wells, and horizontal/extended reach wells, water-based muds occasionally exhibit poor performance (Burke and Veil 1995). In these cases, synthetic drilling muds are often used in place of water-based drilling muds (Burke and Veil 1995). A wide array of chemicals including internal olefins, esters, linear alpha-olefins, poly alpha-olefins, and linear paraffins can serve as the basal fluid in synthetic drilling muds. Prior to their injection into shale formations, water-based and synthetic drilling muds are amended with a variety of prepackaged powdered components (Table 1). These components are added to the muds in order to adjust their density, viscosity, pH and filtration, circulation, and hole stabilization properties. Several of the components can also serve as nutrient sources for microorganisms (Table 1). Components that are used to add viscosity and control the filtration and circulation properties of mud often serve as sources of carbon for microorganisms (Table 1). Components that are used to add weight, reduce viscosity, and stabilize the hole can often serve as sources of sulfate or phosphate for microorganisms (Table 1). Water-based and synthetic muds are prepared and mixed at elevated temperatures in tanks that are not protected from their surroundings (Darley and Gray 1988; Struchtemeyer et al. 2011). Therefore, any microorganisms that are present in the mud components or introduced from the surrounding environment during the drilling process will likely have an opportunity to grow and proliferate before the mud is injected into the subsurface. Large volumes of drilling mud are also routinely lost in the formation during drilling, which will lead to the introduction of exogenous nutrients and microorganisms into oil- and natural gas-producing shales (Darley and Gray 1988).

Despite the fact that drilling muds are routinely lost in shale formations and contain a variety of nutrients, which are known to support microbial growth, very few studies

Table 1 Drilling mud additives that can serve as nutrient sources for microorganisms

Name of mud component[a]	Purpose in drilling mud[a]	Nutrient(s) supplied to microorganisms[a]
Barite ($BaSO_4$)	Weighing agent	Sulfur
Lignosulfonates	Thinning agent	Sulfur
Potassium sulfate (K_2SO_4)	Hole stabilizer	Potassium/sulfur
Polyphosphates	Thinning agent	Phosphorous
Cellulose	Increases mud viscosity	Carbon
Starch	Increases mud viscosity	Carbon
Xanthan gum	Increases mud viscosity	Carbon
Wood fiber blends	Lost circulation material	Carbon
Nut hulls	Lost circulation material	Carbon

[a]All of the names of mud components and the information regarding the purpose of individual mud components and the nutrients supplied to microorganisms by mud components were taken from previous studies that examined the chemical properties of drilling muds (Prasad and Katiyar 2010; Struchtemeyer et al. 2011)

have examined their potential influence on the microbiology of shale formations. The microbiological impacts of water-based drilling muds on shale formations were recently examined at seven newly constructed wells in the Barnett shale (Struchtemeyer et al. 2011). The results of quantitative real time PCR and most probable number dilutions from this study showed that the addition of powdered mud components to drilling water caused an increase in numbers of total bacteria, aerobic heterotrophic bacteria, acid-producing bacteria, and sulfate-reducing bacteria (Struchtemeyer et al. 2011). The microbial communities in drilling water and the final mud mixtures, which were pumped into the formation, were also compared at each of these seven newly constructed well sites using pyrosequencing-based surveys of 16S rRNA genes (Struchtemeyer et al. 2011). The results of this work showed that a substantial decrease in microbial diversity occurred as a result of the mud formulation process (Struchtemeyer et al. 2011). The microbial communities in drilling water were highly diverse and were dominated by 16S rRNA sequences that are typically observed in freshwater ecosystems including members of the phyla/classes *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Firmicutes*, *Gammaproteobacteria*, *Verrucomicrobia*, *Cyanobacteria*, *Planctomycetes*, *Acidobacteria*, and *Gemmatimonadetes* (Struchtemeyer et al. 2011). The microbial communities in drilling muds were almost completely distinct from and much less diverse than the drilling water samples (Struchtemeyer et al. 2011). The microbial communities in six out of the seven mud samples were dominated by 16S rRNA sequences affiliated with *Firmicutes* and *Gammaproteobacteria* (Struchtemeyer et al. 2011). The microbial community in the seventh mud sample was dominated by 16S rRNA sequences that were affiliated with *Firmicutes* and *Betaproteobacteria* (Struchtemeyer et al. 2011).

The drastic shifts that were observed in the microbial communities from the water-based Barnett shale drilling mud samples appeared to reflect physical changes associated with the mud formulation process (Struchtemeyer et al. 2011). Many of the 16S rRNA sequences that were present at higher abundance in drilling mud than drilling water were affiliated with strict and facultative anaerobes (Struchtemeyer et al. 2011). This was likely attributable to the increased viscosity and lower oxygen concentrations associated with the drilling muds (Struchtemeyer et al. 2011). 16S rRNA sequences that were affiliated with polymer-degrading genera including *Acetovibrio*, *Ruminococcus*, *Eubacterium*, and *Clostridium* were also much more abundant in drilling mud than drilling water (Struchtemeyer et al. 2011). These genera of bacteria were likely stimulated by the large quantities of different polysaccharides (nut hulls, cedar fiber, and xanthan gum) that were used to make drilling muds at these sites (Struchtemeyer et al. 2011). A variety of sulfide-producing lineages including *Desulfotomaculum*, *Desulfovibrio*, *Desulfomicrobium*, *Desulfobacterium*, *Desulfitobacterium*, *Thermodesulfobacterium*, *Thermoanaerobacter*, and *Thermotoga* were also much more abundant in drilling mud than drilling water (Struchtemeyer et al. 2011). Several of the sulfide-producing lineages that were present at higher abundance in drilling mud than drilling water (*Desulfotomaculum*, *Thermodesulfobacterium*, *Thermoanaerobacter*, and *Thermotoga*) were also thermophilic (Struchtemeyer et al. 2011). These observations coupled with microcosm

studies, which showed that the addition of powdered mud components to drilling water stimulated sulfide producers at temperatures observed in Barnett shale natural gas wells, provided evidence that drilling mud can contribute to incidences of sulfidogenesis in shale formations (Struchtemeyer et al. 2011). In addition to thermophilic sulfide producers, several genera of non-sulfide-producing, thermophilic microorganisms (e.g., *Geobacillus* and *Petrobacter*) were also much more abundant in drilling mud than drilling water (Struchtemeyer et al. 2011). The increased abundance of thermophilic lineages in drilling mud relative to drilling water was attributed to the elevated temperatures (up to 50 °C) associated with the mud-making process (Struchtemeyer et al. 2011).

The results of the water-based drilling fluid studies in the Barnett shale clearly showed that the drilling mud formulation process stimulated microbial communities which are capable of surviving the anoxic conditions and elevated temperatures encountered in many shale formations (Struchtemeyer et al. 2011). The microbial communities stimulated in water-based drilling muds also appear to be capable of contributing to deleterious microbiologically related processes (e.g., sulfidogenesis and corrosion) that have been previously reported in oil- and natural gas-producing shale formations (Struchtemeyer et al. 2011). Several of the microbial lineages that were observed in the water-based mud samples from the Barnett shale, including *Desulfobacterium*, *Desulfovibrio*, *Thermotoga*, *Petrobacter*, *Thermodesulfobacterium*, and *Thermoanaerobacter*, have been previously observed in production water samples from high temperature oil and natural gas wells (Magot 2005; Salinas et al. 2004). In many cases these microorganisms were assumed to be indigenous to high temperature oil and natural gas wells since they had been routinely observed in geographically distinct reservoirs and possessed physiological properties (optimum growth temperature and salt tolerance range) that were consistent with in situ reservoir conditions (Magot 2005). However, the observation that these microorganisms were stimulated in water-based muds during the drilling mud formulation process in newly constructed Barnett shale natural gas wells, suggests that they may, in fact, be of exogenous origin (Struchtemeyer et al. 2011). Even though it is clear that water-based drilling muds impact the microbiology of oil- and natural gas-producing shale formations, the impact of synthetic drilling fluids on the microbiological properties of these formations is less clear. A recent study conducted in the Marcellus shale was unable to extract detectable levels of DNA or amplify 16S rRNA sequences from a synthetic oil-based drilling mud that was used to construct a new natural gas well in this formation (Murali Mohan et al. 2013). The authors performed a series of control experiments in which they spiked the synthetic oil-based drilling mud with *E. coli* DNA to ensure that the mud components did not contain inhibitors that would prevent the extraction of DNA and the amplification of 16S rRNA gene sequences (Murali Mohan et al. 2013). The authors were able to extract *E. coli* DNA and amplify its 16S rRNA gene sequence from this spiked synthetic oil-based drilling mud sample (Murali Mohan et al. 2013). This observation provided conclusive proof that the lack of DNA in this drilling mud sample was due to the absence of microorganisms rather than the presence of inhibitors (Murali Mohan et al. 2013). The authors were unable to conclude if the absence of

microorganisms in this synthetic oil-based drilling mud was attributable to the presence of long chain hydrocarbons, which are not readily degradable by microorganisms, or biocide treatments that occurred (Murali Mohan et al. 2013). Thus, it remains unclear if synthetic oil-based drilling fluids impact the microbiology of other oil- and natural gas-producing shale formations. However, it is important to note that a variety of studies have observed microbial degradation of synthetic oil-based drilling fluids (Benka-Coker and Olumagin 1996; Nweke and Okpokwasili 2003; Okpokwasili and Nnubia 1999). Therefore, it would seem likely that these synthetic oil-based drilling fluids could potentially harbor active populations of microorganisms, which would impact the microbiological properties of shale formations.

4 The Influence of Hydraulic Fracturing on the Microbiology of Oil- and Natural Gas-Producing Shale Formations

Since shale formations are extremely impermeable, drilling alone will not allow for the economic recovery of oil and natural gas (Arthur et al. 2008; Veil 2010). Therefore, newly constructed wells in shale formations must be hydraulically fractured (Arthur et al. 2008; Veil 2010). During the fracturing process, large volumes of fluid are pumped into a predrilled portion of the formation at high pressure, which creates fractures in the shale (Arthur et al. 2008; Veil 2010). These fractures increase the permeability of the shale and allow for the recovery of economic volumes of oil and natural gas (Arthur et al. 2008; Veil 2010). Water from municipal sources or wells, which contains low levels of bacteria, is the primary component that is used to make fracturing fluids (Fichter et al. 2009). This water is hauled to and stored in man-made ponds (Fig. 1) for prolonged periods of time prior to the start of the fracturing process (Fichter et al. 2009). During this storage period, the frac water is exposed to air, rainfall, and runoff, which often leads to high levels of microbial contamination (Fichter et al. 2009). Once the fracturing process starts, frac water is pumped from the pond into frac tanks (Fig. 1) where biocide is added to control microbial growth (Struchtemeyer and Elshahed 2012). After the frac water is treated with biocide, it is pumped to a blender (Fig. 1) where a mixture of chemicals and sand are added (Struchtemeyer and Elshahed 2012). Some of the notable chemicals that are added to the frac water in the blender include sugar-based polymers which increase the viscosity of the frac fluids (Arthur et al. 2008). These polymers can also serve as growth substrates for any microorganisms that survive biocide treatment (Arthur et al. 2008). The mixture of water (approximately 90.6%), chemicals (approximately 0.45%), and sand (approximately 8.95%) that leaves the blender (Fig. 1) represents the final frac fluid mixture (Arthur et al. 2008; Struchtemeyer and Elshahed 2012). These frac fluids are pumped from the blender into the formation at high pressure (Fig. 1), which creates fractures in the shale (Arthur et al. 2008).

After the fractures are created in the shale, the frac fluids are often left in the formation for a period of time ranging anywhere from 1 day to several months in length (Struchtemeyer and Elshahed 2012). Newly fractured wells then undergo a

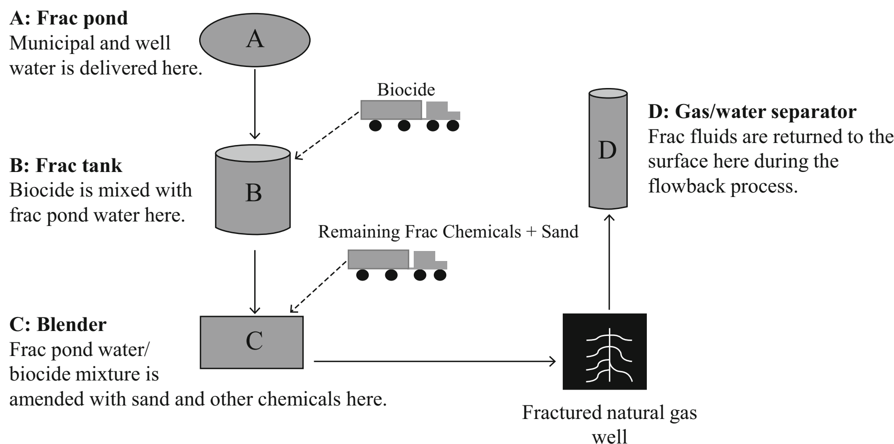


Fig. 1 Generalized overview of the events that occur during the hydraulic fracturing process in shale formations (Struchtemeyer and Elshahed 2012). The *solid arrows* represent the direction that fracturing fluids flow during the fracturing and flowback processes. The *dashed lines* are used to highlight points where biocides, sand, and other fracturing chemicals are introduced to the system during the process

process referred to as flowback (Fig. 1), which involves the removal of the liquid portion of the frac fluids from the formation (Struchtemeyer and Elshahed 2012). Previous work has shown that the flowback process only recovers between 30% and 70% of the fracturing fluids that were pumped into the formation (Veil 2010). The large volumes of frac fluids that are left behind in the formation are exposed to high temperatures, become anaerobic, and leach chlorides, iron, and organic matter from the shale (Blauch et al. 2009; Houston et al. 2009). These conditions will likely allow for the growth and proliferation of indigenous microorganisms that are present in the shale and any microorganisms in the frac fluids that survived biocide treatments (Blauch et al. 2009; Houston et al. 2009). The sand that was added to the frac fluids is also left behind in the shale and holds open the newly created fractures, which allows gas to flow to the wellbore (Arthur et al. 2008; Veil 2010).

In spite of the importance of hydraulic fracturing, the microbiological properties of frac fluids have only been investigated recently. Studies conducted at newly constructed natural gas wells in the Marcellus and Barnett shale formations have used 16S rRNA gene sequencing methodologies to examine the microbial communities in samples of preinjection frac fluids and flowback waters (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). The microbial communities in pre-injection frac fluids from both shale formations were highly diverse and dominated by 16S rRNA sequences affiliated with aerobic *Actinobacteria*, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, and *Cyanobacteria*, which have previously been observed in freshwater ecosystems (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012; Wu et al. 2007; Zwart et al. 2002). These observations were not surprising since freshwater was the

primary component used to prepare fracturing fluids in these shale formations (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). The microbial communities in flowback water samples from these two shale formations were much less diverse than the microbial communities present in the preinjection frac fluids (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). The flowback water communities from these two shale formations also bore little, if any, resemblance to the preinjection frac fluid communities (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). As the flowback process progressed in each of these two shale formations, the microbial communities in flowback waters became dominated by 16S rRNA gene sequences that were affiliated with the phylum *Firmicutes* (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). Many of the 16S rRNA gene sequences that were more abundant in flowback waters than the preinjection frac fluids were affiliated with facultative/obligate anaerobes, endospore-forming microorganisms, thermophilic microorganisms, halophilic microorganisms, and sulfide-producing microorganisms (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). The increased abundance of these different groups of microorganisms in flowback waters was attributed to a variety of physical (e.g., increased temperature) and chemical (e.g., reduced oxygen concentrations, biocide exposure, increased salt concentrations, and increased sulfur concentrations) changes that these fluids were exposed to during the fracturing process (Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012).

One of the major drawbacks associated with 16S rRNA sequencing-based studies is their inability to provide insight on the metabolic capabilities of microorganisms within a specific microbial community (Mohan et al. 2014). Traditionally these studies have required the use of assumptions regarding the metabolic capabilities of specific microbial community members (Mohan et al. 2014). These assumptions are typically made based on the physiological properties of the closest cultured microorganisms that are identified in 16S rRNA sequence databases (Mohan et al. 2014). Recent advances in the field of metagenomics allow researchers to simultaneously gain insight on the taxonomic and metabolic profiles of microorganisms within a specific community. Metagenomic studies were recently conducted with the same preinjection frac fluids and flowback waters from the Marcellus shale that had previously been subjected to 16S rRNA sequence analyses (Mohan et al. 2014; Murali Mohan et al. 2013). The microbial communities that were detected in these fluids using metagenomics were consistent with those obtained using 16S rRNA gene-based surveys (Mohan et al. 2014; Murali Mohan et al. 2013). The metabolic profile from these samples demonstrated that increases in relative abundance and functional changes occurred for genes responsible for carbohydrate metabolism, respiration, sporulation and dormancy, iron acquisition and metabolism, stress response, and sulfur metabolism in flowback water relative to the preinjection frac fluids (Mohan et al. 2014). As was the case with 16S rRNA sequencing surveys, the results of this metagenomic study seemed to suggest that the populations of microorganisms in flowback water were adapted to survive the elevated temperatures, reduced oxygen concentrations, biocide amendments, and increased concentrations of salt, iron, and sulfur, which

are routinely encountered in shale formations (Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012).

Even though 16S rRNA and metagenomic studies have provided strong evidence that fracturing stimulates populations of microorganisms which could theoretically survive in shale formations, it is important to point out that these cultivation-independent methodologies cannot be used to identify active microbial communities or processes. However a variety of cultivation-based studies, which were conducted at newly fractured well sites in shale formations, have produced results that are similar to what has been observed using these cultivation-independent approaches (Harrington 2015; Liang et al. 2016). A wide variety of bacterial isolates were recently obtained from pre-injection frac fluids and flowback waters from the Bakken shale formation in North Dakota (Harrington 2015). Taxonomic comparisons of the isolates from these water samples, which involved the use of 16S rRNA gene sequences, indicated that the microbial community in these fluids underwent drastic changes during the fracturing process and was accompanied by a significant decrease in microbial diversity (Harrington 2015). The 16S rRNA sequencing data also indicated that the majority of the isolates from the flowback water were affiliated with *Firmicutes* (Harrington 2015). All of these observations are consistent with what has been observed in other shale formations using cultivation-independent approaches (Harrington 2015; Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). Many bacterial isolates from flowback water collected in the Bakken and Niobrara shale formations possessed physiological properties, including the ability to grow at high temperatures under anaerobic conditions, produce spores, resist biocide treatments, and tolerate high salt concentrations, which are all consistent with genes that displayed increased abundance in metagenomic studies of flowback water (Harrington 2015; Mohan et al. 2014). The phylogeny of the majority of these bacterial isolates (e.g., *Bacilli* and *Gammaproteobacteria*) was also highly similar to 16S rRNA sequences that have been observed in other flowback water samples (Harrington 2015; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). An isolate from the genus *Halanaerobium*, which is one of the most commonly observed genera of halophilic bacteria in flowback waters from shale formations, was also recently recovered from a natural gas-producing field in the Barnett shale that underwent hydraulic fracturing (Liang et al. 2016; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). This microorganism coupled the degradation of guar gum, which is a polysaccharide commonly used to increase the viscosity of hydraulic fracturing fluids, to the production of sulfide when thiosulfate was present (Liang et al. 2016). The ability of this *Halanaerobium* sp. to degrade one of the major components in fracturing fluids, along with the fact that members of this genus have been repeatedly observed in flowback waters from multiple and distinct formations, suggests that these microorganisms are likely stimulated by and survive the hydraulic fracturing process. The production of sulfide by this *Halanaerobium* sp. is also consistent with metagenomic studies from flowback waters, which showed that sulfur-metabolizing genes were more abundant in flowback water relative to the preinjection frac fluids (Liang et al. 2016; Mohan et al. 2014).

The results of previous work have clearly shown that biocide treatments do not kill all of the microorganisms in frac fluids prior to their injection into shale formations (Struchtemeyer and Elshahed 2012). This observation is extremely concerning since large volumes of frac fluids are routinely lost to shale formations during the fracturing process (Veil 2010). The collective results of cultivation-independent and cultivation-based studies all seem to show that the hydraulic fracturing process stimulates populations of microorganisms, which appear to be adapted to survive the conditions that are encountered in shale formations (Harrington 2015; Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer and Elshahed 2012). Many of these microorganisms (e.g., sulfide-producing microorganisms) are known to contribute to deleterious processes (e.g., reservoir plugging/souring and corrosion) in oil and natural gas wells (Fichter et al. 2009; Kermani and Harrop 1996; Youssef et al. 2009). Thus it appears highly likely that the fracturing process, much like the drilling process, contributes to many of the deleterious microbially induced processes that have been observed in shale oil and natural gas wells.

5 Research Needs

Even though more recent studies have begun to shed light on the microbiology of oil- and natural gas-producing shale formations, additional research is needed to obtain a more complete understanding of these complex ecosystems. The microbiological properties of shale formations clearly need to be studied more extensively. However, the collection of shale samples can be quite challenging due to a variety of factors (e.g., the requirement for sophisticated drilling equipment to prevent outside microbial contamination), which have been documented previously (Magot 2005). Many of the factors that make the collection of shale samples difficult lead to cost, personnel, and time constraints (Magot 2005). These issues have prevented the oil and natural gas industries from using optimized drilling methods to collect shale samples that are free of drilling mud contamination (Magot 2005). The impacts of the drilling and hydraulic fracturing processes on the microbiological properties of shale formations also need to be evaluated further. To date, the microbiology of the fluids used during these two processes has primarily been characterized using 16S rRNA sequencing and metagenomic analyses (Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer et al. 2011; Struchtemeyer and Elshahed 2012). The results of this work have clearly shown that drastic shifts in microbial communities occur during these two processes. However, these types of studies provide very little insight on which populations of microorganisms are active in these fluids. Metatranscriptome analysis of drilling and fracturing fluids both pre- and postinjection would provide valuable insight on the active populations of microorganisms that are stimulated by these two processes.

Recent insight on the microbiology of oil- and natural gas-producing shale formations has led to a surge in efforts to control microbial growth in these ecosystems. Numerous studies have conducted laboratory-based experiments to

evaluate the effectiveness of chemical biocides against microorganisms that are commonly found in hydraulic fracturing fluids (Gaspar et al. 2014; Kahrilas et al. 2014; Struchtemeyer et al. 2012). The results of this work have shown that nonoxidizing biocides are more suitable for controlling microbial growth in shale formations than oxidizing biocides (Kahrilas et al. 2014). This has been attributed to the fact that nonoxidizing biocides are generally more stable and less likely to react with the components in fracturing fluids than oxidizing biocides (Kahrilas et al. 2014). The results of laboratory-based studies of chemical biocides have also provided valuable insight on factors that influence biocide efficacy and the mechanisms that microorganisms use to resist biocide treatments (Gaspar et al. 2014; Kahrilas et al. 2014; Struchtemeyer et al. 2012). However, very few studies have conducted long-term in situ examinations of the effects of biocide type, biocide concentration, and frequency of biocide applications on microbial growth in shale formations. This type of work would likely provide valuable insight on how to control microbial growth in oil- and natural gas-producing shale formations, which cannot be obtained through the use of laboratory-based studies alone. For example, recent studies showed that the practice of biocide rotation in hospitals and cooling towers led to decreased biocide resistance (Bentham and Broadbent 1995; Murtough et al. 2001, 2002; Rangel et al. 2011). In situ examinations of biocide rotations in shale formations may produce similar results and help control populations of microorganisms that are typically difficult to treat (e.g., spore formers) in shale formations (Gaspar et al. 2014). In addition to biocide treatments, many other operational practices should also be reevaluated in order to better control microbial growth in oil- and natural gas-producing shale formations. For example, the use of barite as a weighing agent in water-based drilling muds is problematic because it has been shown to stimulate the growth of sulfide-producing microorganisms (Struchtemeyer et al. 2011). Studies have shown that this issue can be avoided by using dolomite in place of barite in water-based drilling muds (Badrul et al. 2007). The amount of time that passes between the frac and flowback processes in newly constructed oil- and natural gas-producing wells in shale formations is also highly variable (Struchtemeyer et al. 2012). The extended incubation of frac fluids in shale formations appears to stimulate the growth and proliferation of microbial communities that are known to cause deleterious processes including sulfidogenesis and corrosion (Mohan et al. 2014; Murali Mohan et al. 2013; Struchtemeyer et al. 2012). This observation is problematic since recycled flowback waters are being used more frequently during the fracturing process at newly constructed oil- and gas-producing wells in shale formations (Arthur et al. 2008; Veil 2010). Additional well construction practices that contribute to microbial-related issues in shale formations need to be identified and studied using long-term, comprehensive, and statistical analyses to prevent their occurrence in newly constructed wells that are used for the production of oil and natural gas.

Acknowledgments I would like to thank Mrs. Ramona Witcher for the editing and proofreading assistance with this chapter.

References

Alexander T et al (2011) Shale gas revolution. Oilfield Rev 23:40–55
Arthur JD, Bohm B, Layne M Hydraulic fracturing considerations for natural gas wells of the Marcellus Shale. Ground Water Protection Council Annual Forum, 2008
Badrul M, Chiou L, Azlina Z, Juliana Z (2007) Dolomite as an alternative weighting agent in drilling fluids. J Eng Sci Technol 2(2):164–176
Benka-Coker M, Olumagin A (1996) Effects of waste drilling fluid on bacterial isolates from a mangrove swamp oilfield location in the Niger Delta of Nigeria. Bioresour Technol 55 (3):175–179
Bentham RH, Broadbent CR (1995) Field trial of biocides for control of *Legionella* in cooling towers. Curr Microbiol 30(3):167–172
Blauch ME, Myers RR, Moore T, Lipinski BA, Houston NA Marcellus shale post-frac flowback waters-where is all the salt coming from and what are the implications? SPE Eastern Regional Meeting, 2009
Bowker KA (2003) Recent development of the Barnett Shale play, Fort Worth Basin. W Tex Geol Soc Bull 91:523–533
Bowker KA (2007) Barnett Shale gas production, Fort Worth Basin: issues and discussion. AAPG Bull 91(4):523–533
Boyer C, Clark B, Jochen V, Lewis R, Miller CK (2011) Shale gas: a global resource. Oilfield Rev 23(3):28–39
Bruner K, Smosna R (2011) A comparative study of the Mississippian Barnett shale, Fort Worth Basin, and Devonian Marcellus shale, Appalachin Basin, DOE/NETL-2011/1478. National Energy Technology Laboratory, Morgantown
Burke CJ, Veil JA (1995) Synthetic-based drilling fluids have many environmental pluses. Oil Gas J 93(48):59–71
Cook T, Perrin J (2016) Hydraulic fracturing accounts for about half of current U.S. crude oil production Today in Energy. U.S. Energy Information Administration, http://www.eia.gov/todayinenergy/detail.cfm?id=25372
Curtis JB (2002) Fractured shale-gas systems. AAPG Bull 86(11):1921–1938
Darley HC, Gray GR (1988) Composition and properties of drilling and completion fluids. Gulf Professional Publishing, Houston
Fichter J, Johnson K, French K, Oden R (2009) Biocides control Barnett Shale fracturing fluid contamination. Oil Gas J 107:38–44
Foght J (2010) Microbial communities in oil shales, biodegraded and heavy oil reservoirs, and bitumen deposits. In: Timmis K (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 2159–2172
Fredrickson J et al (1997) Pore-size constraints on the activity and survival of subsurface bacteria in a late cretaceous shale-sandstone sequence, northwestern New Mexico. Geomicrobiol J 14 (3):183–202
Gaspar J et al (2014) Microbial dynamics and control in shale gas production. Environ Sci Technol Lett 1(12):465–473
Grassia GS, McLean KM, Glénat P, Bauld J, Sheehy AJ (1996) A systematic survey for thermophilic fermentative bacteria and archaea in high temperature petroleum reservoirs. FEMS Microbiol Ecol 21(1):47–58
Harrington A (2015) Cultivation-dependent analysis of microorganisms associated with various hydraulic fracturing fluids. University of Nevada Las Vegas Theses, Dissertations, Professional Papers, and Capstones. Paper 2479. Las Vegas
Head I et al (2010) Hydrocarbon degradation in petroleum reservoirs. In: Timmis K (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 3097–3109
Houston NA, Blauch ME, Weaver DR, Miller D, O'Hara D Fracture-stimulation in the Marcellus Shale-lessons learned in fluid selection and execution. SPE Eastern Regional Meeting, 2009
Hu D, Xu S (2013) Opportunity, challenges and policy choices for China on the development of shale gas. Energ Policy 60:21–26

Jones D et al (2008) Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature 451(7175):176–180

Kahrilas GA, Blotevogel J, Stewart PS, Borch T (2014) Biocides in hydraulic fracturing fluids: a critical review of their usage, mobility, degradation, and toxicity. Environ Sci Technol 49(1):16–32

Kermani M, Harrop D (1996) The impact of corrosion on oil and gas industry. SPE Prod Facil 11:186–189

Kerr RA (2010) Natural gas from shale bursts onto the scene. Science 328(5986):1624–1626

Kleikemper J, Schroth MH, Sigler WV, Schmucki M, Bernasconi SM, Zeyer J (2002) Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. Appl Environ Microbiol 68(4):1516–1523

Krumholz LR, McKinley JP, Ulrich F, Suflita JM (1997) Confined subsurface microbial communities in Cretaceous rock. Nature 386(6620):64–66

Liang R et al (2016) Metabolic capability of a predominant *Halanaerobium* sp. in hydraulically fractured gas wells and its implication in pipeline corrosion. Front Microbiol. https://doi.org/10.3389/fmicb.2016.00988

Loucks RG, Ruppel SC (2007) Mississippian Barnett Shale: lithofacies and depositional setting of a deep-water shale-gas succession in the Fort Worth Basin, Texas. AAPG Bull 91(4):579–601

Magot M (2005) Indigenous microbial communities in oil fields. In: Ollivier B, Magot M (eds) Petroleum microbiology. ASM Press, Washington, DC, pp 21–33

Martini A, Walter L, Budai J, Ku T, Kaiser C, Schoell M (1998) Genetic and temporal relations between formation waters and biogenic methane: Upper Devonian Antrim Shale, Michigan Basin, USA. Geochim Cosmochim Acta 62(10):1699–1720

Martini AM, Walter LM, McIntosh JC (2008) Identification of microbial and thermogenic gas components from Upper Devonian black shale cores, Illinois and Michigan basins. AAPG Bull 92(3):327–339

Meckenstock RU et al (1999) 13C/12C isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ Microbiol 1(5):409–414

Meckenstock RU, Morasch B, Griebler C, Richnow HH (2004) Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers. J Contam Hydrol 75(3):215–255

Mohan AM, Bibby KJ, Lipus D, Hammack RW, Gregory KB (2014) The functional potential of microbial communities in hydraulic fracturing source water and produced water from natural gas extraction characterized by metagenomic sequencing. PLoS ONE 9:e107682. https://doi.org/10.1371/journal.pone.0107682

Murali Mohan A, Hartsock A, Bibby KJ, Hammack RW, Vidic RD, Gregory KB (2013) Microbial community changes in hydraulic fracturing fluids and produced water from shale gas extraction. Environ Sci Technol 47(22):13141–13150

Murtough S, Hiom S, Palmer M, Russell A (2001) Biocide rotation in the healthcare setting: is there a case for policy implementation? J Hosp Infect 48(1):1–6

Murtough S, Hiom S, Palmer M, Russell A (2002) A survey of rotational use of biocides in hospital pharmacy aseptic units. J Hosp Infect 50(3):228–231

Nweke C, Okpokwasili G (2003) Drilling fluid base oil biodegradation potential of a soil *Staphylococcus* species. Afr J Biotechnol 2(9):293–295

Okpokwasili G, Nnubia C (1999) Biodegradation of drilling fluid by marine bacteria from below an oil rig. J Sci Eng Technol 6:1420–1428

Onstott T et al (1998) Observations pertaining to the origin and ecology of microorganisms recovered from the deep subsurface of Taylorsville Basin, Virginia. Geomicrobiol J 15(4):353–385

Prasad M, Katiyar S (2010) Drill cuttings and fluids of fossil fuel exploration in north-eastern India: environmental concern and mitigation options. Curr Sci 98(12):1566–1569

Rangel KM, Delclos G, Emery R, Symanski E (2011) Assessing maintenance of evaporative cooling systems in legionellosis outbreaks. J Occup Environ Hyg 8(4):249–265

Riebeek H (2011) The carbon cycle. In: Earth observatory, NASA. http://earthobservatory.nasa.gov/Features/CarbonCycle/page2.php. Accessed 5 July 2016

Röling WF, Head IM, Larter SR (2003) The microbiology of hydrocarbon degradation in subsurface petroleum reservoirs: perspectives and prospects. Res Microbiol 154(5):321–328
Salinas MB et al (2004) *Petrobacter succinatimandens* gen. nov., sp. nov., a moderately thermophilic, nitrate-reducing bacterium isolated from an Australian oil well. Int J Syst Evol Microbiol 54(3):645–649
Schoell M (1980) The hydrogen and carbon isotopic composition of methane from natural gases of various origins. Geochim Cosmochim Acta 44(5):649–661
Struchtemeyer CG, Elshahed MS (2012) Bacterial communities associated with hydraulic fracturing fluids in thermogenic natural gas wells in North Central Texas, USA. FEMS Microbiol Ecol 81(1):13–25
Struchtemeyer CG, Davis JP, Elshahed MS (2011) Influence of the drilling mud formulation process on the bacterial communities in thermogenic natural gas wells of the Barnett Shale. Appl Environ Microbiol 77(14):4744–4753
Struchtemeyer CG, Morrison MD, Elshahed MS (2012) A critical assessment of the efficacy of biocides used during the hydraulic fracturing process in shale natural gas wells. Int Biodeterior Biodegrad 71:15–21
Tseng H-Y, Onstott TC (1997) A tectogenetic origin for the deep subsurface microorganisms of Taylorsville Basin: thermal and fluid flow model constraints. FEMS Microbiol Rev 20(3–4):391–397
Veil J (2010) Water management technologies used by Marcellus Shale Gas Producers. Final Report ANL/EVS/R-10/3 prepared for United States Department of Energy. Argonne National Laboratory (ANL), Lemont
Vengosh A, Warner N, Jackson R, Darrah T (2013) The effects of shale gas exploration and hydraulic fracturing on the quality of water resources in the United States. Procedia Earth Planet Sci 7:863–866
Vengosh A, Jackson RB, Warner N, Darrah TH, Kondash A (2014) A critical review of the risks to water resources from unconventional shale gas development and hydraulic fracturing in the United States. Environ Sci Technol 48(15):8334–8348
Wang Q, Chen X, Jha AN, Rogers H (2014) Natural gas from shale formation–the evolution, evidences and challenges of shale gas revolution in United States. Renew Sustain Energy Rev 30:1–28
Wilhelms A, Larter S, Head I, Farrimond P, Di-Primio R, Zwach C (2001) Biodegradation of oil in uplifted basins prevented by deep-burial sterilization. Nature 411(6841):1034–1037
Wu X, Xi W, Ye W, Yang H (2007) Bacterial community composition of a shallow hypertrophic freshwater lake in China, revealed by 16S rRNA gene sequences. FEMS Microbiol Ecol 61(1):85–96
Youssef N, Elshahed MS, McInerney MJ (2009) Microbial processes in oil fields: culprits, problems, and opportunities. Adv Appl Microbiol 66:141–251
Zwart G, Crump BC, Kamst-van Agterveld MP, Hagen F, Han S-K (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol 28(2):141–155

Cometabolic Bioremediation

14

Terry C. Hazen

Contents

1 Introduction 234
2 Methanotrophs 239
3 Ammonium Oxidizers 243
4 Research Needs 243
References 244

Abstract

Cometabolic bioremediation is probably the most underappreciated bioremediation strategy currently available. Cometabolism strategies stimulate only indigenous microbes with the ability to degrade the contaminant and cosubstrate, e.g., methane, propane, toluene, and others. This highly targeted stimulation insures that only those microbes that can degrade the contaminant are targeted, thus reducing amendment costs, well and formation plugging, etc. Cometabolic bioremediation has been used on some of the most recalcitrant contaminants, e.g., PCE, TCE, MTBE, TNT, dioxane, atrazine. Methanotrophs have been demonstrated to produce methane monooxygenase, an oxidase that can degrade over 1000 compounds. Cometabolic bioremediation also has the advantage of being able to degrade contaminants to trace concentrations, since the biodegrader is not dependent on the contaminant for carbon or energy. In the Gulf of Mexico and in the Arctic Tundra, we have recently found that natural attenuation can be a

T. C. Hazen (✉)
Department of Civil and Environmental Engineering, Department of Earth and Planetary Sciences, Department of Microbiology, Institute for Secure and Sustainable Environment, University of Tennessee, Knoxville, TN, USA

Oak Ridge National Laboratory, Oak Ridge, TN, USA
e-mail: tchazen@utk.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_5

cometabolic process also. Increasingly we are finding that in order to protect human health and the environment that we must remediate to lower and lower concentrations, especially for compounds like endocrine disrupters and trace organics, thus cometabolism may be the best and may be the only possibility that we have to bioremediate some contaminants.

1 Introduction

Cometabolism is the process by which a contaminant is fortuitously degraded by an enzyme or cofactor produced during microbial metabolism of another compound. Typically, there is no apparent benefit to the microorganism involved. Bioremediation strategies that use electron donors that only stimulate a specific group of microorganisms that can degrade the contaminants of concern are ideal for many applications. Many electron donors used as amendments for bioremediation can broadly stimulate many members of the indigenous microbial community, most of which do not have the ability to degrade or completely degrade the contaminants of concern. Indeed, this often creates problems of excess biomass (e.g., plugging the aquifer around the injection site), incomplete degradation of contaminants, transformation of contaminants to more recalcitrant or toxic daughter products, higher costs (amendment/contaminant), and inability of the amendment to stimulate biodegradation at low contaminant concentrations. Cometabolic bioremediation enables remediation strategies that stimulate biodegradation of the contaminants at contaminant concentrations that are way below the concentration that could be of carbon or energy benefit to the biodegrader. Thus, cometabolic bioremediation has the added advantage of allowing scrubbing of environmental contaminants down to undetectable concentrations, e.g., <parts per trillion. Cometabolic bioremediation has been applied both aerobically and anaerobically to a wide variety of contaminants in different environments. The first mention of cometabolic bioremediation was by Wilson and Wilson (1985) and was later defined by McCarty (1987). Cometabolic bioremediation has been used in the field for more than 30 years on some of the most recalcitrant contaminants, e.g., chlorinated alkenes, PAHs, halogenated aliphatic and aromatic hydrocarbons, MTBE, explosives, dioxane, PCBs, pesticides, and pharmaceuticals.

Microorganisms are versatile in their ability to exist in a variety of habitats and live in hostile environments having a wide range of pH, temperature, heavy metal concentrations, oxygen concentrations, barometric pressures, salinity, and radiation. Under these diverse conditions, a number of microbial types have been isolated that cometabolize contaminants and their daughter products. Ensley (1991) demonstrated a linkage between TCE degradation and aromatic metabolism in *P. cepacia* G4, *P. mendocina*, and *P. putida*. Ensign et al. (1992) reported that pure cultures of *Xanthobacter* sp. cometabolized TCE with the utilization of propylene as a substrate using the enzyme alkene monooxygenase. It is well recognized that TCE and other chlorinated aliphatic compounds can be degraded by selected methanogens (Bouwer and McCarty 1984), methanotrophs

(Little et al. 1988), species of *Pseudomonas* (*P. cepacia, P. mendocina* and *P. putida*), and nitrifiers (Vannelli et al. 1990; Hyman et al. 1988) capable of degrading aromatic compounds (Nelson et al. 1988). Additionally, aerobic conditions do not appear to support the formation of undesirable metabolites, such as c-DCE, t-DCE or VC that are dehalogenation products of anaerobic degradation of TCE. Mahendra et al. (2007) demonstrated that monooxygenase-containing bacteria could degrade 1,4-dioxane. Methyl tert-butyl ether (MTBE) has also been remediated cometabolically (Chen et al. 2006), as has TNT (Yasin et al. 2008), PCBs (Lajoie et al. 1994), and atrazine (Ghosh and Philip 2004) (Table 1). More recently emerging trace organic contaminants (Liu et al. 2015), carbazole (Shi et al. 2015), dibenzofuran (Shi et al. 2013), pharmaceuticals (Gauthier et al. 2010), 1,1,2,2-tetrachloroehane (Cappelletti et al. 2018), lincomycin (Li et al. 2016), tetrabromobisphenol (Gu et al. 2016), and decolorization of textile dyes (Karim et al. 2017) have all been shown to biodegrade cometabolically.

The aerobic cometabolic biodegraders are dependent upon oxygenases, e.g., methane monooxygenase, toluene dioxygenase, toluene monooxygenase, and ammonia monooxygenase. These enzymes are extremely strong oxidizers, e.g., methane monooxygenase is known to degrade over 1000 different compounds. However, like any bioremediation process, the proper biogeochemical conditions are necessary to maximize and maintain biodegradation, e.g., maintaining oxygen levels or other terminal electron acceptors that the cometabolic biodegrader is dependent (Table 2 and Hazen 1997; Hazen and Sayler 2016, and ▶ Chap. 12, "In Situ Groundwater Bioremediation" in this book). In addition, cometabolic biostimulation may require pulsing of electron donor or electron acceptor to reduce competitive inhibition between the substrate the microbe can use and the contaminant. Pulsing of methane was found to significantly improve biodegradation of TCE rates by methanotrophs (Hazen et al. 2009). Indeed, during the Deep Water Horizon (DWH) leak (Hazen et al. 2010), there was evidence that in the Gulf of Mexico where episodic releases methane have occurred for millions of years from natural seeps that this pulsing of methane may be degrading oil and other organics via cometabolic biodegradation. The methane oxidizers bloomed during the DWH leaked above 400 m once the well was capped (Dubinsky et al. 2013; Redmond and Valentine 2012; Reddy et al. 2012). This suggests that intrinsic cometabolic bioremediation or cometabolic natural attenuation may be a serious phenomenon in the ocean and in arctic tundra (Stackhouse et al. 2017). It has also been found that significant background biodegradation reactions can occur during injection of terminal electron acceptors like oxygen. Enzien et al. (1994) demonstrated that in a bulk aerobic environment being injected with methane and air significant amount of reductive dechlorination of PCE to TCE could occur in anaerobic niches in the aquifer sediment. Rates of PCE and TCE oxidation are inversely different depending on the number of Cl (Fig. 1).

Given the diverse body of literature on cometabolic bioremediation processes, we will focus in detail on the two groups that have been most well studied, i.e., methanotrophs and ammonium oxidizers.

Table 1 Cometabolic bioremediation substrates, enzymes, and contaminants

Cosubstrates	Methane, methanol, propane, propylene biphenyl (aerobic)	Ammonia, nitrate (aerobic)	Toluene, butane, phenol, citral, cumin aldehyde, cumene, and limonene (aerobic)	Methanol (anaerobic)	Glucose, acetate, lactate, sulfate, pyruvate (anaerobic)
Enzymes (microbes)	Methane monooxygenase, methanol dehydrogenase, alkene monooxygenase, catechol dioxygenase (*Methylosinus, Ralstonia, Rhodococcus*)	Ammonia monooxygenase (*Nitrosomonas, Nitrobacter, ammonia oxidizing archaeal*)	Toluene monooxygenase, toluene dioxygenase (*Rhodococcus, Pseudomonas, Arthrobacter, Comamonas*)	Alcohol dehydrogenases (*Pseudomonas, Streptomyces, Corynebacterium*)	Dehalogenase, AtzA, dichloromethane dehalogenase (*Dehalococcoides, Dehalobacter, Methanogens, Desulfovibrio, Clostridium, Geobacter, Clavibacter, Aspergillus*)
Contaminants	TCE, DCE, VC, PAHs, PCBs, MTBE, creosote, 1,4 dioxane, >1000 different compounds	TCE, DCE, VC, TNT, emerging trace organic contaminants	TCE, DCE, VC, 1,1-DCE, 1,1,1-TCA, MTBE, TBBPA, carbazole, dibenzofuran, pharmaceuticals	PCE, TCE, DCE, VC, hexachloro-cyclohexane	BTEX, PCE, PAHs, pyrene, atrazine, TNT, lincomycin decolorization, etc.

Table 2 Performance monitoring parameters for cometabolic biodegradation

Performance parameter	Method	Data use	Performance expectation	Recommended frequency of analysis
Chemicals of concern (CoCs)	EPA SW-846: 8260B (VOC) or 8270D (SVOC) (laboratory). Field gas chromatography (GC) or GC/ mass spectroscopy (MS)	Background and source/plume for comparison following treatment. Also used to determine if there are compounds that may inhibit the cometabolic process	CoCs and degradation products are expected to decline to below regulatory compliance levels within the treatment zone after substrate addition	Baseline and recommended for each groundwater sampling round
Primary substrate	EPA SW-846: 8260B (VOC) or 8270D (SVOC) (laboratory). Field gas chromatography (GC) or GC/ mass	Used to determine the extent and availability of substrate for consumption by bacteria	Downward trend in substrate concentrations should track downward trend in CoC concentrations	Site specific – baseline and all sampling events thereafter
Appropriate cometabolic degrading microorganisms	Quantified by molecular techniques such as polymerase chain reaction – specialty laboratory	Used to determine presence and quantity of appropriate microorganism at baseline period or after bioaugmentation	Appropriate microorganisms will be detected and increase as a consequence of adding electron acceptors and/or donors	Baseline prior to remedy initiation and quarterly. Once a high titer is measured and growth is ensured, the test is not critical
Oxygen	Bacharach Fyrite® Gas Analyzer (soil gas). DO meter (APHA 1992: 4500-O G) (field). Downhole probe or flow-through cell (groundwater)	For aerobic cometabolism determines aerobic conditions exist. For anaerobic cometabolism determines absence of oxygen	Determines if consumption of oxygen requires supplementation or if increase in oxygen will require substrate to reduce it	Site specific – baseline and as appropriate thereafter
pH	Field probe with direct-reading meter (APHA 1992: 4500-H+ B)	Used to confirm pH conditions are stable or to identify trends of concern (EPA 2004)	Enhanced aerobic bioremediation pH range of 5–9 pH units (EPA 2004)	For active systems daily for the startup phase (7–10 days) and weekly to monthly thereafter (EPA 2004)

(*continued*)

Table 2 (continued)

Performance parameter	Method	Data use	Performance expectation	Recommended frequency of analysis
Carbon dioxide	APHA et al. 1992: 4500-CO_2 C (titrimetric) or 4500-CO_2 D (calculation requiring total alkalinity and pH)	Used as an indicator that microbial activity has been stimulated	Indicator parameter	Optional for active system (ITRC 2008)
Oxidation reduction potential (ORP)	Direct-reading meter, A2580B, or USGS A6.5 (field)	Used with other geochemical parameters to determine if groundwater conditions are optimal for aerobic or anaerobic biodegradation	Positive ORP values (>0.0 mV) with elevated DO and absence of TOC/DOC can indicate that additional substrate is needed for anaerobic biodegradation	Baseline and typically measured at the wellhead using a flow-through cell to protect samples from exposure to oxygen

Modiified after https://clu-in.org/techfocus/default.focus/sec/Bioremediation/cat/Cometabolic_Aerobic_and_Anaerobic_Bioremediation

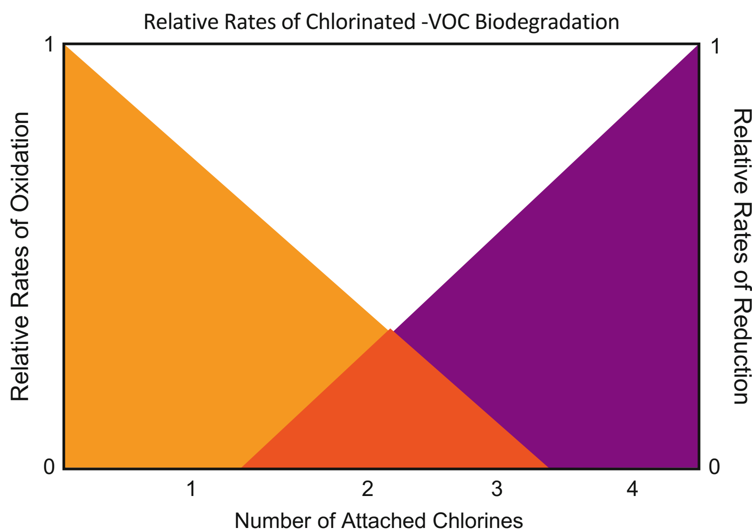


Fig. 1 Aerobic and anaerobic biodegradation rates

2 Methanotrophs

Methanotrophs, methane-oxidizing bacteria, oxidize methane via a series of enzymes that are unique to this group (Koh et al. 1993). The primary enzyme in this oxidation chain is methane monooxygenase. Methane monooxygenase is an extremely powerful oxidizer, thus giving it the capability of oxidizing a wide variety of normally recalcitrant compounds including TCE (Cardy et al. 1991). Wackett (Newman and Wackett 1991; Tsien et al. 1989) and others (Chaudhry and Chapalamadugu 1991; Wilson and Wilson 1985; Fogel et al. 1986; Little et al. 1988) demonstrated that soluble methane monooxygenase induces formation of TCE-epoxide from TCE. TCE-epoxide is extremely unstable and therefore spontaneously breaks down to simpler compounds like formate, etc. All of the daughter compounds are either unstable or small and easily metabolizable compounds, thus making the final and almost immediate end products of TCE-epoxide formation, carbon dioxide, and chloride salts, unlike anaerobic dechlorination which can stall at daughter products like vinyl chloride which are more toxic than the original contaminant, e.g., PCE and TCE (Fig. 2).

Methanotrophic bacteria (methanotrophs) are bacteria that use methane as a sole source of carbon. The first enzyme involved in the oxidation of methane to methanol by methanotrophs is methane monooxygenase (MMO). Two forms of MMO have been reported: soluble methane monooxygenase (sMMO), found mainly in the

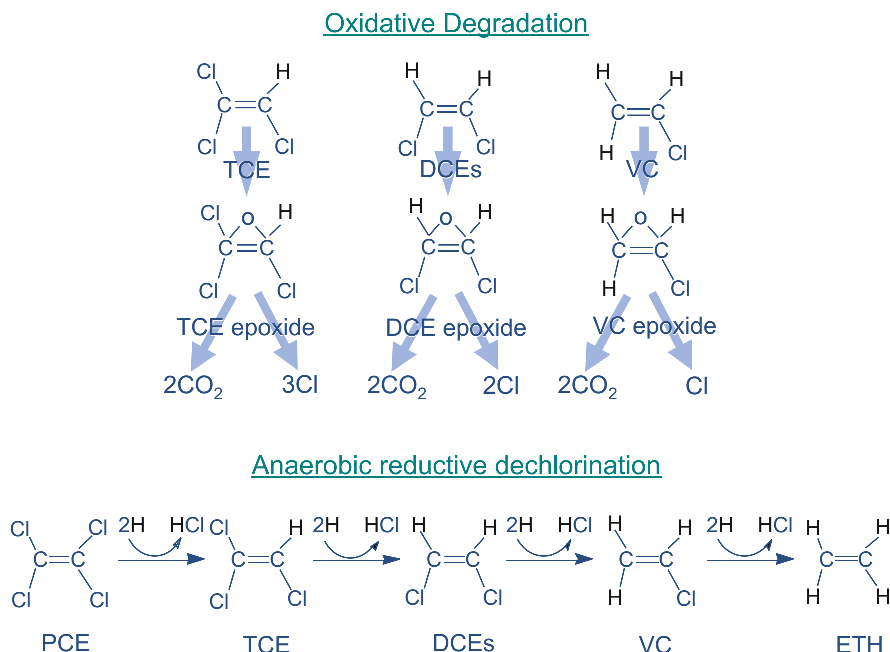


Fig. 2 Aerobic and anaerobic cometabolic pathways

cytoplasm and particulate methane monooxygenase (pMMO) which is associated with the cell membrane. Studies related to these two enzymes have mainly been studied in two methanotrophs, namely, *Methylococcus capsulate* (Bath) and *Methylococcus trichosporium* OB3b. Numerous groups have studied sMMO in great detail with regard to isolation and characterization as well as crystal structure. Since pMMO is membrane bound, this enzyme losses activity upon lysis making it difficult to isolate and purify resulting in fewer details regarding this enzyme. The two enzymes can coexist in methanotrophs; however, their activities have been directly reported to be dependent on the copper ion to biomass ratio in *M. capsulate* (Bath). A low copper ion to biomass ratio expresses sMMO, while a high copper ion to biomass ratio expresses pMMO (Stanley et al. 1983). While pMMO is found in most methanotrophic bacteria, sMMO is present only in a few select methanotrophs. Both MMOs oxidize methane to methanol and are capable of cometabolizing chlorinated aliphatic hydrocarbons, namely, chloroform, dichloromethane, *trans*-dichloroethene, *cis*-1,2-dichloroethene, 1,1-dichloroethene, trichloroethene at various rates and to different extents. Therefore, methanotrophs are a useful tool for commercial purposes mainly cleanup of sites contaminated with toxic pollutants. However, sMMO being nonspecific has a broader substrate specificity in comparison to pMMO, some substrates like cyclohexane or naphthalene cannot be oxidized by pMMO, and both enzymes do not oxidize perchloroethylene. Methanotrophs have also been reported to be useful for production of bulk chemicals and as methane sinks (Oremland and Culbertson 1992). Mixed cultures expressing pMMO have shown to degrade t-DCE, VC, c-DCE, TCE, and 1,1-DCE. Transformation of t-DCE and VC by pMMO was 20 times greater than those reported for sMMO, while transformation of the other three compounds was either similar or less, indicating the importance of this enzyme over sMMO for bioremediation.

One of the many uses of methanotrophs has been in the bioremediation of trichloroethylene (TCE), which is most commonly found in groundwater along with other halogenated compounds. The first product formed in the oxidation of TCE is an epoxide which is then converted to glyoxylic acid with chloride being released. Glyoxylic acid is then oxidized to carbon dioxide. Although TCE is known to be degraded by several other bacteria, e.g., various species of *Pseudomonas*, containing oxygenases, the rate of degradation by methanotrophs expressing sMMO is many times faster than pMMO and other oxygenases making it favorable for use in bioremediation. For efficient bioremediation, it is important to optimize enzyme/enzymes activity responsible for the transformation, as well as to maintain the activity for extended period of time. This has been studied in detail for *M. trichosporium* OB3 by Sayler et al. (1995). Their study showed that specific sMMO activity was directly proportional to the concentration of dissolved methane. Addition of formate (20 mM) significantly increased sMMO activity. Nitrate, phosphate, iron, and magnesium also had remarkable effect on growth as well as sMMO activity. Addition of vitamins also effected sMMO activity; however, excessive vitamins proved to be harmful. Such studies are necessary and prove useful when designing a bioremediation process.

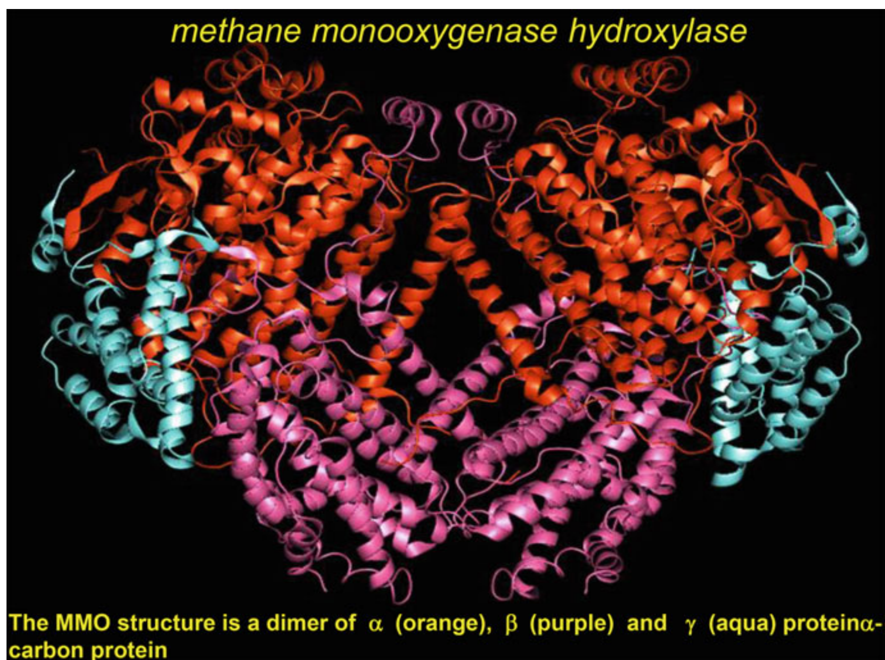


Fig. 3 Methane monooxgenase 3D molecular structure

sMMO from *M. capsulatus* (Bath) and *M. trichosporium* OB3b was shown to consist of three components: protein A, a hydroxylase made-up of three subunits α, β, γ, of molecular masses 60, 45, and 20 kDa, respectively; protein B which is 16 kDa, a regulatory protein; and protein C 39 kDa, a reductase (Paulsen et al. 1994). The crystal structure of sMMO hydroxylase has also been determined (Rosenzweig et al. 1993) (Figs. 3 and 4). In both organisms, the genes encoding for soluble methane monooxygenase enzyme complexes have been found to be clustered on the chromosome. The complete DNA sequences of both gene clusters have been determined and they show considerable homology (Murrell 1992). Detailed studies of the genes encoding sMMO, the DNA sequence, have led to the development of sMMO probes which have been used to detect MMO gene-specific DNA and methanotrophs in mixed cultures and in natural environmental samples (Hazen et al. 2009). The genes for Protein B and Protein C of *Methylococcus* have been expressed in *E. coli* and the proteins obtained were functionally active. Cloning of sMMO genes has led to construction of sMMO mutants of *M. trichosporium* OB3b.

Anderson and McCarty (1997) have reported higher yields of t-DCE and VC degradation by methanotrophs expressing pMMO as compared to sMMO. Also the fact that pMMO are present in most methanotrophs seems logical to develop systems that can enhance this activity for the purpose of treatment of sites contaminated with these compounds. Although sMMO and pMMO are known to coexist in

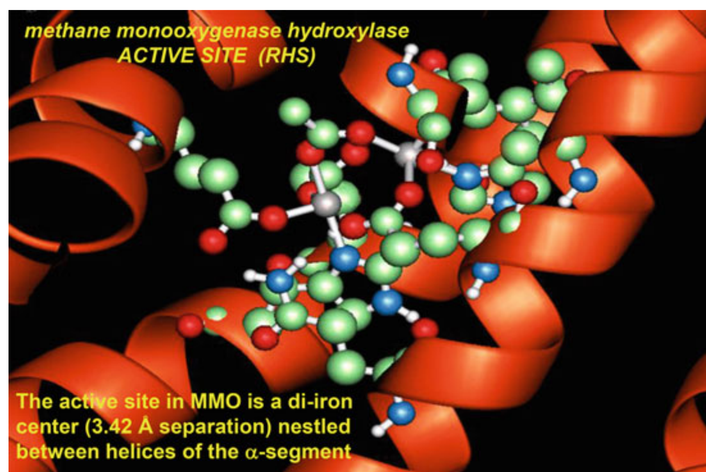


Fig. 4 MMO reaction site

methanotrophs, the fact that pMMO is membrane bound has made it difficult to purify this enzyme unlike sMMO and perform detailed studies like sMMO. Several groups have attempted and are still pursuing this aspect of pMMO and to date only a few reports are available.

Isolation of active pMMO from methanotrophs has been difficult since it loses activity once it has been separated from the membrane. The loss of pMMO activity has been reported to be overcome by addition of a nonionic detergent followed by removal of the detergent and reconstitution of lipid vesicle. Activity of pMMO in the membrane fraction was also stabilized by increasing the concentration of copper in growth medium. Other factors favoring pMMO activity were increased iron and copper concentration, maintaining the pH of buffer at 7.0 and anaerobic conditions during solubilization. Addition of copper ions has resulted in enhanced pMMO activity; however, it has not prolonged the activity nor does it reactivate the enzyme once activity is lost (Zahn et al. 1996). The isolation and characterization of pMMO from *M. capsulatus* (Bath) have been reported by Nguyen et al. (1998). They have obtained active stable pMMO from *M. capsulatus* (Bath) by maintaining high copper levels and methane stress conditions in growth medium. Membrane solubilization was achieved under anaerobic conditions and by addition of dodecyl beta-D-maltoside. The active extract was then purified by chromatography. By switching the growth conditions to favor pMMO activity over sMMO, the same group has reported three polypeptides of 46, 35, 26 kDA and has shown a trinuclear copper center in pMMO by EPR. They have reported pMMO to be copper requiring and sensitive to dioxygen similar to the results of Zahn et al. (1996). The switch between sMMO and pMMO gene expression has been suggested to involve a common regulatory pathway. Chan et al. (2004) have shown pMMO from *M. capsulatus* (Bath) to be a copper-containing three-subunit enzyme. The role of copper in pMMO has been reported to be in the active site of pMMO rather than a structural one.

3 Ammonium Oxidizers

Nitrification is the bacterial mediated process in which ammonia is oxidized sequentially to nitrite then to nitrate. In soils and fresh and saline waters, ammonia is oxidized to nitrite by nitrite-oxidizing bacteria such as the chemolithoautotrophic bacterium, *Nitrosomonas europaea*. Nitrite is oxidized to nitrate by nitrate-oxidizing bacteria such as *Nitrobacter agilis* and *N. winogradskyi* (Fliermans et al. 1974). Nitrifying bacteria are ubiquitous components of the soil and sediment microbial populations. Their activities are stimulated in agricultural soils following the application of ammonia- or urea-based fertilizers.

The oxidation of ammonia to nitrite by *Nitrosomonas europaea* is initiated by the enzyme ammonia monooxygenase (AMO). Because of the broad substrate range of AMO (Arciero et al. 1989), nitrifiers such as *N. europaea* can be used in the bioremediation of contaminated soils, sediments, and groundwaters (Yang et al. 1999). AMO catalyzes the oxidation of ammonia to hydroxylamine which is subsequently oxidized to nitrite (NO_2) by hydroxylamine oxidoreductase (Wood 1986) with the release of four electrons. Two of the electrons are transferred to AMO in order to activate the O_2 and maintain a steady state for ammonia oxidation. AMO in *Nitrosomonas europaea* also catalyzes the oxidation of several alternate substrates including hydrocarbons and halogenated hydrocarbons (Rasche et al. 1990). These oxidations require a reductant which can be supplied by the simultaneous oxidation of ammonia.

Both CH_4 and C_2H_4 competitively inhibit ammonia oxidation by *N. europaea*, since it appears that these compounds bind predominantly to the same binding site as ammonia (Keener and Arp 1993). The competitive character of the inhibition of CH_4, C_2H_4, C_2H_6, CH_3Cl, and CH_3Br is supported by the optimal N_2H_4 requirements that decrease with increasing concentrations of ammonia. Thus, it is not likely that the stimulation of TCE degrading bacteria of the genus *Nitrosomonas* would occur with the injection of methane or other substrates that were competitively inhibitory to the AMO enzyme. Under bioremediation techniques that injected methane, a loss of the *Nitrosomonas* population that has the ability to degrade TCE would be inhibited. Such a phenomenon was observed through the use of species-specific fluorescent antibodies (Fliermans et al. 1994; Hazen et al. 1994).

The AFCEE *IRP Aerobic Cometabolic In Situ Bioremediation Technology Guidance Manual and Screening Software User's Guide* provides specific guidance on well placement and technology design.

See Hazen and Sayler (2016) for examples and methods for Environmental Systems Biology of Contaminated Sites.

4 Research Needs

Cometabolic bioremediation is extremely underappreciated as a bioremediation strategy, though it has been used for an extremely wide variety of contaminants in different environments with different cosubstrates. Indeed, it has also been

underappreciated as a natural attenuation phenomenon as recently demonstrated by studies in the arctic tundra and the Gulf of Mexico (DWH). Much more research needs to be done on modeling life cycle costs of various remediation strategies, including treatment trains and grading into natural attenuation or intrinsic bioremediation. These models need to be tested and verified in full-scale deployments. Cometabolic processes quite often can easily be graded into natural attenuation, e.g., air injection alone at sites with methane or other cometabolic substrate to increase degradation rate and transition into a stable aerobic or microaerophilic environment that can sustain natural attenuation of any residual contaminant. Research on bioaugmentation strategies using cometabolic biodegraders and synthetic biology to produce unique, high rate, and highly specific biodegraders could vastly improve our environmental stewardship in the future.

References

American public health association (APHA) (1992) Standard methods for examination of water and waste 18th ed. American Public health Association, Washing ton DC

Anderson JE, McCarty PL (1997) Transformation yields of chlorinated ethenes by a methanotrophic mixed culture expressing particulate methane monooxygenase. Appl Environ Microbiol 63:687–693

Arciero DM, Vannelli T, Logan M, Hooper AB (1989) Degradation of trichloroethylene by the ammonia-oxidizing bacterium, *Nitrosomonas europaea*. Biochem Biophys Res Commun 159:640–643

Bouwer EJ, McCarty PL (1984) Modeling of trace organics biotransformation in the subsurface. Ground Water 22:433–440

Cappelletti M, Pinelli D, Fedi S, Zannoni D, Frascari D (2018) Aerobic co-metabolism of 1,1,2,2-tetrachloroethane by *Rhodococcus* aetherivorans TPA grown on propane: kinetic study and bioreactor configuration analysis. J Chem Technol Biotechnol 93:155–165

Cardy DNL, Laidler V, Salmond GPC, Murrell JC (1991) Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b. Mol Microbiol 5:1261–1264

Chan SI, Chen KHC, Yu SSF, Chen CL, Kuo SSJ (2004) Toward delineating the structure and function of the particulate methane monooxygenase from methanotrophic bacteria. Biochemistry 43:4421–4430

Chaudhry GR, Chapalamadugu S (1991) Biodegradation of halogenated organic-compounds. Microbiol Rev 55:59–79

Chen KF, Kao CM, Chen TY, Weng CH, Tsai CT (2006) Intrinsic bioremediation of MTBE-contaminated groundwater at a petroleum-hydrocarbon spill site. Environ Geol 50:439–445

Dubinsky EA, Conrad ME, Chakraborty R, Bill M, Borglin SE, Hollibaugh JT, Mason OU, Piceno YM, Reid FC, Stringfellow WT, Tom LM, Hazen TC, Andersen GL (2013) Succession of hydrocarbon-degrading bacteria in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. Environ Sci Technol 47:10860–10867

EPA (2004) How to Evaluate Alternative Cleanup Technologies for Underground Storage Tank Sites: A Guide for Corrective action plan reviewers. United States Environmental Protection Agency, Agency USEP, Washington, DC

Ensign SA, Hyman MR, Arp DJ (1992) Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacter* strain. Appl Environ Microbiol 58: 3038–3046

Ensley BD (1991) Biochemical diversity of trichloroethylene metabolism. Annu Rev Microbiol 45:283–299

Enzien MV, Picardal F, Hazen TC, Arnold RG, Fliermans CB (1994) Reductive dechlorination of trichloroethylene and tetrachloroethylene under aerobic conditions in a sediment column. Appl Environ Microbiol 60:2200–2205

Fliermans CB, Bohlool BB, Schmidt EL (1974) Detection of *Nitrobacter* in natural habitats using fluorescent antibodies. Appl Microbiol 27:124–129

Fliermans CB, Dougherty JM, Franck MM, McKinsey PC, Hazen TC (1994) Immunological techniques as tools to characterize the subsurface microbial community at a trichloroethylene contaminated site. In: Hinchee RE et al (eds) Applied biotechnology for site remediation. Lewis Publishers, Boca Raton, pp 186–203

Fogel MM, Taddeo AR, Fogel S (1986) Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl Environ Microbiol 51(4):720–724

Gauthier H, Yargeau V, Cooper DG (2010) Biodegradation of pharmaceuticals by *Rhodococcus rhodochrous* and *Aspergillus niger* by co-metabolism. Sci Total Environ 408:1701–1706

Ghosh PK, Philip L (2004) Atrazine degradation in anaerobic environment by a mixed microbial consortium. Water Res 38:2277–2284

Gu C, Wang J, Liu SS, Liu GF, Lu H, Jin RF (2016) Biogenic Fenton-like reaction involvement in cometabolic degradation of tetrabromobisphenol A by *Pseudomonas* sp fz. Environ Sci Technol 50:9981–9989

Hazen TC (1997) Bioremediation. In: Amy P, Haldeman D (eds) Microbiology of the terrestrial subsurface. CRC Press, Boca Raton, pp 247–266

Hazen TC, Sayler GS (2016) Environmental systems microbiology of contaminated environments. In: Yates M, Nakatsu C, Miller R, Pillai S (eds) Manual of environmental microbiology, 4th edn. ASM Press, Washington, DC, pp 5.1.6-1–5.1.6-10

Hazen TC, Lombard KH, Looney BB, Enzien MV, Dougherty JM, Fliermans CB, Wear J, Eddy-Dilek CA (1994) Summary of in situ bioremediation demonstration (methane biostimulation) via horizontal wells at the Savannah River Site Integrated Demonstration Project. In: Gee GW, Wing NR (eds) Proceedings of thirty-third Hanford symposium on health and the environment: in-situ remediation: scientific basis for current and future technologies. Battelle, Columbus, pp 135–150

Hazen TC, Chakraborty R, Fleming J, Gregory IR, Bowman JP, Jimenez L, Zhang D, Pfiffner SM, Brockman FJ, Sayler GS (2009) Use of gene probes to assess the impact and effectiveness of aerobic in situ bioremediation of TCE. Arch Microbiol 191:221–232

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Borglin SE, Fortney JL, Stringfellow WT, Bill M, Conrad ME, Tom LM, Chavarria KL, Alusi TR, Lamendella R, Joyner DC, Spier C, Baelum J, Auer M, Zemla ML, Chakraborty R, Sonnenthal EL, D'Haeseleer P, Holman HYN, Osman S, Lu ZM, Van Nostrand JD, Deng Y, Zhou JZ, Mason OU (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 330:204–208

Hyman MR, Murton IB, Arp DJ (1988) Interactions of ammonia monoxygenase from *Nitrosomonas europaea* with alkanes, alkenes and alkynes. Appl Environ Microbiol 54: 3187–3190

ITRC (2008) Use of Risk assessment in management of contaminated sites. The Interstate Technology &Regulatory Council, Council TITR, Washington, DC

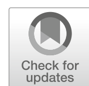
Karim ME, Dhar K, Hossain MT (2017) Co-metabolic decolorization of a textile reactive dye by *Aspergillus fumigatus*. Int J Environ Sci Technol 14:177–186

Keener WK, Arp DJ (1993) Kinetic-studies of ammonia monooxygenase inhibition in *Nitrosomonas europaea* by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay. Appl Environ Microbiol 59:2501–2510

Koh S-C, Bowman JP, Sayler GS (1993) Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, *Methlomonas methanica* 68-1. Appl Environ Microbiol 59:960–967

Lajoie CA, Layton AC, Sayler GS (1994) Cometabolic oxidation of polychlorinated-biphenyls in soil with a surfactant-based field application vector. Appl Environ Microbiol 60:2826–2833
Li YC, Zhou J, Gong BZ, Wang YM, He Q (2016) Cometabolic degradation of lincomycin in a Sequencing Batch Biofilm Reactor (SBBR) and its microbial community. Bioresour Technol 214:589–595
Little CD, Palumbo AV, Herbes SE, Lidstrom ME, Tyndall RL, Gilmer PL (1988) Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl Environ Microbiol 54:951–956
Liu L, Binning PJ, Smets BF (2015) Evaluating alternate biokinetic models for trace pollutant cometabolism. Environ Sci Technol 49:2230–2236
Mahendra S, Petzold CJ, Baidoo EE, Keasling JD, Alvarez-Cohen L (2007) Identification of the intermediates of in vivo oxidation of 1,4-dioxane by monooxygenase-containing bacteria. Environ Sci Technol 41:7330–7336
McCarty PL (1987) Bioengineering issues related to in situ remediation of contaminated soils and groundwater. In: Omenn GS (ed) Environmental biotechnology. Plenum, New York, pp 143–162
Murrell JC (1992) The genetics and molecular biology of obligate methane-oxidizing bacteria. In: Murrell JC, Dalton H (eds) Methane and methanol utilizers. Plenum, New York, pp 115–148
Nelson MJK, Montgomery SO, Prichard PH (1988) Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl Environ Microbiol 54:604–606
Newman LM, Wackett LP (1991) Fate of 2,2,2-trichloroacetaldehyde (chloral hydrate) produced during trichloroethylene oxidation by methanotrophs. Appl Environ Microbiol 57:2399–2402
Nguyen HHT, Elliott SJ, Yip JHK, Chan SI (1998) The particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a novel copper-containing three-subunit enzyme – isolation and characterization. J Biol Chem 273:7957–7966
Oremland RS, Culbertson CW (1992) Importance of methane-oxidizing bacteria in the methane budget as revealed by the use of a specific inhibitor. Nature 356:421–423
Paulsen KE, Liu Y, Fox BG, Lipscomb JD, Munck E, Stankovich MT (1994) Oxidation-reduction potentials of the methane monooxygenase hydroxylase component from *Methylosinus trichosporium* OB3b. Biochemistry 33:713–722
Rasche ME, Hicks RE, Hyman MR, Arp DJ (1990) Oxidation of monohalogenated ethanes and n-chlorinated alkanes by whole cells of *Nitosomonas europaea*. J Bacteriol 172:5368–5373
Reddy CM, Arey JS, Seewald JS, Sylva SP, Lemkau KL, Nelson RK, Carmichael CA, McIntyre CP, Fenwick J, Ventura GT, Van BAS M, Camilli R (2012) Composition and fate of gas and oil released to the water column during the Deepwater Horizon oil spill. Proc Natl Acad Sci U S A 109:20229–20234
Redmond MC, Valentine DL (2012) Natural gas and temperature structured a microbial community response to the Deepwater Horizon oil spill. Proc Natl Acad Sci U S A 109:20292–20297
Rosenzweig AC, Frederick CA, Lippard SJ, Nordlund P (1993) Crystal-structure of a bacterial nonheme iron hydroxylase that catalyzes the biological oxidation of methane. Nature 366: 537–543
Sayler GS, Layton A, Lajoie C, Bowman J, Tschantz M, Fleming JT (1995) Molecular site assessment and process monitoring in bioremediation and natural attenuation. Appl Biochem Biotechnol 54:277–290
Shi SN, Zhang XW, Ma F, Sun TH, Li A, Zhou JT, Qu YY (2013) Cometabolic degradation of dibenzofuran by *Comamonas* sp MQ. Process Biochem 48:1553–1558
Shi SN, Qu YY, Zhou H, Ma Q, Ma F (2015) Characterization of a novel cometabolic degradation carbazole pathway by a phenol-cultivated *Arthrobacter* sp W1. Bioresour Technol 193:281–287
Stackhouse B, Lau MCY, Vishnivetskaya T, Burton N, Wang R, Southworth A, Whyte L, Onstott TC (2017) Atmospheric CH_4 oxidation by Arctic permafrost and mineral cryosols as a function of water saturation and temperature. Geobiology 15:94–111
Stanley SH, Prior SD, Leak DJ, Dalton H (1983) Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms – studies in batch and continuous cultures. Biotechnol Lett 5:487–492

Tsien HC, Brusseau GA, Hanson RS, Wackett LP (1989) Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 55:3155–3161
Vannelli T, Logan M, Arciero D, Hooper AB (1990) Degradation of halogenated aliphatics by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl Environ Microbiol 56:1169–1171
Wilson JT, Wilson BH (1985) Biotransformation of trichloroethylene in soil. Appl Environ Microbiol 29:242–243
Wood PM (1986) Nitrification as a bacterial energy source. In: Prosser JI (ed) Nitrification. Society for General Microbiology (IRL Press), Washington, DC, pp 39–62
Yang L, Chang YF, Chou MS (1999) Feasibility of bioremediation of trichloroethylene contaminated sites by nitrifying bacteria through cometabolism with ammonia. J Hazard Mater 69:111–126
Yasin M, Shah AA, Hameed A, Ahmed S, Hasan F (2008) Use of microorganisms for the treatment of trinitrotoluene (TNT) containing effluents. J Chem Soc Pak 30:442–448
Zahn JA, Arciero DM, Hooper AB, DiSpirito AA (1996) Cytochrome c′ of *Methylococcus capsulatus* Bath. Eur J Biochem 240:684–691



Anaerobic Biodegradation of Hydrocarbons: Metagenomics and Metabolomics

15

Lisa M. Gieg and Courtney R. A. Toth

Contents

1 Introduction 250
2 Anaerobic Hydrocarbon Metabolism: A General Overview 251
3 Insights on Anaerobic Hydrocarbon Biodegradation Using Metagenomic Approaches 259
4 Genomes of Anaerobic Hydrocarbon-Degrading Isolates and Enrichment Cultures as Benchmarks for Metagenomes 260
5 Hydrocarbon-Impacted Environments: Targeted Metagenomics (Amplicon Sequencing) 262
6 Hydrocarbon-Impacted Environments: Shotgun Metagenomics 265
6.1 Fossil Energy Reservoirs 265
6.2 Hydrocarbon Seeps and Hydrothermal Vents 271
6.3 Contaminated Marine Environments 272
6.4 Contaminated Terrestrial Environments 273
7 Hydrocarbon Metabolomics 274
8 Research Needs 275
References 276

Abstract

The biodegradation of hydrocarbons under anaerobic conditions is a significant process that is now known to occur in diverse environments. Understanding this process has important implications for the bioremediation of hydrocarbon-contaminated terrestrial and marine environments, for enhanced energy recovery from deep subsurface fossil energy reservoirs, and for climate change effects related to the release of methane and other hydrocarbons from natural seeps and hydrothermal vents. While much understanding of anaerobic hydrocarbon

L. M. Gieg (✉) · C. R. A. Toth
Department of Biological Sciences, University of Calgary, Calgary, AB, Canada
e-mail: lmgieg@ucalgary.ca; ctot@ucalgary.ca

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_16

metabolism has been gleaned from cultivation-based studies, cultivation-independent meta-omics approaches such as metagenomics can offer new insights into the process in more complex, natural hydrocarbon-containing environments. Further, a metabolomic approach that seeks specific metabolites diagnostic of anaerobic hydrocarbon biodegradation can provide the "ultimate proof" that this process is occurring in situ. This chapter highlights the key pathways of anaerobic hydrocarbon metabolism and summarizes metagenomic information garnered to date from sequencing hydrocarbon degraders, enrichment cultures, and diverse hydrocarbon-containing environmental samples. Further, a brief overview of hydrocarbon metabolomics is presented, along with research needs on this topic.

1 Introduction

Hydrocarbons are ubiquitous across the globe. Comprised of carbon and hydrogen atoms, hydrocarbons are considered natural products; they are synthesized by some algal, plant, and animal species to serve a variety of functions (Harms et al. 1999) and are generated from forest fires and other anthropogenic sources (Vergnoux et al. 2011). By far though, the Earth's terrestrial and marine subsurface environments contain the largest inventory of hydrocarbons (including the smallest hydrocarbon, methane). These are found beneath the ocean floor where they can be naturally emitted via cold seeps or generated within hydrothermal vent systems (Boetius and Wenzhöfer 2013; Teske et al. 2014) or are entrained in crude oil, coal, or shale reservoirs, where hydrocarbons were generated from ancient buried biomass subject to high temperatures and pressures over geologic time. Due to humankind's predominant use of petroleum-based energy, the recovery of gaseous and liquid hydrocarbons from such deposits and its subsequent distribution, refining, storage, and combustion have led to widespread water and land contamination with hydrocarbons. While hydrocarbons are considered chemically inert (Widdel and Musat 2010), it has now been definitively demonstrated that naturally occurring microbial communities contain a multitude of enzymes that can readily biotransform hydrocarbons (including methane) both in the presence and absence of oxygen (Widdel and Musat 2010). Microbial hydrocarbon degradation is now known to occur in surface soils and sediments, shallow groundwater environments, subsurface fossil energy reservoirs, cold seeps, marine waters and sediments, and hydrothermal vent systems. This fact has many important implications for the remediation of hydrocarbon-contaminated areas (Techtmann and Hazen 2016), for crude oil quality in reservoirs or for enhanced energy recovery (Head et al. 2014), and for minimizing greenhouse gas (methane) emissions from natural terrestrial and marine systems (Boetius and Wenzhöfer 2013). This chapter will focus on hydrocarbon biodegradation by anaerobic microorganisms; for information on aerobic hydrocarbon biodegradation, the reader is referred to recent reviews (Rojo 2009; Ghosal et al. 2016).

Knowledge regarding anaerobic hydrocarbon biodegradation has been gained mainly from research conducted in the last 30 years. Such research has led to the isolation of dozens of anaerobic hydrocarbon-utilizing isolates (nitrate-reducing, metal-reducing, sulfate-reducing bacteria and archaea) and enriched methanogenic consortia (e.g., Tables 1 and 2; Widdel et al. 2010; Jiménez et al. 2016) along with the description of numerous anaerobic hydrocarbon-degrading enrichment cultures or processes (e.g., a search on PubMed for the term "anaerobic hydrocarbon biodegradation" yielded >7000 references, accessed October 2016). Thus, as the topic of anaerobic hydrocarbon biodegradation is vast, this chapter will provide a general overview as to how recent cultivation-independent "omics" approaches are starting to be applied to our understanding of this process. Many omics/meta-omics technologies have emerged in recent years that examine biological molecules at various levels of information flow: from DNA ((meta)genomics, genetic potential for activity) to RNA ((meta)transcriptomics, identifying expressed genes) to proteins ((meta)proteomics, detecting enzymes or other proteins actually made to catalyze biochemical reactions) and finally to metabolites (metabolomics, identifying biochemical products of expressed genes and enzymes). Here, we highlight how metagenomic (and metatranscriptomics or metaproteomics in some cases) approaches have been used to understand anaerobic hydrocarbon biodegradation in general and to gain insight into the ecological principles governing hydrocarbon-containing environments including in hydrocarbon resource environments (oil reservoirs, coal seams, and shale formations), in natural hydrocarbon-releasing cold seeps and hydrothermal vents, and in hydrocarbon-contaminated marine and terrestrial environments. A discussion on the use of metabolomics to assess anaerobic in situ biodegradation in hydrocarbon-containing environments is included here as well, but the reader is referred to a more detailed consideration of this topic in this manual (Gieg and Toth 2016) and to other recent reviews (Agrawal and Gieg 2013; Callaghan 2013a).

2 Anaerobic Hydrocarbon Metabolism: A General Overview

Recent publications (including several chapters in this manual, e.g., *Biochemistry of Anaerobic Degradation of Hydrocarbons*) describe in detail the anaerobic biodegradation of various classes of hydrocarbons including alkanes (Callaghan 2013b; Musat 2015; Rabus et al. 2016), monoaromatic hydrocarbons (Foght 2008), and polycyclic aromatic hydrocarbons (PAHs) (Meckenstock et al. 2016), so here we only highlight some of the major hydrocarbon activation mechanisms. Notably, the principles governing anaerobic hydrocarbon metabolism have been mainly determined by studying either highly enriched cultures or pure cultures isolated from hydrocarbon-bearing environments. Compilations of such isolates are available (Widdel et al. 2010; Heider and Schühle 2013; Stagars et al. 2016) and include nitrate-reducing, iron-reducing, and sulfate-reducing bacteria (and one sulfate-reducing archaeum); some examples are provided in Tables 1 and 2. Highly enriched

Table 1 Summary of anaerobic hydrocarbon-degrading isolates for which a whole genome sequence is available

Isolate	Electron acceptor	Hydrocarbons utilized	Genome status and accession number	Relevance of study	References
Saturated hydrocarbon degraders					
Gamma proteobacterium HdN1	NO_3^-	Alkanes C_6–C_{30}	Complete, NC_014366	Failure to detect an *ass*- or *mas*-like gene offered evidence that fumarate-independent mechanisms of anaerobic alkane activation were indeed possible, as proposed by previous studies	Ehrenreich et al. (2000) and Zedelius et al. (2011)
Desulfosarcina sp. BuS5	SO_4^{2-}	Alkanes C_3–C_4	Whole genome shotgun sequence, NZ_AXAM00000000	Demonstrated fumarate addition to propane at the C_1 and C_2 position. Genome analysis identified a single putative *masD* gene, suggesting that the same gene is responsible for C_3 and C_4 activation	Kniemeyer et al. (2007), and Sievert et al. (unpublished – submitted 2013)
Desulfococcus oleovorans Hxd3	SO_4^{2-}	*n*-Alkanes C_{12}–C_{20}; *n*-1-alkanes C_{14}–C_{17}	Complete, NC_009943	Genome analysis did not detect homologs to *ass* genes, supporting the theory that an alternate mechanism of alkane activation occurs in this isolate	Aeckersberg et al. (1998), So et al. (2003), and Copeland et al. (unpublished – submitted 2007)
Desulfatibacillum alkenivorans AK-01	SO_4^{2-}	*n*-Alkanes C_{13}–C_{18}, 1-hexadecene and 1-pentadecene	Complete, NC_011768	Confirmed physiological evidence of metabolic processes involved in anaerobic alkane degradation, such as fumarate regeneration, and successfully predicted this strain's ability to grow syntrophically with methanogens	So and Young (1999) and Callaghan et al. (2012)
Archaeoglobus fulgidus VC-16	SO_4^{2-}, $S_2O_3^{2-}$	C_{10}–C_{21}, *n*-alk-1-enes C_{12}–C_{21}	Complete, AE000782	First sequenced genome of a sulfate-reducing archaeum. Predicted to metabolize a variety of hydrocarbons due to the presence of 57 β-oxidation genes. This was recently experimentally verified, and a fumarate addition gene was also detected	Klenk et al. (1997) and Khelifi et al. (2010, 2014)

Aromatic hydrocarbon degraders					
Dechloromonas aromatica RCB	NO_3^-	Benzene, toluene, xylene, chlorobenzoate	Complete, NC_007298	Homologs to known anaerobic monoaromatic hydrocarbon-degrading genes (e.g., *bss*, *pcr*, *bcr*) were notably absent, though aerobic genes were present, leading to the suggestion that benzene degradation was coupled to oxygen produced during nitrate reduction	Coates et al. (2001) and Salinero et al. (2009)
Azoarcus toluclasticus MF63	NO_3^-	Toluene	Whole genome shotgun sequence, NZ_ARJX00000000	Part of JGI's efforts to sequence 1000 genomes (proposal ID: 733)	Song et al. (1999) and Kyrpides et al. (unpublished – submitted 2013)
Azoarcus sp. CIB	NO_3^-	Toluene, xylene	Complete, CP011072	Metabolic flexibility suggests both aerobic and anaerobic monoaromatic hydrocarbon biodegradation is possible and that the strain can adapt to varying environmental stressors, making it a potential candidate for future remediation studies	Martín-Moldes et al. (2015)
Aromatoleum aromaticum EbN1	NO_3^-	Toluene, ethylbenzene	Complete, NC_006513 Two plasmids also sequenced; NC_006823, NC_006824	First sequenced genome of an anaerobic aromatic-degrading member of the Betaproteobacteria. Genes for 10 major peripheral pathways of aromatic metabolism (aerobic and anaerobic) were detected, including the *bss* operon and *ebdABC*	Rabus and Widdel (1995) and Rabus et al. (2005)
Geobacter metallireducens GS-15	Fe(III)	Toluene	Complete, NC_007517 One plasmid also sequenced; NC_007515	*G. metallireducens* was predicted to have greater metabolic diversity than *G. sulfurreducens*, including the ability to degrade toluene (experimentally verified). Physiology and gene regulation are also predicted to be substantially different from other *Geobacteraceae*	Aklujkar et al. (2009)

(*continued*)

Table 1 (continued)

Isolate	Electron acceptor	Hydrocarbons utilized	Genome status and accession number	Relevance of study	References
Geobacter sp. Ben	Fe(III)	Toluene, benzene	Draft genome, unpublished	Second sequenced genome of an anaerobic benzene-degrading isolate. Genomic insight confirmed aerobic benzene degradation is not possible	Zhang et al. (2012)
Ferroglobus placidus AEDII12DO	Fe(II)	Benzene	Complete, NC_013849	Sequenced genome of an anaerobic benzene-degrading thermophile	Holmes et al. (2011) and Anderson et al. (2011)
Desulfobacula toluolica Tol2	SO_4^{2-}	Toluene	Complete, NC_018645	First sequenced genome of an anaerobic aromatic-degrading marine sulfate reducer. Predicted the metabolism of toluene (experimental verified and metabolites detected) and carboxylated/hydroxylated derivatives. Another notable feature was the genome's unusually high plasticity, which seems to correlate with flexible substrate catabolism	Rabus et al. (1993) and Wöhlbrand et al. (2013)
Deltaproteobacterium NaphS2	SO_4^{2-}	2-Methyl-naphthalene	Whole genome shotgun sequence, NZ_ADZZ00000000	Predication of ring reduction genes (*ncr*) for naphthoyl-CoA in a manner analogous to *bcr* (for anaerobic benzoate ring reduction)	Galushko et al. (1999) and DiDonato et al. (2010)

Table 2 Summary of anaerobic hydrocarbon-degrading enrichment cultures for which a metagenomic sequence is available

Enrichment culture	Electron accepting process	Hydrocarbons utilized	Metagenome accession #	Relevance of study	References
Deltaproteobacterium N47	SO_4^{2-}	2-Methyl-naphthalene, naphthalene	Available across 17 contigs, FR695864–FR695880	Combined metagenomics and proteomics to predict/identify novel genes expressed during anaerobic growth on PAHs	Bergmann et al. (2011)
m-Xylene-degrading	SO_4^{2-}	*m*-Xylene	European Nucleotide Archive, PRJEB11632	Supported previous hypotheses that Epsilonproteobacteria do not initiate xylene degradation in this enrichment culture. Rather, this group was suggested to be mixotrophic and thought to scavenge acetate within the syntrophic consortium	Bozinovski et al. (2012, 2014) and Keller et al. (2015)
Benzene degrading	Methanogenesis	Benzene	Short Read Archive, PRJNA281117	Confirmed *Deltaproteobacterium* ORM2 as a key benzene degrader; metagenomic surveys identified sequences belonging Parcubacteria (candidate division OD1) not detected by qPCR. This organism was present in all benzene-degrading cultures evaluated (after suitable primers were designed) and may be a key player in benzene degradation	Luo et al. (2016)
SCADC	Methanogenesis	Short-chain alkanes (C_6–C_{10}, mixture also contains traces of 2-methylpentane and methylcyclopentane)	Short Read Archive, SRX831148 Draft genomes also available	Initial genomic surveys failed to detect sequence homologs for *assA* and *bssA* despite further targeted gene analysis confirming their presence (Tan et al. 2015b). A secondary genomic survey showed improved homology for *assABC* (Tan et al. 2015a), in addition to detecting other anaerobic hydrocarbon activation genes	Tan et al. (2013, 2015a, b)

(*continued*)

Table 2 (continued)

Enrichment culture	Electron accepting process	Hydrocarbons utilized	Metagenome accession #	Relevance of study	References
NAPDC	Methanogenesis	Naphtha, a mixture of monoaromatics and C_6–C_{10} alkanes	Short Read Archive, SRX831147	Much like SCADC, reported sequence homology for several anaerobic hydrocarbon activation genes across several hydrocarbon classes, albeit not all with high sequence homology	Tan et al. (2015a)
TOLDC	Methanogenesis	Toluene	Short Read Archive, SRX831099	Despite being enriched solely on toluene for >10 years, several anaerobic hydrocarbon activation genes were found to be present (including *bss*A and *ass*A)	Fowler et al. (2012) and Tan et al. (2014, 2015a)
n-C_{16} degrading	Methanogenesis	Hexadecane	DDBJ/EMBL/ Genbank, LNQE00000000	Used metagenomic binning, metatranscriptomic analysis, and metabolic modeling to deduce interspecies interactions between taxa driving syntrophy in methanogenic communities	Embree et al. (2014, 2015), Tan et al. (2014)
n-C_{28} degrading	Methanogenesis	Octacosane (C_{28}), $C_{10–18}$, C_{40}, C_{50}	Short Read Archive, PRJNA293354 Draft genomes also available	Revealed *Smithella* as key degrader by fumarate addition and genes associated with syntrophic interactions with methanogens	Wawrik et al. (2016)

methanogenic cultures, wherein syntrophic partnerships are needed to convert hydrocarbons to methane, have also been studied in order to understand the bio-conversions of specific hydrocarbons under these conditions (e.g., Gieg et al. 2014; Jiménez et al. 2016). The pathways for anaerobic hydrocarbon metabolism and the corresponding genes and enzymes were largely identified prior to the widespread use of "omics" approaches; typically, cultures were incubated with the substrate of interest, and processes were tracked using analytical chemistry (e.g., to identify pathway intermediates) and classical molecular biology/genetic approaches (e.g., Sanger sequencing, closing, gene selection, etc.). From these studies, hydrocarbon activation mechanisms including fumarate addition, carboxylation, and hydroxylation have been identified (along with a couple of studies suggesting activation by methylation of unsubstituted aromatics, Ulrich et al. 2005; Safinowski and Meckenstock 2006). Of these, the addition of hydrocarbons to the double bond of fumarate (simply deemed "fumarate addition") is the most widespread and understood mechanism catalyzed by a glycyl radical enzyme and occurs for the activation of alkylated aromatics and linear and cyclic alkanes (Fig. 1), with a couple of notable exceptions (ethylbenzene degradation under nitrate-reducing conditions occurs via hydroxylation, Rabus et al. 2005; *n*-alkane degradation may also occur via hydroxylation and/or carboxylation; So et al. 2003; Callaghan et al. 2009; Callaghan 2013b). Carboxylation and hydroxylation are the primary mechanisms proposed for the activation of non-substituted aromatics such as benzene, naphthalene, and phenanthrene (Meckenstock et al. 2016). Most evidence to date supports carboxylation as the most likely mechanism for the activation of non-substituted aromatics (Zhang and Young 1997; Holmes et al. 2011; Abu Laban et al. 2010; Mouttaki et al. 2012; Luo et al. 2014), with the exception of an iron-reducing species (*Geobacter metallireducens*) that appears to activate benzene by hydroxylation, yielding phenol (Zhang et al. 2013). Figure 1 summarizes the most widely reported mechanisms for the activation of different classes of hydrocarbons under anoxic conditions. Along with identifying the metabolites (intermediates) in hydrocarbon degradation pathways, studies have also revealed the key genes and characterized several of the enzymes associated with these pathways. For example, benzylsuccinate synthase (BSS) was identified as the enzyme catalyzing toluene activation over 20 years ago, encoded by the *bss*A gene; dozens of publications now describe this novel and uniquely anaerobic biochemical reaction (as reviewed in Foght 2008; Widdel et al. 2010; Heider and Schühle 2013). For alkane activation by fumarate addition, alkylsuccinate synthase (ASS, also called (1-methyl)alkylsuccinate synthase, MAS) is the key activation enzyme, encoded by the *ass*A/*mas*D gene (different designations for the same gene identified concurrently by two different research groups; Callaghan et al. 2008; Grundmann et al. 2008). Similarly, the *nms*A gene encodes the naphthyl-2-methylsuccinate synthase enzyme subunit that activates 2-methylnaphthalene by addition to fumarate (Meckenstock et al. 2016). Most recently, a thermophilic anaerobic consortium (enriched from the Guaymas Basin hydrothermal vent samples) dominated by an archaeal phylotype closely related to *Methanosarcinales* was proposed to activate *n*-butane (and *n*-propane) via an alkyl-coenzyme M mechanism in a manner analogous to that demonstrated for anaerobic

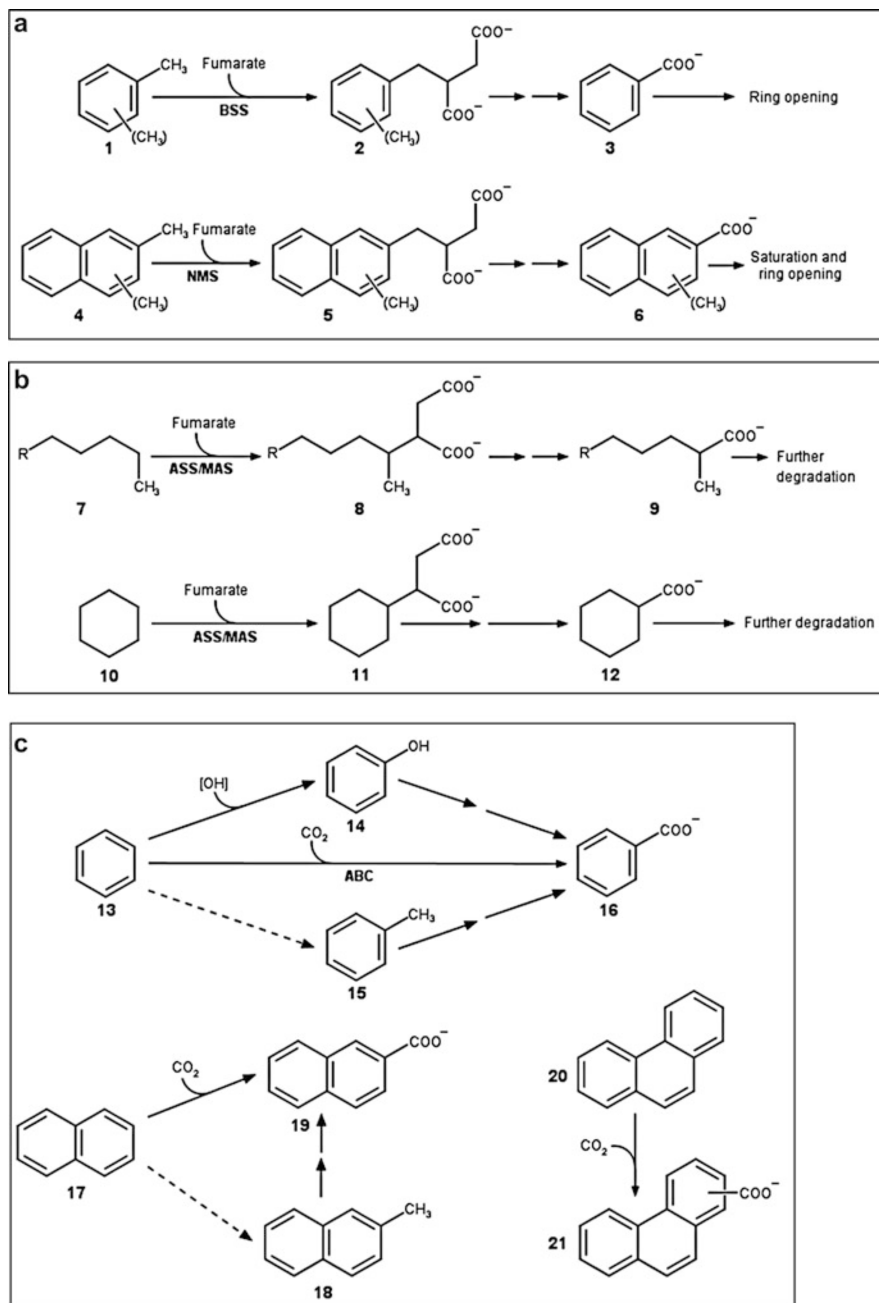


Fig. 1 Overview of the major hydrocarbon activation mechanisms under anaerobic conditions. Metabolites are shown as free acids. Multiple *arrows* represent more than one enzymatic step; *dashed arrows* represent an unknown reaction. *Structure nomenclature*: *1* toluene (or xylene);

methane oxidation operating via reverse methanogenesis (Laso-Pérez et al. 2016). Although further work is needed to understand and confirm this mechanism, this finding has revealed a potential novel mechanism of hydrocarbon activation and expands upon the substrate range of methanogen-like organisms. The key hydrocarbon activation genes and metabolites identified through these laboratory-based physiological studies have important applications in assessing in situ hydrocarbon biodegradation processes in various environments (see sections below).

3 Insights on Anaerobic Hydrocarbon Biodegradation Using Metagenomic Approaches

Even though several anaerobic hydrocarbon-utilizing isolates have been described within the last 30 years, obtaining pure isolates is usually difficult, tedious, and time-consuming. Further, it is unclear as to whether laboratory isolates truly represent the diversity of hydrocarbon degraders that may be found in various natural environments. Genomics has emerged as a field of science that examines the genetic material, or genomes, of organisms in a culture-independent manner and thus can greatly expand our knowledge of microbial processes occurring in diverse environments. The term "metagenome" refers to the genetic (genomic) compositions of *all* organisms in a given sample (e.g., of the total microbial community) without the need for cultivation; this field of science is referred to as metagenomics. Metagenomic approaches are typically conducted in either a targeted or nontargeted fashion. Targeted metagenomics is more commonly known as amplicon sequencing; here, a gene of interest is amplified by PCR using primer sets specific to that gene in order to capture multiple sequences of that gene in a given sample. For example, targeting a portion of the small subunit ribosomal RNA gene (the 16S rRNA gene for prokaryotes) is the most common way to obtain taxonomic information, or microbial community composition, of a given sample. Other genes diagnostic of particular microbial groups (e.g., *dsr*AB for sulfate reducers or *mcr*A for methanogens, Johnson et al. 2015; Gründger et al. 2015) or encoding functional genes (e.g., for anaerobic hydrocarbon biodegradation, *ass*A, *bss*A, *nms*A) are often utilized as well (von Netzer et al. 2013, 2016). In nontargeted metagenomics, or shotgun metagenomics, all genetic material is sequenced in order to capture the complete suite of genes within a given sample in order to gain an understanding of the overall

Fig. 1 (continued) 2 (methyl)benzylsuccinate; *3* benzoate; *4* 2-methylnaphthalane/dimethylnaphthalene; *5* naphthylmethylsuccinate; *6* (methyl)-2-naphthoate; *7* *n*-alkane; *8* (2-methyl)alkylsuccinate; *9* 2-alkylmalonate; *10* cyclohexane; *11* cyclohexyl succinate; *12* cyclohexanecarboxylate; *13* benzene; *14* phenol; *15* toluene; *16* benzoate; *17* naphthalene; *18* 2-methylnaphthalene; *19* 2-naphthoate; *20* phenanthrene; and *21* phenanthrene carboxylate. *Enzyme nomenclature*: **BSS** catalytic subunit of benzylsuccinate synthase, **NMS** catalytic subunit of naphthyl-2-methylsuccinate synthase; **ASS/MAS** catalytic subunit of alkylsuccinate synthase, **ABC** putative gene encoding anaerobic benzene carboxylase

functional potential of the microbial ecosystem. To conduct metagenomic analysis, DNA is extracted from the requisite culture(s) or environmental sample(s), and the sample is processed for sequencing using a parallel sequencing approach (e.g., Illumina MiSeq or HiSeq is currently the most widely employed sequencing approach although Ion Torrent and Pac Bio are also used; most analyses, e.g., prior to ~2014, used the pioneering Roche 454 pyrosequencing system which is no longer supported). Once sequences are obtained, they are subjected to quality control analyses and processed using a variety of software tools that not only allow for the identification of genes, but can also assign, or bin, genes to specific taxa (Segata et al. 2013; Pérez-Wohlfeil et al. 2016; Anantharaman et al. 2016). Reconstructing individual genomes within metagenomic datasets can provide enormously valuable information regarding the potential functionalities and interactions between community members within an ecosystem and can also lead to the discovery of new, previously unclassified taxa (Anantharaman et al. 2016).

4 Genomes of Anaerobic Hydrocarbon-Degrading Isolates and Enrichment Cultures as Benchmarks for Metagenomes

Although metagenomic approaches do not require cultivation, analyzing and interpreting metagenomic datasets benefit greatly by comparing sequences to reference genes and genomes for easier gene annotation and identification. Thus, publishing the genome sequences of anaerobic hydrocarbon-degrading isolates not only provide insights into the overall functional capabilities of these types of microorganisms but also serve as invaluable “blueprints” for interpreting metagenomic datasets from hydrocarbon-impacted environments. The genomes of several hydrocarbon-degrading nitrate reducers, iron reducers, and sulfate reducers have now been described (see Table 1 for examples) providing important information about the physiological characteristics of anaerobic hydrocarbon degraders. For example, genomic analysis of *Desulfatibacillum alkenivorans* AK-01 identified 2 loci harboring the alkylsuccinate synthase gene and putative genes for an alkyl-CoA ligase, mutase, and carboxyltransferase (presumably needed for further metabolic transformations of alkylsuccinates; Callaghan et al. 2012). Further, the AK-01 genome revealed several genes believed to be important for its growth on alkanes under syntrophic conditions an activity that was experimentally confirmed (Callaghan et al. 2012). Target gene amplification and genomic sequencing of the nitrate-reducing strain HdN1, in addition to hydrocarbon metabolite analysis, failed to detect evidence for an *ass*A/*mas*D-like gene which suggested an alternate mechanism of *n*-alkane activation (Zedelius et al. 2011). Physiological tests revealed that alkane metabolism may instead be coupled to the denitrification intermediates NO_2^- and NO wherein O_2 is generated to serve as a co-reactant for hydroxylation (Zedelius et al. 2011; Callaghan 2013b) similar to that shown during methane oxidation under nitrate-reducing conditions (Ettwig et al. 2010). *Desulfococcus oleovorans* Hxd3 is another sulfate-reducing alkane degrader that does not utilize fumarate addition for *n*-alkane activation (So et al. 2003). Instead, its genome sequence revealed the

presence of an ethylbenzene dehydrogenase-like complex that may allow for alkane hydroxylation (Callaghan 2013b). Thus, sequencing the genomes of isolates greatly helps to elucidate the biochemical reactions associated with anaerobic hydrocarbon metabolism (along with other physiological processes) and provides benchmarking information to help reconstruct individual genomes from metagenomic datasets.

The biodegradation of hydrocarbons under methanogenic communities is a syntrophic process wherein different microorganisms (e.g., fermentative bacteria, syntrophs, and methanogens) collectively carry out this reaction in a thermodynamically interdependent manner (Sieber et al. 2012; Gieg et al. 2014). Therefore, a shotgun metagenomic approach is essential to determine the genetic composition of these culture systems. To date, the metagenomes of six methanogenic hydrocarbon-degrading cultures have been sequenced and described in publications; one methanogenic benzene-degrading culture (Luo et al. 2016), two cultures enriched from oil sands tailings ponds that degrade naphtha (NAPDC; naphtha is refinery product consisting of monoaromatics and C_6-C_{10} alkanes and is present in many oil sands tailings ponds) or short-chain alkanes (SCADC; C_6-C_{10} *n*-alkanes with small proportions of 2-methylpentane and methylcyclopentane) (Tan et al. 2013, 2015a), one culture enriched from a gas condensate-contaminated aquifer on toluene (TOLDC) (Fowler et al. 2012; Tan et al. 2015a), one culture enriched on *n*-hexadecane (Zengler et al. 1999; Embree et al. 2014), and one culture enriched from San Diego Bay sediments on *n*-octacosane (Wawrik et al. 2016). A comparative metagenomic analysis of SCADC, NAPDC, and TOLDC revealed that all cultures harbored fumarate addition genes to degrade a variety of hydrocarbon types along with genes for H_2- and acetate-utilizing methanogenesis (Tan et al. 2015a). Further, a comparison of these metagenomes with 41 other environmental metagenomic datasets revealed an enrichment of genes associated with anaerobic hydrocarbon biodegradation, syntrophy, and methanogenesis, suggesting these are hallmark features of this process (Tan et al. 2015a). In metagenomic sequencing of one methanogenic benzene-degrading culture, combined with other molecular methods used to analyze 15 other cultures, Luo et al. (2016) unequivocally identified a *Deltaproteobacterium* designated ORM2 as the key benzene degrader under methanogenic conditions. Studies with the *n*-hexadecane-degrading culture helped to reveal the key taxa involved in hydrocarbon degradation (*Smithella*, *Desulfovibrio*, *Methanoculleus*, *Methanocalculus*, and *Methanosaeta*) and key hydrocarbon biodegradation genes (associated with *Smithella*, Tan et al. 2014) as well as potential interactions among members of the syntrophic community (Embree et al. 2014, 2015). Similar findings were discovered following the metagenomic sequencing of an *n*-octacosane-degrading culture; a *Smithella* phylotype harbored the *ass*A genes needed for *n*-alkane activation along with the genes required for syntrophic interactions and energy conservation (Wawrik et al. 2016). Although work with isolates has shown that nitrate or sulfate reducers can completely mineralize hydrocarbons (e.g., Table 1), there are also several examples wherein syntrophic consortia are required to utilize hydrocarbons under nitrate- or sulfate-reducing conditions (Gieg et al. 2014). For example, Luo et al. (2014) used a metatranscriptomic approach to reveal that benzene degradation by a

nitrate-reducing culture requires at least two partner organisms, one of which appears to contain the putative genes associated with syntrophic benzene activation.

5 Hydrocarbon-Impacted Environments: Targeted Metagenomics (Amplicon Sequencing)

By far, the most commonly used genomic approach in the field of environmental microbiology is taxonomic (phylogenetic) profiling of a given sample based on amplifying a portion of the 16S rRNA gene for prokaryotic identification (or the 18S rRNA gene for eukaryotes). Prior to ~10 years ago, assessing microbial diversity in a cultivation-independent manner was usually performed using clone libraries and Sanger sequencing or techniques such as denaturing gradient gel electrophoresis or T-RFLP (terminal-restriction fragment length polymorphism) analysis. While these approaches are sometimes still used (especially T-RFLP; von Netzer et al. 2013; Gründger et al. 2015; Luo et al. 2016), the majority of publications regarding anaerobic hydrocarbon-containing environments now describe the use of amplicon sequencing (primarily based on the 16S rRNA gene) to determine microbial diversity. Here, DNA is extracted from mixed samples and subject to a PCR reaction using a primer set that targets specific variable regions of the 16S rRNA gene (often the V3-V4 or V6–V8 regions; http://jgi.doe.gov/our-science/science-programs/metagenomics/; An et al. 2013a). Although there are limitations to this approach (such as primer bias, not all diversity captured, Singer et al. 2016), targeted metagenomic sequencing has become very affordable per base pair sequenced (www.genome.gov/sequencingcosts) and is now usually included as a standard approach for most environmental microbiology studies to identify key taxa within a given community and predict their putative functions. In general, the majority of proposed anaerobic hydrocarbon-degrading bacteria have been found to affiliate within the Proteobacteria or Firmicutes, but genera within these phyla can vary substantially (Head et al. 2014; Mouser et al. 2016). Other members of diverse phyla (e.g., *Chloroflexi*, *Spirochaetes*, *Bacteroidetes*) are usually associated with mixed hydrocarbon-impacted communities (Strąpoć et al. 2011; Kleinsteuber et al. 2012; Mouser et al. 2016). In methanogenic hydrocarbon-degrading environments, the archaeal taxa are predominantly methanogens that utilize hydrocarbons, acetate, and/or methylated/methoxy substrates (Strąpoć et al. 2011; Kleinsteuber et al. 2012; Head et al. 2014; Mayumi et al. 2016). In hot environments such as thermogenic oil reservoirs (>50 °C), bacterial members such as those affiliating with *Thermotogales*, *Synergistales*, *Deferribacterales*, or *Thermoanaerobacterales* and thermophilic methanogens like *Methanothermobacter* are believed to be involved in anaerobic hydrocarbon degradation (Orphan et al. 2000; Gieg et al. 2010), but definitive evidence is required. However, it has been experimentally verified that the thermophilic archaeon *Archaeoglobus fulgidus* can metabolize long-chain alkanes and alkenes under sulfate-reducing conditions (Khelifi et al. 2010, 2014).

Obtaining 16S rRNA gene-based community profiles across a variety of similar environments can help determine whether similar taxa can be considered

characteristic of that habitat; e.g., does a core microbiome exist? A few studies have addressed this question using phylogenetic profiling data collected from similar environments. For example, Wilson et al. (2016) conducted a comparative analysis of 95 different anoxic samples collected from six different oil sands tailings ponds (managed by three different operators in different ways, e.g., by treatment with different chemical additives) to determine whether a core microbiome was associated with these highly engineered anaerobic hydrocarbon-degrading environments. Each individual tailings pond contained its own core biome that presumably reflected the selective pressures placed on the extant communities due to different pond management strategies (Wilson et al. 2016). An analysis of all 95 tailings pond samples revealed that the core microbiome consisted of only two to five OTUs that included *Comamonadaceae*, *Hydrogenophilaceae*, and/or *Anaerolineaceae* as the bacterial members and *Methanosaeta* and *Methanoregula* as the archaeal members. It was postulated that these limited taxa play key roles in the various anaerobic processes and/or harbor functional abilities that are common across all tailings ponds such as hydrocarbon degradation and methanogenesis (Wilson et al. 2016). To examine whether shale-associated fluids (typically saline in nature) harbor similar or distinctive taxa, Mouser et al. (2016) conducted a nonparametric multidimensional scaling (NMDS) analysis of 16S rRNA gene sequences retrieved from the limited datasets available for this environment, including data collected before and following a hydraulic fracturing operation (a method used to recover gas or fluids from shale formations). They found that microbial communities in the source waters used for fracturing were very different and revealed few halotolerant organisms. However, there was a large shift in the microbial community profiles in the flowback waters in as little as 1–14 days post fracturing (Mouser et al. 2016) wherein the dominant taxa were primarily known halotolerant microorganisms such as *Marinobacter*, *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Arcobacter*, and *Marinilabilia*. After 2–4 weeks post fracturing, the diversity was found to decrease substantially, with *Halanaerobium* (a firmicute) becoming heavily enriched – this trend was seen across all fracturing operations analyzed. Other taxa such as *Halomonas* and *Marinobacter* were found to be more broadly distributed throughout the course of fluid flowback and were hypothesized to be active degraders of hydrocarbons or related substrates associated with shales (Mouser et al. 2016; Daly et al. 2016).

Network or co-occurrence analysis is another approach often used to determine potential interactions between microbial community members and can be based on 16S rRNA gene sequences (Barberán et al. 2012; Williams et al. 2014) or metagenomic datasets (Li et al. 2016). Common correlation coefficients used to identify positive or negative interactions between pairs of microorganisms within each dataset include Pearson's r (a measure of the linear dependence) and Spearman's ρ (a nonparametric measure of rank) (Barberán et al. 2012). While it not has yet met widespread application to study hydrocarbon biodegradation (e.g., An et al. 2013a; Fowler et al. 2016), network analysis may offer new insight to help understand community functions, especially with the increasing number of metagenomes available for sequencing comparison. For example, in

an assessment of 160 phylogenetic datasets generated from oil reservoirs, coal seams, oil sands, and oil sands tailings ponds (~14,000 OTUs), An et al. (2013a) conducted a 16S rRNA co-occurrence analysis on the order level and found two major positive co-occurrence networks that metabolize hydrocarbons in a mutually exclusive manner: network A that consisted almost entirely of anaerobic taxa and network B that consisted of facultative anaerobes and aerobes. Accompanying metagenomic surveys also detected a higher frequency of aerobic catabolic genes in samples harboring greater proportions of network B taxa, calling into reconsideration the notion that hydrocarbon resource environments are exclusively anoxic (An et al. 2013a). It should be noted that metagenomic-based network analysis requires additional computational considerations due to the high complexity and (sometimes) incomplete nature of the datasets (Li et al. 2016).

In lieu of determining microbial diversity based on amplification of the 16S rRNA gene, several researchers have used a targeted metagenomics approach based on the key hydrocarbon activation genes for benzylsuccinate synthase (*bss*A) and alkylsuccinate synthase (*ass*A/*mas*D) to identify the anaerobic hydrocarbon-degrading potential in hydrocarbon-containing environments. Beller and colleagues initially designed a primer set based on a Betaproteobacterial (nitrate reducer) *bss*A sequence in order to interrogate a hydrocarbon-contaminated site for toluene biodegradation potential (Beller et al. 2002). Subsequently, Winderl et al. (2007) expanded on this work by designing primer sets that more broadly included diverse *bss*A genes associated with iron and sulfate reducers and successfully used these to identify hydrocarbon degraders in a tar oil-contaminated aquifer system (Winderl et al. 2008). Similarly, following the discovery of the *ass*A/*mas*D genes responsible for alkane activation, Callaghan et al. (2010) designed several primer sets based on the AK-01 *ass*A and *bss*A gene sequences and were used to successfully detect both genes in variety of enrichment cultures, river sediments, and contaminated aquifer samples. Johnson et al. (2015) also found *ass*A and *bss*A genes in Chesapeake Bay estuarine sediments revealing the potential for anaerobic alkane and aromatic hydrocarbon biodegradation in these environments. The *ass*A genes have been detected in a variety of produced waters from crude oil reservoirs where putative hydrocarbon-degrading anaerobic taxa and/or putative metabolites were also detected (Li et al. 2012; Zhou et al. 2012; Bian et al. 2015) and in coalbed methane site fluids (Wawrik et al. 2012). von Netzer et al. (2013) further refined the *bss*A primer sets to be applicable to be even more diverse sequences associated with a broader range of environments including hydrocarbon-contaminated aquifers, cold seeps, and hydrothermal vent systems. Gittel et al. (2015) and Stagars et al. (2016) independently developed new primer sets for the *ass*A/*mas*D genes based on a variety of known alkane-degrading isolates and available sequences and used these to interrogate numerous different hydrocarbon seep environments from across the globe, revealing that these environments harbor an unprecedented diversity of anaerobic alkane-degrading ability.

6 Hydrocarbon-Impacted Environments: Shotgun Metagenomics

While the majority of studies to date have used targeted metagenomics to assess microbial community composition or specific functional properties, there are now some reports on the use of shotgun metagenomics to determine the overall potential functionality of communities within anoxic hydrocarbon-containing environments. Table 3 provides examples of where a shotgun metagenomic approach was used to assess the genetic composition of samples from a variety of such environments. The importance of anaerobic hydrocarbon biodegradation in these environments and findings resulting from their metagenomic sequencing are briefly described below.

6.1 Fossil Energy Reservoirs

It is well known that deep subsurface crude oil-containing reservoirs harbor thriving subsurface microbial communities wherein a variety of saturate and aromatic hydrocarbons can serve as the key carbon and energy substrates (Head et al. 2014). In fact, most of the world's crude oil in reservoirs have been biodegraded to some extent over geological time; this is believed to have primarily occurred under syntrophic, methanogenic conditions because isotopic signatures of methane in gas caps overlying reservoirs (where biodegraded oil is found) is primarily biogenic in nature (Head et al. 2014). More recently, microorganisms associated with shale or coal reservoirs have been identified (Strąpoć et al. 2011; An et al. 2013a; Lawson et al. 2015; Mouser et al. 2016). The carbon substrates within these fossil energy reservoirs are less defined but also contain complex, organic carbon-rich substrates; organic solvent extracts of coal or shale have revealed a variety of components such as alkanes, PAHs, heterocyclic compounds, aromatic acids and alcohols, and alkanoic acids that can feasibly support extant microbial communities (Orem et al. 2010; Strąpoć et al. 2011; Lawson et al. 2015). In addition, Mayumi et al. (2016) recently discovered that methoxy compounds found in coal can be used directly by some methanogens to produce methane. Understanding the microbial activities within petroliferous reservoirs not only provides insight into life processes occurring within the deep subsurface but can have important applications for microbially enhanced energy recovery wherein entrained hydrocarbon or related substrates can be converted to CH_4 as a clean-burning energy source (An et al. 2013a; Head et al. 2014; Lawson et al. 2015).

While the microbial community compositions (16S rRNA gene analysis) for several crude oil reservoirs, coal seams, and shale gas systems have been determined (e.g., reviews by Head et al. 2014; Mouser et al. 2016), several reports now describe a metagenomic analysis of samples from these hydrocarbon-containing environments (Table 1). Kotlar et al. (2011) and Lewin et al. (2014) were among the first to perform metagenomic sequencing of two hot (80–85 °C), deep (~ 2.5 km below the

Table 3 Examples of anoxic hydrocarbon-containing environmental samples studied using a shotgun metagenomic approach and a summary of key findings related to anaerobic hydrocarbon biodegradation

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
Fossil energy reservoirs			
Crude oil reservoir produced water from 2.5 km below sea floor (85 °C and 250 bar); not exposed to seawater injection (well I) Norwegian continental shelf Microbial diversity of hot oil reservoirs	Fluids retrieved from deep hot sediments (well I) were dominated by sulfate/sulfur-reducing bacteria, with lesser abundance of methanogenic (primarily *Methanococcus*) taxa	Not reported	Kotlar et al. (2011)
Crude oil reservoir produced water from 2.5 km below sea floor (85 °C and 250 bar); not exposed to seawater injection (well II) Norwegian continental shelf Microbial diversity of hot oil reservoirs, comparisons between well I (Kotlar et al. 2011) and well II	Fluids retrieved from deep hot sediments from well II, physically separated from well I, were dominated by archaea (*Thermococcus* and *Pyrococcus*, both noted for S metabolism) with lesser abundance of bacteria (primarily Deltaproteobacteria); both wells I and II showed similar taxa overall but in different abundances and similar gene compositions	Not reported	Lewin et al. (2014)
Crude oil reservoir, produced water sample, 30 °C, water flooded Medicine Hat, Alberta, Canada Functional microbial potential within hydrocarbon resource environments	Dominant taxa affiliated with *Clostridiales*, *Syntrophobacterales*, *Methanomicrobiales*, and *Methanosarcinales*, all known to be associated with methanogenic oil biodegradation; gene analysis revealed an enrichment of anaerobic hydrocarbon-degrading genes compared to aerobic hydrocarbon-degrading genes	Short Read Archive, SRX210984	An et al. (2013a)
Crude oil reservoir, produced water samples Alaska North Slope oilfields, Alaska, USA Physiological potential of microbial communities in petroleum reservoirs	Reconstructed genomes for several anaerobic microbes including candidate phyla; identified multiple *ass*A, *bss*A, and benzoate reductase genes indicating potential for anaerobic hydrocarbon biodegradation; nitrogen-fixing genes associated with methanogens	Raw reads deposited at Genbank, SRP057267	Hu et al. (2016)
Crude oil reservoir, crude oil samples Qinghai and Daqing oilfields, China Microbial community composition and functioning in oil reservoirs; comparisons with metagenomes from 2 oil reservoirs and other environments (948 total)	Bacteria dominated by Proteobacteria (*Pseudomonas, Acinetobacter, Marinobacter*) and Firmicutes while Archaea dominated by methanogens; genes for both aerobic and anaerobic hydrocarbon biodegradation found in oilfield samples	Short Read Archive, BioProject PRJNA251580	Nie et al. (2016)

(*continued*)

Table 3 (continued)

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
Deep shale formations disturbed by hydraulic fracturing, sampled over a period of 328 days Appalachian basin shales – Marcellus (Pennsylvania) and Utica (Ohio), USA Subsurface microbiology and biogeochemistry in deep engineered systems; changes following hydraulic fracturing	Enrichment of halotolerant communities following hydraulic fracturing; reconstruction of 31 genomes demonstrated potential for methanogenesis from methylated substrates, for fermentation of chemical additives used for hydraulic fracturing (that can include hydrocarbons), and for sulfur cycling; only aerobic hydrocarbon-degrading genes were detected	Short Read Archive BioProject PRJNA308326	Daly et al. (2016)
Shale samples from Marcellus shale, including source water, and flowback water after 1 and 9 days Pennsylvania, USA Assess microbial community response following hydraulic fracturing	Flowback water samples were more enriched in anaerobic taxa than in original source water, along with an increase in gene abundances for numerous metabolic processes; hydrocarbon-degrading gene analysis not examined	MG-RAST, 4525703.3, 4525704.3, 4525705.3	Mohan et al. (2014)
Coal seams, 4 produced water and 3 cuttings samples from different depths Alberta, Canada Functional microbial potential within hydrocarbon resource environments	An analysis for aerobic and anaerobic hydrocarbon-degrading genes revealed a predominance of aerobic genes, suggesting that aerobic processes are more common in deep coal seams than expected	Short Read Archive, SRX210870, SRX210867, SRX210868, SRX210875, SRX210869, SRX211003, SRX211004	An et al. (2013a)
Deep bituminous coal seam sample from the Mannville Group Alberta, Canada Understanding microbial communities and functions in coal seams	Metagenomic sequencing allowed for the reconstruction of a major taxon associated with this coal sample (*Celeribacter*) which harbored genes for aerobic degradation of aromatics, glycogen storage, and fermentation pathways suggesting aerobic/anaerobic scheme for coal substrate metabolism leading to methanogenic substrates	Short Read Archive, SRR573886	Lawson et al. (2015)
Deep-sea cold seeps and hydrothermal vents			
Crude oil seep, core samples from 0–4 cm and 10–15 cm depths Tonya seep, Coal Oil Point in Santa Barbara Channel, California, USA Potential for aerobic and anaerobic oxidation of methane and other hydrocarbons	Genes for aerobic methane oxidation found at 0–4 cm, while genes for (reversed) methanogenesis found at 1–15 cm depth along with anaerobic methane oxidizing taxa (ANME-1, ANME-2, ANME-3) and sulfate-reducing syntrophic partners	Genbank, SRP005641	Håvelsrud et al. (2011)

(*continued*)

Table 3 (continued)

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
Crude oil cold seep sample Coal Oil Point in Santa Barbara Channel, California, USA Microbial diversity and processes around hydrocarbon seeps	Dominance of anaerobic methane oxidizers (ANME-1, ANME-2, ANME-3) and requisite genes for (reverse) methanogenesis were found	IMG/M project ID 45292	Hawley et al. (2014a, b)
Pockmarked sediments (potential hydrocarbon-releasing cold seeps) overlying a known petroleum reservoir (Troll field) and "control" sediments with no hydrocarbon influence North Sea, Troll (hydrocarbon) and Oslofjord (no hydrocarbon) areas, Norway Microbial community understanding in relation to geochemical parameters in cold seep systems; potential for anaerobic methane/ hydrocarbon degradation	Some known hydrocarbon biodegradation genes were overrepresented in the pockmarked samples compared to the "control" sediments. Several were for aerobic processes but also included genes for benzoyl-CoA reductase which catalyzes aromatic ring reduction. In contrast, genes for AOM were not enriched, suggesting little methane is occurring from the pockmarked sediments. Overabundance of autotrophic nitrifiers was evident in pockmarked vs. control sediments	Genbank, SRP009243	Håvelsrud et al. (2012)
Oil-immersed, hydrocarbon-releasing deep-sea hydrothermal vent Guaymas Basin, California, USA Ecological functions and activities of microbial communities in hydrothermal vents	Several thermophilic sulfate reducers were identified along with *bss*A and *ass*A genes demonstrating the potential for anaerobic hydrocarbon biodegradation; enhanced expression of these genes was also found in a subsequent metatranscriptomic analysis	MG-RAST, 4510962.3	He et al. (2013, 2015)
Hydrothermal vent microbial mats (red mat, 18 °C; white mat 70 °C) Hellenic Volcanic Arc, Greece Microbial diversity and processes around hydrothermal vents	Detected a variety of genes associated with the anaerobic biodegradation of aromatic hydrocarbons including for fumarate addition (*bss*A), hydroxylation (ethylbenzene dehydrogenase), and benzoyl-CoA reductases; these genes were more abundant in the cooler red mat	IMG/M, 3300002231 & 3300002242	Oulas et al. (2016)
Hydrocarbon-impacted marine environments			
Marine sediments near Deepwater Horizon blowout, and one distant sample unimpacted by hydrocarbons Gulf of Mexico, USA Characterize microbial communities and assess	Metagenomic analysis of cored anoxic seabed sediments (2) collected from near the blowout site, as compared to a distant unaffected sample (1) revealed an enriched in sulfate-reducing taxa, and an enrichment/detection of anaerobic hydrocarbon-degrading genes (e.g., *bss*A) in a sample closest to the blowout site; PCR amplification	*ass*A and *bss*A sequences deposited in GenBank, JX135105-JX135128	Kimes et al. (2014)

(*continued*)

Table 3 (continued)

Environmental sample, location, and process investigated	Key findings	Metagenome accession #	References
potential for hydrocarbon metabolism in anoxic marine sediments associated with hydrocarbon-contamination	revealed the presence of *ass*A and *bss*A only in the hydrocarbon-contaminated sediments		
Hydrocarbon-impacted terrestrial environments			
Oil sands tailings ponds, anoxic zone, 3 samples Northeastern Alberta, Canada Functional microbial potential within hydrocarbon resource environments	Taxa affiliated primarily with known syntrophs and methanogens; genes associated with both anaerobic and aerobic aromatic hydrocarbon biodegradation were detected	Short Read Archive, SRX210980, SRX210872, SRX210871	An et al. (2013a)
Oil sands tailings, anoxic zone Northeastern Alberta, Canada Assessment of anaerobic hydrocarbon biodegradation potential	Metagenomic analysis of unenriched anoxic tailings samples revealed genes associated with acetate- and H_2-using methanogenesis, fumarate addition to aromatic hydrocarbons, (*bss*A gene), and genes for the ATP-independent ring reduction of benzoate	MG-RAST, 4492774.3; Short Read Archive, SRX210871	An et al. (2013b)
Hydrocarbon and chlorinated solvent-contaminated aquifer sediments, 15 m depth Kwazulu-Natal, South Africa Determine microbial communities and functions in contaminated aquifers	Dominance of *Bacteroides* and Betaproteobacteria; protein recruitment plots showed close similarities to known hydrocarbon degraders strain EbN1 and *Dechloromonas aromatica* RCB; some anaerobic benzoate degradation genes detected	Not reported	Abbai and Pillay (2013)

seafloor) crude oil reservoir samples retrieved from the Norwegian North Sea that were ~3 km apart and physically separated, and analysis focused mainly on the recovered taxa. They found similar gene profiles and anaerobic taxa across both wells, albeit the microbial members were of different relative abundances (Lewin et al. 2014). Nie et al. (2016) performed metagenomic sequencing on crude oils obtained from two distinct Chinese oilfields and compared these with the Norwegian metagenomic datasets (Kotlar et al. 2011; Lewin et al. 2014). The Chinese field metagenomes were found to be abundant in lipid metabolism genes, along with genes for known aerobic (e.g., *alk*B) and anaerobic (*ass*A/*bss*A) hydrocarbon metabolism in accordance with the identification of both aerobic and anaerobic taxa. In contrast, the Norwegian samples that were characterized primarily by anaerobic taxa contained only the anaerobic hydrocarbon-degrading genes (Nie et al. 2016). Hu et al. (2016) conducted shotgun metagenomic sequencing on several produced water samples from Alaska North Slope oilfields characterized by varying temperatures (24–27 °C or 80–83 °C) and whether or not they had a history of

souring. Immense diversity was discovered but decreased as fluid temperature increased. Dozens of nearly complete genomes were reconstructed from this metagenomics dataset. In the hottest sample (80–83 °C), *Thermoanaerobacter*, *Desulfonautics*, *Archaeoglobus*, and *Thermodesulfobacterium* were the primary organisms detected, for which near-complete genomes were reconstructed and were not found to contain any genes associated with anaerobic hydrocarbon metabolism. In contrast, reconstructed genomes from the cooler reservoir samples revealed the presence of several taxa known to be associated with anaerobic hydrocarbon biodegradation (*Clostridia*, *Clostridiales*, *Desulfotomaculum*, *Syntrophobacterales*) along with a relatively high abundance of candidate phyla such as OP9 (Atribacteria) and OD1 (Parcubacteria). Notably, many gene sequences for benzylsuccinate synthase, alkylsuccinate synthase, and benzoyl-CoA reductase were associated with these taxa, indicating the potential for in situ anaerobic hydrocarbon biodegradation in these anoxic crude oil reservoirs (Hu et al. 2016).

Some shale and coal reservoir samples have also been subject to metagenomic sequencing (Table 3). Although some coal-bearing environments are dominated by anaerobic signatures (taxa and genes) (Wawrik et al. 2012; Gründger et al. 2015), some of these have exhibited aerobic signatures in addition to anaerobic signatures, going against the more commonly accepted belief that these are exclusively anoxic environments. In a 16S rRNA gene survey and metagenomic analysis of hydrocarbon resource environments (that included coal cuttings, cores, and produced waters, crude oil reservoir produced water, and samples from oil sands and oil sands tailings ponds), taxa with known aerobic respiration along with aerobic hydrocarbon-degrading genes (e.g., for mono- and dioxygenases) were detected to some extent in all samples. Unexpectedly, the coal samples had the highest gene counts for aerobic hydrocarbon biodegradation, including genes for aerobic methane oxidation (An et al. 2013a). Based on these findings, it was proposed that oxygen may intermittently be available to subsurface environments through meteoric waters or through the slow diffusion of oxygen from the coal itself. Metagenomic analyses of other coalbed methane samples have also found genes for the aerobic transformation of a variety of monoaromatic compounds (that are known coal degradation by-products) along with genes for fermentative and methanogenic pathways (Lawson et al. 2015). The reconstruction of a nearly complete genome of a *Celeribacter* sp. that had genes for the aerobic degradation of aromatics, glycogen storage, and fermentation pathways led the authors to propose that such bacteria can degrade coal components during oxygen ingress, store the carbon as glycogen, and then ferment this during periods of anoxia producing methanogenic substrates leading to subsequent methane production from coalbeds (Lawson et al. 2015). In fractured shale formations, it also appears that aerobic, but not anaerobic, hydrocarbon-degrading genes are present (Daly et al. 2016); thus some aerobic hydrocarbon metabolism may occur in these kinds of reservoirs. However, genes for fermentation of a variety of other substances (including chemicals used in fracturing operations) and for methanogenesis indicate that methane-producing consortia are also key in shale deposits (Daly et al. 2016).

6.2 Hydrocarbon Seeps and Hydrothermal Vents

In contrast to confined fossil energy reservoirs (that are bound by impermeable cap rocks allowing for the accumulation of economically recoverable amounts of crude oil and gas), hydrocarbons can also be steadily released through natural cold seeps or hydrothermal vent systems (Farwell et al. 2009; Boetius and Wenzhöfer 2013; Teske et al. 2014). These unconfined structures release either gaseous hydrocarbons (primarily methane, or C_1–C_4 alkanes) or a mix of gaseous and liquid alkanes ($>C_4$) that, along with other potential substrates (such as inorganic sulfur compounds), support the proliferation of both macro- and microbiological life-forms (Boetius and Wenzhöfer 2013, Teske et al. 2014). Notably, the process of anaerobic methane oxidation (AOM) has been well documented at both cold seeps and at hydrothermal vents (Orcutt et al. 2008; Knittel and Boetius 2009; Teske et al. 2014). The microorganisms involved in AOM are vital for keeping global methane emissions from oceans at bay by acting as natural methane biofilters (Knittel and Boetius 2009; Boetius and Wenzhöfer 2013); this process has been estimated to consume ~300 Tg methane/year within marine systems (Hawley et al. 2014a).

To date, metagenomic analyses have been performed on samples recovered from some natural hydrocarbon-releasing systems. Coal Oil Point in the Santa Barbara Channel (California, USA) has been a well-studied natural hydrocarbon seep area because it releases heavy oil that creates oil slicks on the ocean surface, along with significant amounts of methane (Hornafius et al. 1999; Farwell et al. 2009). In order to study the potential for AOM at this seep area, both Håvelsrud et al. (2011) and Hawley et al. (2014a, b) conducted a metagenomic analysis of seep sediments. Both studies revealed the presence of known AOM taxa (anaerobic methanotrophic archaea, or ANME, and sulfate reducers) along with genes associated with sulfate reduction and with “reverse” methanogenesis, a known mechanism of anaerobic methane oxidation (Hallam et al. 2004). These studies thus showed the potential for AOM at this oil-releasing cold seep.

Guaymas Basin, located in the Gulf of California (Baja California), harbors an active hydrothermal vent system (Teske et al. 2016). Here, buried sedimentary organic matter is hydrothermally transformed to a variety of hydrocarbons (and other components such as organic acids and non-hydrocarbon gases) that are continuously emitted from hydrothermal vent features. This hydrothermal vent area is of high microbiological interest as it is characterized by steep temperature gradients and diverse redox zones that can feasibly support diverse microbial processes including AOM (Teske et al. 2014; Kleindienst et al. 2014). The Guaymas Basin hydrothermal vent system has also been the source of several hydrocarbon-degrading sulfate-reducing isolates (Table 1) including strain BuS5 (propane and butane utilizer, activation by fumarate addition; Kniemeyer et al. 2007), *Desulfothermus naphthae* TD3 (alkane utilizer; Rueter et al. 1994), strain EbS7 (ethylbenzene utilizer, activation by fumarate addition; Kniemeyer et al. 2003), and the newly reported butane-degrading mixed culture that activates *n*-butane via CoM (Laso-Pérez et al. 2016). In an investigation of sulfate-reducing diversity and anaerobic hydrocarbon-utilizing functions at the Guaymas Basin and a cooler seep (Amon mud volcano), Kleindienst

et al. (2014) showed that seep-associated sulfate reducers capable of utilizing either butane or dodecane were phylogenetically affiliated with the *Desulfosarcina*/*Desulfococcus* clade (to which BuS5 belongs, along with sulfate-reducing partners associated with AOM). Indeed, several reports have now also identified diverse *ass*A genes associated with the anaerobic oxidation of higher alkanes both in the Guaymas Basin and in other hot and cold seeps (von Netzer et al. 2013; Stagars et al. 2016). Recent metagenomic sequencing of a Guaymas Basin vent sample revealed the presence of a number of thermophilic sulfate- or sulfur-utilizing taxa including members of the *Archaeoglobaceae*, *Thermococcaceae*, *Desulfobacteraceae*, and *Thermodesulfobacteraceae* along with several *ass*A and *bss*A gene sequences associated with known sulfate-reducing hydrocarbon-degrading strains (He et al. 2013, 2015). Collectively, these studies have clearly demonstrated that this hydrothermal vent system is an area of active anaerobic hydrocarbon biodegradation beyond AOM. Further, the detection of anaerobic hydrocarbon-degrading genes in a geographically distant hydrothermal vent system (Hellenic Volcanic Arc; Oulas et al. 2016) has underlined the importance of anaerobic hydrocarbon-biodegrading activities associated with hydrocarbon-releasing thermal vent areas.

6.3 Contaminated Marine Environments

It is now firmly established that the world's oceans host diverse microbial life (Hazen et al. 2016). Owing to the fact that approximately 600,000 tons of hydrocarbons leak into marine systems through natural seeps each year, microbial communities in many marine environments have the ability to utilize hydrocarbons and hence are "primed" to respond to oil influxes (Kimes et al. 2014; Hazen et al. 2016). This phenomenon was clearly observed following the Deepwater Horizon blowout in the Gulf of Mexico in 2010 that released approximately four million barrels of crude oil and ~250,000 metric tons of natural gas (mostly methane, with lesser amounts of C_2–C_4 alkanes) at a depth of ~1500 m below the sea surface. The Gulf of Mexico is a marine system that is rife with natural seeps (emitting an estimated 0.4–1 million barrels oil/year); thus the extant microbial community responded rapidly to the released oil (Orcutt et al. 2008; Hazen et al. 2016). To date, most research has focused on the response of aerobic microorganisms (for detailed reviews, see Kimes et al. 2014; Joye et al. 2014; King et al. 2015; Hazen et al. 2016). For example, multiple lines of evidence demonstrated that within a few weeks following the accident, microorganisms within the impacted deepwater column were found to have aerobically biodegraded several of the spilled light oil components (Hazen et al. 2010). Members of the *Oceanospirillales*, *Colwellia*, and *Cycloclasticus* were among the first taxa enriched in the deep oil plume, differing substantially from uncontaminated waters collected at the same depths, followed by a succession of other taxa (Kimes et al. 2014; Hazen et al. 2016). Further metagenomic and metatranscriptomic studies of samples collected following the spill revealed the key aerobic microbial players that contributed to the aerobic biodegradation of particular crude oil components in the water column, identifying, for example, that members of the *Oceanospirillales*

were key *n*-alkane and cyclic alkane degraders and *Colwellia* were key ethane and propane degraders (Redmond and Valentine 2012; Mason et al. 2012). Studies also showed that some of the released hydrocarbons were deposited onto the seafloor either as hydrocarbons or as marine snow (a mixture of oil, microorganisms, and extracellular polymeric substances; Joye et al. 2014; Kimes et al. 2014; Chanton et al. 2015). Metagenomic sequencing of 64 seabed surface sediment samples revealed that the oiliest sediments contained the greatest enrichment of *Colwellia* and an unclassified Gamma proteobacterium, along with genes for aerobic aliphatic hydrocarbon biodegradation, demonstrating a capacity for aerobic hydrocarbon degradation at the seafloor surface sediments (Mason et al. 2014). In a separate study, *Cycloclasticus* was found to be associated with the snow floc areas on the seabed, along with members of the *Desulfobacteraceae* and *Desulfobulbaceae* in some samples, suggesting the development of anaerobic "patches" in the sediments (Yang et al. 2016). To date, only one study has reported on potential anaerobic hydrocarbon transformations in seabed sediments associated with the Deepwater Horizon spill. Kimes et al. (2013) collected three core samples (1.5–3 cm below seabed surface) from near the blowout wellhead and conducted a metagenomic analysis to assess the response of the hydrocarbon-exposed sediment communities (compared to a hydrocarbon-free sample). Most notably, the metagenomic survey revealed an enrichment of several *bss*A and *ass*A genes associated with the sample collected closest to the wellhead along with the detection of primarily Deltaproteobacteria, showing that the anoxic sediments harbored the potential for anaerobic hydrocarbon degradation. Metabolite determinations also revealed the presence of benzylsuccinates in the sediments, strengthening the argument that anaerobic hydrocarbon degradation was active in the hydrocarbon-impacted sediments associated with the Deepwater Horizon blowout (Kimes et al. 2013).

6.4 Contaminated Terrestrial Environments

Although there have been numerous studies examining the anaerobic in situ bioremediation of hydrocarbons in contaminated groundwater aquifers (e.g., Beller 2000; Beller et al. 1995, 2002, 2008; Gieg et al. 1999; Griebler et al. 2004; Winderl et al. 2007, 2008; Parisi et al. 2009; Callaghan et al. 2010; Jobelius et al. 2011; Essaid et al. 2011; Morasch et al. 2011; Meckenstock et al. 2015), there have been surprisingly few reports to date describing a metagenomic dataset from this kind of environment (Table 3). Abbai and Pillay (2013) used a metagenomics approach to examine the microbiological and functional composition of two borehole samples retrieved from an aquifer system contaminated with industrial chemicals including aromatic hydrocarbons. While the majority of taxa and hydrocarbon-degrading genes detected were aerobic in nature (e.g., oxygenases), some identified taxa were mostly closely associated with known hydrocarbon-degraders although anaerobic hydrocarbon-degrading genes (e.g., *ass*A, *bss*A) were not found or reported (Abbai and Pillay 2013). Although not aquifer systems, terrestrial-based oil sands tailings ponds that store solid and liquid wastes from surface bitumen mining in northeastern Alberta,

Canada, are predominantly anoxic (Penner and Foght 2010; Ramos-Padrón et al. 2011), harboring methanogenic consortia capable of biodegrading a variety of alkanes and aromatic hydrocarbons as demonstrated in several enrichment cultures (e.g., Siddique et al. 2006, 2007; Tan et al. 2015a, b; Abu Laban et al. 2015) and in situ (Stasik et al. 2015). In accordance with observations in enrichments, a metagenomic analysis of unenriched anoxic tailings samples revealed genes associated with acetotrophic and hydrogenotrophic methanogenesis, fumarate addition (*bss*A), and ATP-independent ring reduction of benzoate (An et al. 2013b). These metagenomic findings provided additional evidence that methanogenic consortia are capable of biotransforming hydrocarbons associated with oil sands tailings ponds.

7 Hydrocarbon Metabolomics

The term "metabolome" has been defined as a collection of all of the biochemical molecules produced by a given cellular system, and the field of science involving the analysis of the metabolome is called "metabolomics." In the field of anaerobic hydrocarbon metabolism, "hydrocarbon metabolomics" or "hydrocarbon metabolite profiling" generally refers to evaluating environmental samples for the presence of specific, signature hydrocarbon metabolites that are only detected if anaerobic biodegradation is occurring within a given environment. Thus, while metagenomics reveals the genetic *potential* for biochemical reactions, metabolomics can be considered as the "ultimate proof" that a biochemical reaction has occurred. Using a metabolomic approach to deduce in situ anaerobic hydrocarbon biodegradation has been a topic of recent reviews (e.g., Agrawal and Gieg 2013; Callaghan 2013a) and is the focus of a separate chapter in this manual (Gieg and Toth 2016), so here we present only a high-level overview on this topic.

There are a handful of anaerobic mechanisms known to mediate anaerobic hydrocarbon activation for which signature metabolites (and in some cases genes and enzymes) have been identified, including fumarate addition, carboxylation, and hydroxylation (see above and reviews by Widdel and Musat 2010; Heider and Schühle 2013; Musat 2015; Rabus et al. 2016). Of these pathways, metabolites stemming from fumarate addition reactions (benzylsuccinates, alkylsuccinates and naphthylmethylsuccinates; Fig. 1) can be considered as the best diagnostic indicators of anaerobic in situ hydrocarbon metabolism (in part) due to their unequivocal relationship with their parent molecule, their absence in fuel mixtures, and their relative stability in the environment (NRC 1993; Beller 2000). As such, finding these signature metabolites in hydrocarbon-containing environments provides unequivocal evidence that anaerobic hydrocarbon biodegradation is occurring. Fumarate addition metabolites have now been detected in an array of hydrocarbon-containing environments, including in groundwater systems (e.g., Beller 2000; Beller et al. 1995, 2002, 2008; Elshahed et al. 2001; Gieg and Suflita 2002; Martus and Püttman 2003; Griebler et al. 2004; Gieg et al. 2009; Parisi et al. 2009; Jobelius et al. 2011), petroleum reservoirs (e.g., Duncan et al. 2009; Gieg et al. 2010; Bian et al. 2015), coal seams (Wawrik et al. 2012), and in oil-contaminated marine sediments (Kimes

et al. 2013). Note, however, that fumarate addition reactions are not known to occur for unsubstituted aromatic hydrocarbons like benzene, naphthalene, or other unsubstituted PAHs and heterocycles, making it difficult to use a metabolomic approach to diagnose their in situ biodegradation. For example, anaerobic benzene biodegradation yields either benzoate or phenol as an early intermediate (Fig. 1). Since both of these intermediates can also be formed aerobically (Assinder and Williams 1990), and benzoate is a central metabolic intermediate of numerous aromatic substrates (Fuchs et al. 2011), their detection in a field site cannot be definitively linked to the anaerobic biodegradation of benzene. Similarly, down-gradient metabolites following initial fumarate addition reactions (e.g., toluic acids from xylenes, naphthoic acids from naphthalenes, and fatty acids from alkanes) can also be products of aerobic metabolism (Mahajan et al. 1994). Thus, conclusively diagnosing the in situ anaerobic metabolism for some hydrocarbons can be inherently challenging.

Another of the most important limitations to consider when employing hydrocarbon metabolomics is that the absence of intermediate products cannot be interpreted as an absence of degradation, as metabolites are transient during active catabolism and therefore can be difficult to detect by conventional instrumentation (Callaghan 2013a; Gieg and Toth 2016). Therefore, it is critical that multiple diagnostic approaches be employed when evaluating in situ anaerobic biodegradation in field investigations (Gieg et al. 1999; Weiss and Cozzarelli 2008; Beller 2000; Bombach et al. 2010; Morasch et al. 2011). For example, using a functional gene approach, such as the detection or quantification of *ass*A or *bss*A (by PCR or qPCR), can also compliment a metabolomic analysis and has been used to determine the prospects for in situ anaerobic biodegradation in some hydrocarbon-containing environments (Beller et al. 2002, 2008; Callaghan et al. 2010; Oka et al. 2011; Wawrik et al. 2012; Li et al. 2012; Zhou et al. 2012; Bian et al. 2015). Along with biomarker and genomic tools, recent studies have also evaluated the application of proteomics and metatranscriptomics to characterize anaerobic hydrocarbon biodegradation (e.g., Selesi et al. 2010; Konopka and Wilkins 2012; Embree et al. 2014). These approaches offer real-time snapshots of the functional expression of hydrocarbon-catabolizing genes, but have yet to be widely applied to assessing anaerobic hydrocarbon biotransformation in situ.

8 Research Needs

The earliest investigations into anaerobic hydrocarbon biodegradation focused primarily on understanding this process in anoxic hydrocarbon-contaminated environments such as groundwater aquifers. However, through biogeochemical observations and the use of molecular biology and genomics approaches, it is now apparent that this process occurs in highly diverse environments that additionally include subsurface fossil energy reservoirs, marine sediments, hydrocarbon seeps, and hydrothermal vent systems. The study of isolates or highly enriched cultures obtained from several of these environments has shed enormous light on the

mechanisms of hydrocarbon metabolism in the absence of oxygen, bolstered by information garnered from their genome (and/or transcriptome/proteome) sequences. However, the roles of and interactions among microorganisms in most natural environments, including hydrocarbon-containing ecosystems, are poorly understood. Metagenomics (and other meta-omics approaches) can provide an abundance of information to help define and understand natural biogeochemical processes. While there are now some available metagenomic datasets for microbial communities inhabiting diverse hydrocarbon-containing environments (Table 3), there is still a great need for many more datasets generated from these hydrocarbon-containing environments in order to better understand their governing ecological principles. For example, while the anaerobic hydrocarbon biodegradation within contaminated aquifers has been studied for a long time and in great detail, only one metagenomic dataset has been described for this environment (Abbai and Pillay 2013). Recently, Anantharaman et al. (2016) conducted a terabase-scale metagenomic study of aquifer sediments (not hydrocarbon impacted) from which they were able to reconstruct >2500 individual genomes that allowed for the discovery of many new phyla and a proposed understanding of how microbial community members interact to carry out critical biogeochemical reactions; conducting these sorts of studies for hydrocarbon-containing environments is a clear research need. Further, amassing a large number of metagenomic datasets from environments with similar ecological pressures (such as anoxia and the presence of hydrocarbons) can, for example, allow for comparative analyses in order to elucidate the metabolic traits that define these environments (such as syntrophy, Tan et al. 2015a; Oberding and Gieg 2016).

That said, it should be noted that while metagenomics can provide enormous amounts of informative genetic information, it is an approach that describes metabolic *potential* – experimentation is still required to observe this potential. Thus, obtaining hydrocarbon-degrading isolates or highly enriched cultures and characterizing their functions through physiological experimentation, along with defining their genomes, transcriptomes, proteomes, and metabolomes, are ongoing research needs. Such information from model hydrocarbon degraders can help guide the interpretation of metagenomic datasets and "ground-truth" metagenomic-based predictions through experimentation. In all, metagenomic approaches are only starting to be used to assess anaerobic hydrocarbon biodegradation in many environments but coupled with model experimental systems have the potential to reveal a more comprehensive understanding about this process in diverse hydrocarbon-containing ecosystems.

References

Abbai NS, Pillay B (2013) Analysis of hydrocarbon-contaminated groundwater metagenomes as revealed by high-throughput sequencing. Mol Biotechnol 54:900–912

Abu Laban N, Selesi D, Rattei T, Tischler P, Meckenstock RU (2010) Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. Environ Microbiol 12:2783–2796

Abu Laban N, Dao A, Semple K, Foght J (2015) Biodegradation of C_7 and C_8 *iso*-alkanes under methanogenic conditions. Environ Microbiol 17:4898–4915
Aeckersberg F, Rainey FA, Widdel F (1998) Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Arch Microbiol 170:361–369
Agrawal A, Gieg LM (2013) In situ detection of anaerobic alkane metabolites in subsurface environments. Front Microbiol 4:140
Aklujkar M, Krushkal J, DiBartolo G, Lapidus A, Land ML, Lovley DR (2009) The genome sequence of *Geobacter metallireducens*: features of metabolism, physiology and regulation common and dissimilar to *Geobacter sulfurreducens*. BMC Microbiol 9:109. https://doi.org/10.1186/1471-2180-9-109
An D, Brown D, Chatterjee I, Dong X, Ramos-Padron E, Wilson S, Bordenave S, Caffrey SM, Gieg LM, Sensen CW, Voordouw G (2013a) Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond. Genome 56:612–618
An D, Caffrey SM, Soh J, Agrawal A, Brown D, Budwill K, Dong X, Dunfield PF, Foght J, Gieg LM, Hallam SJ, Hanson NW, He Z, Jack TR, Klassen J, Konwar KM, Kuatsjah E, Li C, Larter S, Leopatra V, Nesbø CL, Oldenburg T, Pagé AP, Ramos-Padron E, Rochman FF, Saidi-Mehrabad A, Sensen CW, Sipahimalani P, Song YC, Wilson S, Wolbring G, Wong ML, Voordouw G (2013b) Metagenomics of hydrocarbon resource environments indicates aerobic taxa and genes to be unexpectedly common. Environ Sci Technol 47: 10708–10717
Anantharaman K, Brown CT, Hug LA, Sharon I, Castelle CJ, Probst AJ, Thomas BC, Singh A, Wilkins MJ, Karaoz U, Brodie EL, Williams KH, Hubbard SS, Banfield JF (2016) Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. Nat Commun 7:13219. https://doi.org/10.1038/ncomms13219
Anderson I, Risso C, Holmes D, Lucas S, Copeland A, Lapidus A, Cheng JF, Bruce D, Goodwin L, Pitluck S, Saunders E, Brettin T, Detter JC, Han C, Tapia R, Larimer F, Land M, Hauser L, Woyke T, Lovley D, Kyrpides N, Ivanova N (2011) Complete genome sequence of *Ferroglobus placidus* AEDII12DO. Stand Genomic Sci 5:50–60
Assinder SJ, Williams PA (1990) The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv Microb Physiol 31:1–69
Barberán A, Bates ST, Casamayor EO, Fierer N (2012) Using network analysis to explore co-occurrence patterns in soil microbial communities. ISME J 6:343–351
Beller HR (2000) Metabolic indicators for detecting in situ anaerobic alkylbenzene degradation. Biodegradation 11:125–139
Beller HR, Ding WH, Reinhard M (1995) Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. Environ Sci Technol 29:2864–2870
Beller HR, Kane SR, Legler TC, Alvarez PJ (2002) A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. Environ Sci Technol 36:3977–3984
Beller HR, Kane SR, Legler TC, McKelvie JR, Lollar BS, Pearson F, Balser L, Mackay DM (2008) Comparative assessments of benzene, toluene, and xylene natural attenuation by quantitative polymerase chain reaction analysis of a catabolic gene, signature metabolites, and compound-specific isotope analysis. Environ Sci Technol 42:6065–6072
Bergmann F, Selesi D, Weinmaier T, Tischler P, Rattei T, Meckenstock RU (2011) Genomic insights into the metabolic potential of the polycyclic aromatic hydrocarbon degrading sulfate-reducing Deltaproteobacterium N47. Environ Microbiol 13:1125–1137
Bian XY, Mbadinga SM, Liu YF, Yang SZ, Liu JF, Ye RQ, Gu JD, Mu BZ (2015) Insights into the anaerobic biodegradation pathway of *n*-alkanes in oil reservoirs by detection of signature metabolites. Sci Rep 5:9801. https://doi.org/10.1038/srep09801
Boetius A, Wenzhöfer F (2013) Seafloor oxygen consumption fuelled by methane from cold seeps. Nat Geosci 6:725–734

Bombach P, Richnow HH, Kästner M, Fischer A (2010) Current approaches for the assessment of in situ biodegradation. Appl Microbiol Biotechnol 86:839–852

Bozinovski D, Herrmann S, Richnow H-H, von Bergen M, Seifert J, Vogt C (2012) Functional analysis of an anaerobic *m*-xylene-degrading enrichment culture using protein-based stable isotope probing. FEMS Microbiol Ecol 81:134–144

Bozinovski D, Taubert M, Kleinsteuber S, Richnow H-H, von Bergen M, Vogt C, Seifert J (2014) Metaproteogenomic analysis of a sulfate-reducing enrichment culture reveals genomic organization of key enzymes in the *m*-xylene degradation pathway and metabolic activity of proteobacteria. Syst Appl Microbiol 37:488–501

Callaghan AV (2013a) Metabolomic investigations of anaerobic hydrocarbon-impacted environments. Curr Opin Biotechnol 24:506–515

Callaghan AV (2013b) Enzymes involved in the anaerobic oxidation of *n*-alkanes: from methane to long-chain paraffins. Front Microbiol 4:89. https://doi.org/10.3389/fmicb.2013.00089

Callaghan AV, Wawrik B, Ní Chadhain SM, Young LY, Zylstra GJ (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. Biochem Biophys Res Commun 366:142–148

Callaghan AV, Tierney M, Phelps CD, Young LY (2009) Anaerobic biodegradation of *n*-hexadecane by a nitrate-reducing consortium. Appl Environ Microbiol 75:1339–1344

Callaghan AV, Davidova IA, Savage-Ashlock K, Parisi VA, Gieg LM, Suflita JM, Kukor JJ, Wawrik B (2010) Diversity of benzyl- and alkylsuccinate synthase genes in hydrocarbon-impacted environments and enrichment cultures. Environ Sci Technol 44:7287–7294

Callaghan A, Morris BE, Pereira IA, McInerney MJ, Austin RN, Groves JT, Kukor JJ, Suflita JM, Young LY, Zylstra GJ, Wawrik B (2012) The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. Environ Microbiol 14:101–113

Chanton J, Zhao T, Rosenheim BE, Joye S, Bosman S, Brunner C, Yeager KM, Diercks AR, Hollander D (2015) Using natural abundance radiocarbon to trace the flux of petrocarbon to the seafloor following the Deepwater Horizon oil spill. Environ Sci Technol 49:847–854

Coates JD, Chakraborty R, Lack JG, O'Connor SM, Cole KA, Bender KS, Achenbach LA (2001) Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. Nature 411:1039–1043

Daly RA, Borton MA, Wilkins MJ, Hoyt DW, Kountz DJ, Wolfe RA, Welch SA, Marcus DN, Trexler RV, MacRae JD, Krzycki JA, Cole DR, Mouser PJ, Wrighton KC (2016) Microbial metabolisms in a 2.5-km-deep ecosystem created by hydraulic fracturing in shales. Nat Microbiol 5:16146. https://doi.org/10.1038/nmicrobiol.2016.146. [Epub ahead of print]

DiDonato RJ Jr, Young ND, Butler JE, Chin KJ, Hixson KK, Mouser P, Lipton MS, DeBoy R, Methé BA (2010) Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene expression during anaerobic growth on naphthalene. PLoS One 5:e14072

Duncan KE, Gieg LM, Parisi VA, Tanner RS, Tringe SG, Bristow J, Suflita JM (2009) Biocorrosive thermophilic microbial communities in Alaskan North Slope oil facilities. Environ Sci Technol 43:7977–7984

Ehrenreich P, Behrends A, Harder J, Widdel F (2000) Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol 173:58–64

Elshahed MA, Gieg LM, McInerney MJ, Suflita JM (2001) Signature metabolites attesting to the insitu attenuation of alkylbenzenes in anaerobic environments. Environ Sci Technol 35:682–689

Embree M, Nagarajan H, Movahedi N, Chitsaz H, Zengler K (2014) Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community. ISME J 8:757–767

Embree M, Liu JK, Al-Bassam MM, Zengler K (2015) Networks of energetic and metabolic interactions define dynamics in microbial communities. Proc Natl Acad Sci U S A 112:15450–15455

Essaid HI, Bekins BA, Herkelrath WN, Delin GN (2011) Crude oil at the Bemidji site: 25 years of monitoring, modeling, and understanding. Ground Water 49:706–726

Ettwig KF, Butler MK, Le Paslier D, Pelletier E, Mangenot S, Kuypers MM, Schreiber F, Dutilh BE, Zedelius J, de Beer D, Gloerich J, Wessels HJ, van Alen T, Luesken F, Wu ML, van de Pas-

Schoonen KT, Op den Camp HJ, Janssen-Megens EM, Francoijs KJ, Stunnenberg H, Weissenbach J, Jetten MS, Strous M (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464:543–548

Farwell C, Reddy CM, Peacock E, Nelson RK, Washburn L, Valentine DL (2009) Weathering and the fallout plume of heavy oil from strong petroleum seeps near Coal Oil Point, CA. Environ Sci Technol 43:3542–3548

Foght J (2008) Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects. J Mol Microbiol Biotechnol 15:93–120

Fowler SJ, Dong X, Sensen CW, Suflita JM, Gieg LM (2012) Methanogenic toluene metabolism: community structure and intermediates. Environ Microbiol 14:754–764

Fowler SJ, Toth CR, Gieg LM (2016) Community structure in methanogenic enrichments provides insight into syntrophic interactions in hydrocarbon-impacted environments. Front Microbiol 7:562. https://doi.org/10.3389/fmicb.2016.00562

Fuchs G, Boll M, Heider J (2011) Microbial degradation of aromatic compounds – from one strategy to four. Nat Rev Microbiol 9:803–816

Galushko A, Minz D, Schink B, Widdel F (1999) Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ Microbiol 1:415–420

Ghosal D, Ghosh S, Dutta TK, Ahn Y (2016) Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. Front Microbiol 7:1369. https://doi.org/10.3389/fmicb.2016.01369

Gieg LM, Suflita JM (2002) Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environ Sci Technol 36:3755–3762

Gieg LM, Toth CRA (2016) Signature metabolite analysis to determine in situ anaerobic hydrocarbon biodegradation. In: Timmis K (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, Submitted 19 Oct 2016

Gieg LM, Kolhatkar RV, McInerney MJ, Tanner RS, Harris SH, Sublette KL, Suflita JM (1999) Evidence for intrinsic bioremediation in a gas condensate-contaminated aquifer. Environ Sci Technol 33:2550–2560

Gieg LM, Alumbaugh RE, Field J, Jones J, Istok JD, Suflita JM (2009) Assessing in situ rates of anaerobic hydrocarbon bioremediation. Microb Biotechnol 2:222–233

Gieg LM, Davidova IA, Duncan KE, Suflita JM (2010) Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. Environ Microbiol 12:3074–3086

Gieg LM, Fowler SJ, Berdugo-Clavijo C (2014) Syntrophic biodegradation of hydrocarbon contaminants. Curr Opin Biotechnol 27:21–19

Gittel A, Donhauser J, Røy H, Girguis PR, Jørgensen BB, Kjeldsen KU (2015) Ubiquitous presence and novel diversity of anaerobic alkane degraders in cold marine sediments. Front Microbiol 6:1414

Griebler C, Safinowski M, Vieth A, Richnow HH, Meckenstock RU (2004) Combined application of stable carbon isotope analysis and specific metabolites determination for assessing in situ degradation of aromatic hydrocarbons in a tar oil-contaminated aquifer. Environ Sci Technol 38:617–631

Gründger F, Jiménez N, Thielemann T, Straaten N, Lüders T, Richnow HH, Krüger M (2015) Microbial methane formation in deep aquifers of a coal-bearing sedimentary basin, Germany. Front Microbiol 6:200. https://doi.org/10.3389/fmicb.2015.00200

Grundmann O, Behrends A, Rabus R, Amann J, Halder T, Heider J, Widdel F (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN. Environ Microbiol 10:376–385

Hallam SJ, Putnam N, Preston CM, Detter JC, Rokhsar D, Richardson PM, DeLong EF (2004) Reverse methanogenesis: testing the hypothesis with environmental genomics. Science 305:1457–1462

Harms G, Rabus R, Widdel F (1999) Anaerobic oxidation of the aromatic plant hydrocarbon *p*-cymene by newly isolated denitrifying bacteria. Arch Microbiol 172:303–312

Håvelsrud OE, Haverkamp TH, Kristensen T, Jakobsen KS, Rike AG (2011) A metagenomic study of methanotrophic microorganisms in Coal Oil Point seep sediments. BMC Microbiol 11:221. https://doi.org/10.1186/1471-2180-11-221

Håvelsrud OE, Haverkamp TH, Kristensen T, Jakobsen KS, Rike AG (2012) Metagenomic and geochemical characterization of pockmarked sediments overlaying the Troll petroleum reservoir in the North Sea. BMC Microbiol 12:203. https://doi.org/10.1186/1471-2180-12-203

Hawley ER, Malfatti SA, Pagani I, Huntemann M, Chen A, Foster B, Copeland A, del Rio TG, Pati A, Jansson JR, Gilbert JA, Tringe SG, Lorenson TD, Hess M (2014a) Metagenomes from two microbial consortia associated with Santa Barbara seep oil. Mar Genomics 18(Pt B):97–109

Hawley ER, Piao H, Scott NM, Malfatti S, Pagani I, Huntemann M, Chen A, Glavina Del Rio T, Foster B, Copeland A, Jansson J, Pati A, Tringe S, Gilbert JA, Lorenson TD, Hess M (2014b) Metagenomic analysis of microbial consortium from natural crude oil that seeps into the marine ecosystem offshore Southern California. Stand Genomic Sci 9:1259–1274

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Borglin SE, Fortney JL, Stringfellow WT, Bill M, Conrad ME, Tom LM, Chavarria KL, Alusi TR, Lamendella R, Joyner DC, Spier C, Baelum J, Auer M, Zemla ML, Chakraborty R, Sonnenthal EL, D'haeseleer P, Holman HY, Osman S, Lu Z, Van Nostrand JD, Deng Y, Zhou J, Mason OU (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 330:204–208

Hazen TC, Prince RC, Mahmoudi N (2016) Marine oil biodegradation. Environ Sci Technol 50:2121–2129

He Y, Xiao X, Wang F (2013) Metagenome reveals potential microbial degradation of hydrocarbon coupled with sulfate reduction in an oil-immersed chimney from Guaymas Basin. Front Microbiol 4:148. https://doi.org/10.3389/fmicb.2013.00148

He Y, Feng X, Fang J, Zhang Y, Xiao X (2015) Metagenome and metatranscriptome revealed a highly active and intensive sulfur cycle in an oil-immersed hydrothermal chimney in Guaymas Basin. Front Microbiol 6:1236. https://doi.org/10.3389/fmicb.2015.01236

Head IM, Gray ND, Larter SR (2014) Life in the slow lane; biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. Front Microbiol 5:566. https://doi.org/10.3389/fmicb.2014.00566

Heider J, Schühle K (2013) Anaerobic biodegradation of hydrocarbons including methane. In: Rosenburg E (ed) The prokaryotes – prokaryotic physiology and biochemistry. Springer, Berlin, pp 605–634

Holmes DE, Risso C, Smith JA, Lovley DR (2011) Anaerobic oxidation of benzene by the hyperthermophilic archaeon *Ferroglobus placidus*. Appl Environ Microbiol 77:5926–5933

Hornafius JS, Quigley D, Luyendk BP (1999) The world's most spectacular marine hydrocarbon seeps (coal oil point, Santa Barbara Channel, California): quantification of emissions. J Geophys Res 104:703–720

Hu P, Tom L, Singh A, Thomas BC, Baker BJ, Piceno YM, Andersen GL, Banfield JF (2016) Genome-resolved metagenomic analysis reveals roles for candidate phyla and other microbial community members in biogeochemical transformations in oil reservoirs. MBio 7: e01669–e01615. https://doi.org/10.1128/mBio.01669-15

Jiménez N, Richnow HH, Vogt C, Treude T, Krüger M (2016) Methanogenic hydrocarbon degradation: evidence from field and laboratory studies. J Mol Microbiol Biotechnol 26:227–242

Jobelius C, Ruth B, Griebler C, Meckenstock RU, Hollender J, Reineke A, Frimmel FH, Zwiener C (2011) Metabolites indicate hot spots of biodegradation and biogeochemical gradients in a high resolution monitoring well. Environ Sci Technol 45:474–481

Johnson JM, Wawrik B, Isom C, Boling WB, Callaghan AV (2015) Interrogation of Chesapeake Bay sediment microbial communities for intrinsic alkane-utilizing potential under anaerobic conditions. FEMS Microbiol Ecol 91:114

Joye SB, Teske AP, Kostka JE (2014) Microbial dynamics following the Macondo oil well blowout across Gulf of Mexico environments. Bioscience 64:766–777

Keller AH, Schleinitz KM, Starke R, Bertilsson S, Vogt C, Kleinsteuber S (2015) Metagenome-based metabolic reconstruction reveals the ecophysiological function of *Epsilonproteobacteria*

in a hydrocarbon-contaminated sulfidic aquifer. Front Microbiol 6:1396. https://doi.org/10.3389/fmicb.2015.01396

Khelifi N, Grossi V, Hamdi M, Dolla A, Tholozan JL, Ollivier B, Hirschler-Réa A (2010) Anaerobic oxidation of fatty acids and alkenes by the hyperthermophilic sulfate-reducing archaeon *Archaeoglobus fulgidus*. Appl Environ Microbiol 76:3057–3060

Khelifi N, Amin Ali O, Roche P, Grossi V, Brochier-Armanet C, Valette O, Ollivier B, Dolla A, Hirschler-Réa A (2014) Anaerobic oxidation of long-chain *n*-alkanes by the hyperthermophilic sulfate-reducing archaeon, *Archaeoglobus fulgidus*. ISME J 8:2153–2166

Kimes NE, Callaghan AV, Aktas DF, Smith WL, Sunner J, Golding B, Drozdowska M, Hazen TC, Suflita JM, Morris PJ (2013) Metagenomic analysis and metabolite profiling of deep-sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill. Front Microbiol 4:50. https://doi.org/10.3389/fmicb.2013.00050

Kimes NE, Callaghan AV, Suflita JM, Morris PJ (2014) Microbial transformation of the Deepwater Horizon oil spill – past, present, and future perspectives. Front Microbiol 5:603. https://doi.org/10.3389/fmicb.2014.00603

King GM, Kostka JE, Hazen TC, Sobecky PA (2015) Microbial responses to the Deepwater Horizon oil spill: from coastal wetlands to the deep sea. Annu Rev Mar Sci 7:377–401

Kleindienst S, Herbst FA, Stagars M, von Netzer F, von Bergen M, Seifert J, Peplies J, Amann R, Musat F, Lueders T, Knittel K (2014) Diverse sulfate-reducing bacteria of the *Desulfosarcina/Desulfococcus* clade are the key alkane degraders at marine seeps. ISME J 8:2029–2044

Kleinsteuber S, Schleinitz KM, Vogt C (2012) Key players and team play: anaerobic microbial communities in hydrocarbon-contaminated aquifers. Appl Microbiol Biotechnol 94:851–873

Klenk HP, Clayton RA, Tomb JF, White O, Nelson KE, Ketchum KA, Dodson RJ, Gwinn M, Hickey EK, Peterson JD, Richardson DL, Kerlavage AR, Graham DE, Kyrpides NC, Fleischmann RD, Quackenbush J, Lee NH, Sutton GG, Gill S, Kirkness EF, Dougherty BA, McKenney K, Adams MD, Loftus B, Peterson S, Reich CI, McNeil LK, Badger JH, Glodek A, Zhou L, Overbeek R, Gocayne JD, Weidman JF, McDonald L, Utterback T, Cotton MD, Spriggs T, Artiach P, Kaine BP, Sykes SM, Sadow PW, D'Andrea KP, Bowman C, Fujii C, Garland SA, Mason TM, Olsen GJ, Fraser CM, Smith HO, Woese CR, Venter JC (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. Nature 390:364–370

Kniemeyer O, Fischer T, Wilkes H, Glöckner FO, Widdel F (2003) Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol 69:760–768

Kniemeyer O, Musat F, Sievert SM, Knittel K, Wilkes H, Blumenberg M, Michaelis W, Classen A, Bolm C, Joye SB, Widdel F (2007) Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. Nature 449:898–901

Knittel K, Boetius A (2009) Anaerobic oxidation of methane: progress with an unknown process. Annu Rev Microbiol 63:311–334

Konopka A, Wilkins MJ (2012) Application of meta-transcriptomics and -proteomics to analysis of in situ physiological state. Front Microbiol 3:184

Kotlar HK, Lewin A, Johansen J, Throne-Holst M, Haverkamp T, Markussen S, Winnberg A, Ringrose P, Aakvik T, Ryeng E, Jakobsen K, Drabløs F, Valla S (2011) High coverage sequencing of DNA from microorganisms living in an oil reservoir 2.5 kilometres subsurface. Environ Microbiol Rep 3:674–681

Laso-Pérez R, Wegener G, Knittel K, Widdel F, Harding KJ, Krukenberg V, Meier DV, Richter M, Tegetmeyer HE, Riedel D, Richnow HH, Adrian L, Reemtsma T, Lechtenfeld O, Musat F (2016) Thermophilic archaea activate butane via alkyl-coenzyme M formation. Nature Oct 17. https://doi.org/10.1038/nature20152. [Epub ahead of print]

Lawson CE, Strachan CR, Williams DD, Koziel S, Hallam SJ, Budwill K (2015) Patterns of endemism and habitat selection in coalbed microbial communities. Appl Environ Microbiol 81:7924–7937

Lewin A, Johansen J, Wentzel A, Kotlar HK, Drabløs F, Valla S (2014) The microbial communities in two apparently physically separated deep subsurface oil reservoirs show extensive DNA sequence similarities. Environ Microbiol 16:545–558

Li W, Wang L-Y, Duan R-Y, Liu JF, Gu J-D, Mu B-Z (2012) Microbial community characteristics of petroleum reservoir production water amended with *n*-alkanes and incubated under nitrate-, sulfate-reducing and methanogenic conditions. Int Biodeterior Biodegrad 69:87–96

Li C, Lim KM, Chng KR, Nagarajan N (2016) Predicting microbial interactions through computational approaches. Methods 102:12–19

Luo F, Gitiafroz R, Devine CE, Gong Y, Hug LA, Raskin L, Edwards EA (2014) Metatranscriptome of an anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. Appl Environ Microbiol 80:4095–4107

Luo F, Devine CE, Edwards EA (2016) Cultivating microbial dark matter in benzene-degrading methanogenic consortia. Environ Microbiol 18:2923–2936

Mahajan MC, Phale PS, Vaidyanathan CS (1994) Evidence for the involvement of multiple pathways in the biodegradation of 1- and 2-methylnaphthalene by *Pseudomonas putida* CSV86. Arch Microbiol 161:425–433

Martín-Moldes Z, Zamarro MT, Del Cerro C, Valencia A, Gómez MJ, Arcas A, Udaondo Z, García JL, Nogales J, Carmona M, Díaz E (2015) Whole-genome analysis of *Azoarcus* sp. strain CIB provides genetic insights to its different lifestyles and predicts novel metabolic features. Syst Appl Microbiol 38:462–471

Martus P, Püttman W (2003) Formation of alkylated aromatic acids in groundwater by anaerobic degradation of alkylbenzenes. Sci Total Environ 307:19–33

Mason OU, Hazen TC, Borglin S, Chain PS, Dubinsky EA, Fortney JL, Han J, Holman HY, Hultman J, Lamendella R, Mackelprang R, Malfatti S, Tom LM, Tringe SG, Woyke T, Zhou J, Rubin EM, Jansson JK (2012) Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. ISME J 6:1715–1727

Mason OU, Scott NM, Gonzalez A, Robbins-Pianka A, Bælum J, Kimbrel J, Bouskill NJ, Prestat E, Borglin S, Joyner DC, Fortney JL, Jurelevicius D, Stringfellow WT, Alvarez-Cohen L, Hazen TC, Knight R, Gilbert JA, Jansson JK (2014) Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. ISME J 8:1464–1475

Mayumi D, Mochimaru H, Tamaki H, Yamamoto K, Yoshioka H, Suzuki Y, Kamagata Y, Sakata S (2016) Methane production for coal by a single methanogen. Science 354:222–225

Meckenstock RU, Elsner M, Griebler C, Lueders T, Stumpp C, Aamand J, Agathos SN, Albrechtsen HJ, Bastiaens L, Bjerg PL, Boon N, Dejonghe W, Huang WE, Schmidt SI, Smolders E, Sørensen SR, Springael D, van Breukelen BM (2015) Biodegradation: updating the concepts of control for microbial cleanup in contaminated aquifers. Environ Sci Technol 49:7073–7081

Meckenstock RU, Boll M, Mouttaki H, Koelschbach JS, Cunha Tarouco P, Weyrauch P, Dong X, Himmelberg AM (2016) Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. J Mol Microbiol Biotechnol 26:92–118

Mohan AM, Bibby KJ, Lipus D, Hammack RW, Gregory KB (2014) The functional potential of microbial communities in hydraulic fracturing source water and produced water from natural gas extraction characterized by metagenomic sequencing. PLoS One 9:e107682. https://doi.org/10.1371/journal.pone.0107682

Morasch B, Hunkeler D, Zopfi J, Temime B, Höhener P (2011) Intrinsic biodegradation potential of aromatic hydrocarbons in an alluvial aquifer – potentials and limits of signature metabolite analysis and two stable isotope-based techniques. Water Res 45:4459–4469

Mouser PJ, Borton M, Darrah TH, Hartsock A, Wrighton KC (2016) Hydraulic fracturing offers view of microbial life in the deep terrestrial subsurface. FEMS Microbiol Ecol. Nov 92: fiw166. https://doi.org/10.1093/femsec/fiw166

Mouttaki H, Johannes J, Meckenstock RU (2012) Identification of naphthalene carboxylase as a prototype for the anaerobic activation of non-substituted aromatic hydrocarbons. Environ Microbiol 14:2770–2774

Musat F (2015) The anaerobic degradation of gaseous, nonmethane alkanes – from in situ processes to microorganisms. Comput Struct Biotechnol J 13:222–228

National Research Council (NRC) (1993) In situ bioremediation: when does it work? National Academy Press, Washington, DC

Nie Y, Zhao JY, Tang YQ, Guo P, Yang Y, Wu XL, Zhao F (2016) Species divergence vs. functional convergence characterizes crude oil microbial community assembly. Front Microbiol 7:1254. https://doi.org/10.3389/fmicb.2016.01254

Oberding L, Gieg LM (2016) Metagenomic analyses reveal that energy transfer gene abundances can predict the syntrophic potential of environmental microbial communities. Microorganisms 4:E5. https://doi.org/10.3390/microorganisms4010005

Oka AR, Phelps CD, Zhu X, Saber DL, Young LY (2011) Dual biomarkers of anaerobic hydrocarbon degradation in historically contaminated groundwater. Environ Sci Technol 45:3407–3414

Orcutt B, Samarkin V, Boetius A, Joye S (2008) On the relationship between methane production and oxidation by anaerobic methanotrophic communities from cold seeps of the Gulf of Mexico. Environ Microbiol 10:1108–1117

Orem WH, Voytek MA, Jones EJ, Lerch HE, Bates AL, Corum MD, Warwick PD, Clark AC (2010) Organic intermediates in the anaerobic biodegradation of coal to methane under laboratory conditions. Org Geochem 41:997–1000

Orphan VJ, Taylor LT, Hafenbradl D, Delong EF (2000) Culture-dependent and culture-independent characterization of microbial assemblages associated with high-temperature petroleum reservoirs. Appl Environ Microbiol 66:700–711

Oulas A, Polymenakou PN, Seshadri R, Tripp HJ, Mandalakis M, Paez-Espino AD, Pati A, Chain P, Nomikou P, Carey S, Kilias S, Christakis C, Kotoulas G, Magoulas A, Ivanova NN, Kyrpides NC (2016) Metagenomic investigation of the geologically unique Hellenic Volcanic Arc reveals a distinctive ecosystem with unexpected physiology. Environ Microbiol 18:1122–11236

Parisi VA, Brubaker GR, Zenker MJ, Prince RC, Gieg LM, Da Silva ML, Alvarez PJ, Suflita JM (2009) Field metabolomics and laboratory assessments of anaerobic intrinsic bioremediation of hydrocarbons at a petroleum-contaminated site. Microb Biotechnol 2:202–212

Penner TJ, Foght JM (2010) Mature fine tailings from oil sands processing harbour diverse methanogenic communities. Can J Microbiol 56:459–470

Pérez-Wohlfeil E, Arjona-Medina JA, Torreno O, Ulzurrun E, Trelles O (2016) Computational workflow for the fine-grained analysis of metagenomic samples. BMC Genomics 17(Suppl 8):802

Rabus R, Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol 163:96–103

Rabus R, Nordhaus R, Ludwig W, Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol 59:1444–1451

Rabus R, Kube M, Heider J, Beck A, Heitmann K, Widdel F, Reinhardt R (2005) The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch Microbiol 183:27–36

Rabus R, Boll M, Heider J, Meckenstock RU, Buckel W, Einsle O, Ermler U, Golding BT, Gunsalus RP, Kroneck PM, Krüger M, Lueders T, Martins BM, Musat F, Richnow HH, Schink B, Seifert J, Szaleniec M, Treude T, Ullmann GM, Vogt C, von Bergen M, Wilkes H (2016) Anaerobic microbial degradation of hydrocarbons: from enzymatic reactions to the environment. J Mol Microbiol Biotechnol 26:5–28

Ramos-Padrón E, Bordenave S, Lin S, Bhaskar IM, Dong X, Sensen CW, Fournier J, Voordouw G, Gieg LM (2011) Carbon and sulfur cycling by microbial communities in a gypsum-treated oil sands tailings pond. Environ Sci Technol 45:439–446

Redmond MC, Valentine DL (2012) Natural gas and temperature structured a microbial community response to the Deepwater Horizon oil spill. Proc Natl Acad Sci U S A 109:20292–20297

Rojo F (2009) Degradation of alkanes by bacteria. Environ Microbiol 10:2477–2490

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F (1994) Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature 372:455–458

Safinowski M, Meckenstock RU (2006) Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Environ Microbiol 8:347–352

Salinero KK, Keller K, Feil WS, Feil H, Trong S, Di Bartolo G, Lapidus A (2009) Metabolic analysis of the soil microbe *Dechloromonas aromatica* str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. BMC Genomics 10:351

Segata N, Boernigen D, Tickle TL, Morgan XC, Garrett WS, Huttenhower C (2013) Computational meta'omics for microbial community studies. Mol Syst Biol 9:666

Selesi D, Jehmlich N, von Bergen M, Schmidt F, Rattei T, Tischler P, Lueders T, Meckenstock RU (2010) Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. J Bacteriol 192:295–306

Siddique T, Fedorak PM, Foght JM (2006) Biodegradation of short-chain *n*-alkanes in oil sands tailings under methanogenic conditions. Environ Sci Technol 40:5459–5464

Siddique T, Fedorak PM, MacKinnon M, Foght JM (2007) Metabolism of BTEX and naphtha compounds to methane in oil sands tailings. Environ Sci Technol 41:2350–2356

Sieber JR, McInerney MJ, Gunsalus RP (2012) Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. Annu Rev Microbiol 66:429–452

Singer E, Bushnell B, Coleman-Derr D, Bowman B, Bowers RM, Levy A, Gies EA, Cheng JF, Copeland A, Klenk HP, Hallam SJ, Hugenholtz P, Tringe SG, Woyke T (2016) High-resolution phylogenetic microbial community profiling. ISME J 10:2020–2032

So CM, Young LY (1999) Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl Environ Microbiol 65:2969–2976

So CM, Phelps CD, Young LY (2003) Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. Appl Environ Microbiol 69:3892–3900

Song B, Häggblom MM, Zhou J, Tiedje JM, Palleroni NJ (1999) Taxonomic characterization of denitrifying bacteria that degrade aromatic compounds and description of *Azoarcus toluvorans* sp. nov. and *Azoarcus toluclasticus* sp. nov. Int J Syst Bacteriol 49:1129–1140

Stagars MH, Ruff SE, Amann R, Knittel K (2016) High diversity of anaerobic alkane-degrading microbial communities in marine seep sediments based on (1-methylalkyl)succinate synthase genes. Front Microbiol 6:1511. https://doi.org/10.3389/fmicb.2015.01511

Stasik S, Wick LY, Wendt-Potthoff K (2015) Anaerobic BTEX degradation in oil sands tailings ponds: impact of labile organic carbon and sulfate-reducing bacteria. Chemosphere 138:133–139

Strąpoć D, Mastalerz M, Dawson K, Macalady JL, Callaghan AV, Wawrik B et al (2011) Biogeochemistry of microbial coal-bed methane. Annu Rev Earth Planet Sci 39:617–656

Tan B, Dong X, Sensen CW, Foght J (2013) Metagenomic analysis of an anaerobic alkane-degrading microbial culture: potential hydrocarbon-activating pathways and inferred roles of community members. Genome 56:599–611

Tan B, Nesbø C, Foght J (2014) Re-analysis of omics data indicates *Smithella* may degrade alkanes by addition to fumarate under methanogenic conditions. ISME J 8:2353–2356

Tan B, Fowler SJ, Abu Laban N, Dong X, Sensen CW, Foght J, Gieg LM (2015a) Comparative analysis of metagenomes from three methanogenic hydrocarbon-degrading enrichment cultures with 41 environmental samples. ISME J 9:2028–2045

Tan BF, Semple K, Foght J (2015b) Anaerobic alkane biodegradation by cultures enriched from oil sands tailings ponds involves multiple species capable of fumarate addition. FEMS Microbiol Ecol 91:fiv042

Techtmann SM, Hazen TC (2016) Metagenomic applications in environmental monitoring and bioremediation. J Ind Microbiol Biotechnol 43:1345–1354

Teske A, Callaghan AV, LaRowe DE (2014) Biosphere frontiers of subsurface life in the sedimented hydrothermal system of Guaymas Basin. Front Microbiol 5:362. https://doi.org/10.3389/fmicb.2014.00362

Teske A, de Beer D, McKay LJ, Tivey MK, Biddle JF, Hoer D, Lloyd KG, Lever MA, Røy H, Albert DB, Mendlovitz HP, MacGregor BJ (2016) The Guaymas Basin hiking guide to hydrothermal mounds, chimneys, and microbial mats: complex seafloor expressions of subsurface hydrothermal circulation. Front Microbiol 7:75. https://doi.org/10.3389/fmicb.2016.00075

Ulrich AC, Beller HR, Edwards EA (2005) Metabolites detected during biodegradation of $^{13}C_6$-benzene in nitrate-reducing and methanogenic enrichment cultures. Environ Sci Technol 39:6681–6691

Vergnoux A, Malleret L, Asia L, Doumenq P, Theraulaz F (2011) Impact of forest fires on PAH level and distribution in soils. Environ Res 111:193–198

von Netzer F, Pilloni G, Kleindienst S, Krüger M, Knittel K, Gründger F, Lueders T (2013) Enhanced gene detection assays for fumarate-adding enzymes allow uncovering of anaerobic hydrocarbon degraders in terrestrial and marine systems. Appl Environ Microbiol 79:543–552

von Netzer F, Kuntze K, Vogt C, Richnow HH, Boll M, Lueders T (2016) Functional gene markers for fumarate-adding and dearomatizing key enzymes in anaerobic aromatic hydrocarbon degradation in terrestrial environments. J Mol Microbiol Biotechnol 26:180–194

Wawrik B, Mendivelso M, Parisi VA, Suflita JM, Davidova IA, Marks CR, Van Nostrand JD, Liang Y, Zhou J, Huizinga BJ, Strąpoć D, Callaghan AV (2012) Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. FEMS Microbiol Ecol 81:26–42

Wawrik B, Marks CR, Davidova IA, McInerney MJ, Pruitt S, Duncan KE, Suflita JM, Callaghan AV (2016) Methanogenic paraffin degradation proceeds via alkane addition to fumarate by '*Smithella*' spp. mediated by a syntrophic coupling with hydrogenotrophic methanogens. Environ Microbiol 18:2604–2619

Weiss JV, Cozzarelli IM (2008) Biodegradation in contaminated aquifers: incorporating microbial/molecular methods. Ground Water 46:305–322

Widdel F, Musat F (2010) Diversity and common principles in enzymatic activation of hydrocarbons. In: Timmis K (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 983–1009

Widdel F, Knittel K, Galusko A (2010) Anaerobic hydrocarbon-degrading organisms: an overview. In: Timmis K (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 1997–2021

Williams RJ, Howe A, Hofmockel KS (2014) Demonstrating microbial co-occurrence pattern analyses within and between ecosystems. Front Microbiol 5:358. https://doi.org/10.3389/fmicb.2014.00358

Wilson SL, Li C, Ramos-Padrón E, Nesbø C, Soh J, Sensen CW, Voordouw G, Foght J, Gieg LM (2016) Oil sands tailings ponds harbour a small core prokaryotic microbiome and diverse accessory communities. J Biotechnol 235:187–196

Winderl C, Schaefer S, Lueders T (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bss*A) genes as a functional marker. Environ Microbiol 9:1035–1046

Winderl C, Anneser B, Griebler C, Meckenstock RU, Lueders T (2008) Depth-resolved quantification of anaerobic toluene degraders and aquifer microbial community patterns in distinct redox zones of a tar oil contaminant plume. Appl Environ Microbiol 74:792–801

Wöhlbrand L, Jacob JH, Kube M, Mussmann M, Jarling R, Beck A, Amann R, Wilkes H, Reinhardt R, Rabus R (2013) Complete genome, catabolic sub-proteomes and key metabolites of *Desulfobacula toluolica* Tol2, a marine, aromatic compound-degrading, sulfate-reducing bacterium. Environ Microbiol 15:1334–1355

Yang T, Speare K, McKay L, MacGregor BJ, Joye SB, Teske A (2016) Distinct bacterial communities in surficial seafloor sediments following the 2010 Deepwater Horizon blowout. Front Microbiol 7:1384. https://doi.org/10.3389/fmicb.2016.01384

Zedelius J, Rabus R, Grundmann O, Werner I, Brodkorb D, Schreiber F, Ehrenreich P, Behrends A, Wilkes H, Kube M, Reinhardt R, Widdel F (2011) Alkane degradation under anoxic conditions

by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. Environ Microbiol Rep 3:125–135

Zengler K, Richnow HH, Rosselló-Mora R, Michaelis W, Widdel F (1999) Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 6750:266–269

Zhang X, Young LY (1997) Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl Environ Microbiol 63:4759–4764

Zhang T, Bain TS, Nevin KP, Barlett MA, Lovley DR (2012) Anaerobic benzene oxidation by *Geobacter* species. Appl Environ Microbiol 78:8304–8310

Zhang T, Tremblay P-L, Chaurasia AK, Smith JA, Bain TS, Lovley DR (2013) Anaerobic benzene oxidation via phenol in *Geobacter metallireducens*. Appl Environ Microbiol 79:7800–7806

Zhou L, Li K-P, Mbadinga SM, Yang S-Z, Gu J-D, Mu B-Z (2012) Analyses of *n*-alkanes degrading community dynamics of a high-temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques. Exotoxicology 21:1680–1691

Genomic Responses of *Pseudomonas putida* to Aromatic Hydrocarbons 16

Víctor de Lorenzo and Hiren Joshi

Contents

1 Introduction 288
2 Aromatic Hydrocarbons: Nutrients or Stressors? 290
3 Membrane-Associated Functions 292
4 Effects on Metabolism of Aromatic Compounds 293
5 The Heat Shock Connection 293
6 Genomic Insights into the Control of Expression of the TOL Plasmid Aromatic Degradation Pathways 295
7 Revisiting the Regulation of the TOL System with Deep Transcriptomics 295
8 Metabolic Division of Labor? Stochastic Expression of the Catabolic Operons of TOL Plasmid 297
9 Outlook and Research Needs 299
References 300

Abstract

When *Pseudomonas putida* mt-2 cells face aromatic hydrocarbons, they must make a number of decisions that exemplify virtually every challenge that environmental bacteria need to overcome for thriving in polluted sites. It is thus no surprise that the genome-wide responses of *P. putida* to a variety conditions have been studied in considerable detail. One long-standing issue is how *P. putida* handles nutrient choice when a mixture of substrates coexist in the same site. A complex mechanism reminiscent of catabolite repression is in place to ensure that hydrocarbons are tackled only after virtually all other easy nutrients are

V. de Lorenzo (✉)
Systems Biology Program, Centro Nacional de Biotecnología-CSIC,
Madrid, Spain
e-mail: vdlorenzo@cnb.csic.es

H. Joshi
Bhabha Atomic Research Centre, Kalpakkam, India

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_25

depleted. Once this occurs, typical components of the lighter aromatic fractions of petroleum (e.g., toluene and xylenes) are not only potential nutrients but also strong stressors for *P. putida.* For handling this scenario, simultaneous processing of their presence both as an edible carbon source to benefit from and as a detrimental hassle to keep at bay becomes necessary. A second issue is the metabolic jam caused by the coincident presence in the same cells of competing pathways for *ortho*-cleavage and *meta*-cleavage of the intermediate catechols that degradation of aromatics must necessarily go through. Availability of a suite of genomic, proteomic and reporter technologies has shed light on these questions and exposed a number of surprising solutions that would otherwise look like intractable biochemical and regulatory problems. These include not only specific biochemical and transcriptional devices that overcome intracellular conflicts but also diversification of the population for acquiring different phenotypic roles.

1 Introduction

The genus *Pseudomonas* includes a large group of aerobic Gram-negative γ-proteobacteria that are remarkable for their metabolic versatility and their ability to colonize a very large variety of niches, from plant roots and leaves to soil and human lungs (Nikel et al. 2014a; Belda et al. 2016). Much of this versatility stems from a very robust central metabolic core that is characterized by the glycolytic Entner-Doudoroff (ED) pathway. Unlike the Embden-Meyerhof-Parnas biochemical route, the ED-based EDEMP cycle generates high levels or reducing currency that empowers *Pseudomonas putida* to tolerate higher doses of environmental stress and hosting redox reactions that would be too toxic for other species (Nikel et al. 2015). Furthermore, the frequent occurrence of membrane-bound extrusion pumps enables the genus to withstand many antibiotics and, by the same token, to endure exposure to organic solvents (dos Santos et al. 2004; Ramos et al. 2015). These properties shared by most members of the genus have diversified for the sake of adaptation to specific niches, one of which – soil polluted with aromatic hydrocarbons – is the focus of this Chapter. Different species and strains of *Pseudomonas* have been isolated from such sites because of their capacity to use as sole carbon and energy source compounds like toluene and xylenes. Out of them, the one specimen that has become the experimental model of choice for studying the molecular details of the interaction aromatic hydrocarbons-microbe is strain *P. putida* mt-2.

The origin and credentials and genomic features of this bacterium have been reviewed many times (Belda et al. 2016) and will not be repeated here. But some outstanding features need to be highlighted for the sake of this Chapter. First, *P. putida* mt-2 carries a large catabolic plasmid called pWW0 which endows cells (inter alia) with the ability to completely degrade toluene, *m*-xylene, *p*-xylene, and ethylbenzene (but not *o*-xylene). The process (sketched in Fig. 1) occurs through the action of the enzymes encoded in two biochemical blocks (the *upper* pathway and the *lower* or *meta* pathway) which first convert the head hydrocarbon to the corresponding carboxylic acids and then metabolize the resulting benzoic acid(s),

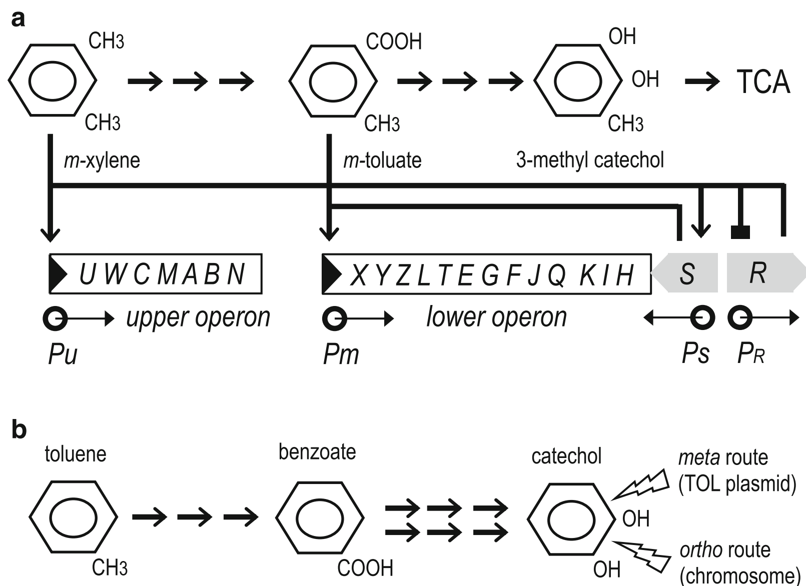


Fig. 1 The TOL regulatory and metabolic network for *m*-xylene and toluene biodegradation borne by the catabolic enzymes in the TOL plasmid. (**a**) Principal actors of the process. In the presence of *m*-xylene or toluene, XylR (expressed from *PR*) activates both the σ^{54}-dependent promoters *Pu* (which transcribes the *upper* pathway operon) and *Ps,* for expression of *xylS* Since the *PR* promoter overlaps the XylR binding sites of the divergent Ps promoter, XylR expression is negatively auto-regulated. In turn, the XylS protein activates *Pm,* the promoter of the *lower* operon. *PR, Ps, Pu* and *Pm* promoters are indicated in front of each cognate gene or operon. By default, XylS is expressed at a low level but its transcription is enhanced by XylR in the presence of *m*-xylene. TCA, tricarboxylic acid cycle. (**b**) benzoate produced by partial metabolism of toluene can be channelled through either the meta ring-cleavage pathway encoded by the *lower* TOL operon or through the *ortho* ring-fission route encoded in the host's chromosome (adapted from Kim et al. 2016)

all the way to pyruvate and acetaldehyde (Domínguez-Cuevas and Marqués 2017). This occurs through formation of an intermediary catechol that is cleaved in *meta* to generate a semialdehyde which is subsequently channeled to the downstream part of the *lower* pathway. Conspicuous features of this so-called TOL system include the intricacy of its genetic arrangement, its regulation, and its interplay with the host's biochemical and genomic networks. Each pathway has its own regulatory protein: the toluene/*m*-xylene responsive transcriptional factor (TF) XylR for the *upper* route and the benzoate/*m*-toluate-responsive TF called XylS for the *lower* part. But both of them are connected, as XylR also upregulates transcription of *xylS,* which is otherwise expressed at low constitutive levels. This is an important detail, as the promoter of the *lower* route (*Pm*) can be activated in two ways: either with a low level of XylS bound to benzoate/*m*-toluate (as it happens when one adds this intermediate effector externally to the medium) or by overproduced XylS without effector (for example when cells are exposed to toluene/*m*-xylene and thus XylR brings about a high level of XylS). Note that over time of exposure to the head

substrate of the TOL pathway, overproduction of XylS is concomitant with intracellular generation of benzoate/*m*-toluate from metabolization of toluene/*m*-xylene and therefore a strong signal amplification takes place that results in high-level transcription of the *lower* route (Domínguez-Cuevas and Marqués 2017).

The regulatory and biochemical scenario described above immediately raises a good number of questions. How do *Pseudomonas* handles toxicity and stress caused by aromatic hydrocarbons while at the same time metabolizes them as nutrients (Reva et al. 2006)? Why are the genes encoding the TOL pathway split in two parts; each with a different TF? Other routes of *Pseudomonas* for degradation or aromatic hydrocarbons (and most metabolic pathways) have just one operon and one regulator. Given that *P. putida* has a separate chromosomally encoded pathway for metabolism of benzoate through an *ortho*-cleavage track of the intermediate catechol, how can the two routes coexist in the same cells? Finally, what is the logic of having such an intricate regulatory network when the same effects could be achieved *prima facie* with a much simpler genetic device? In this article, we argue that these complexities are by no means casual, but they reflect aspects of the *P. putida* lifestyle in their natural niches that may not be evident by just growing the cells in a flask. As discussed below, the main tools for unveiling these qualities involve genomic, proteomic and single-cell reporter technologies which, taken together, expose an unexpected degree of sophistication of these bacteria for dealing with a large number of environmental challenges.

2 Aromatic Hydrocarbons: Nutrients or Stressors?

One of the most intriguing questions showcased by bacteria that thrive in polluted sites is how they manage to metabolize compounds that are inherently harmful to life (de Lorenzo and Loza-Tavera 2011). While some non-degradable pollutants (e.g., heavy metals) are dealt with through their inactivation or pumping out of the cells, potentially edible organic contaminants need to balance a suitable stress response to their detrimental effects with the action of cognate metabolic routes for their assimilation. The starting point is therefore that the same compound (e.g., toluene/*m*-xylene) is both a stressor and a nutrient, each aspect activating different genetic and physiological responses that may compete with each other. How does *P. putida* compute this two-sided signal and decides what to do? This question could start to be addressed only when genomic technologies became available for studying simultaneously the expression profiles of many genes. An early study of the question (Velazquez et al. 2005, 2006) was done with homemade DNA chips printed with a selection of the structural and regulatory DNA sequences of the TOL pathway together with selected descriptors of specific physiological conditions. *P. putida* bearing the TOL plasmid pWW0 was then exposed to *m*-xylene along with stress conditions at doses that did not cause growth effects. The insults included various oxygen tensions, temperatures and nitrogen sources as well as situations of DNA damage, oxidative stress, carbon and iron starvation, respiratory chain damage, and

contact with arsenic – each of them know to elicit a distinct physiological response. The effects of each of the stress classes were categorized in respect to the relative output of *xyl* transcripts. Some results of these experiments were somewhat expected. Given that the metabolic TOL program had to compete with the stress-enduring programs for gene expression resources (ribosomes, RNAP), most of the conditions downregulated the *m*-xylene biodegradation-related genes. Some others, however, appeared to stimulate expression of the TOL-encoded *xyl* genes instead. These included uncouplers of the respiratory chain (azide) and small doses of arsenate. But most important, replacement of $NH4^{+}$ by $NO3^{-}$ as N source increased expression of the TOL cistrons as well. This is of interest given that soils are often amended with fertilizers based on either ammonia or nitrates. To inspect whether such N-regulation observed in the test tube was propagated into actual catabolism of *m*-xylene under natural conditions, a test-tube-to-soil model system was developed which verified that that $NO3^{-}$ had a stimulating effect on *xyl* genes expression and *m*-xylene mineralization in microcosms as compared to $NH4^{+}$. This type of information will surely be useful for planning future bioremediation interventions. In a further screw turn, *P. putida* mt-2 cells were subject to the multiple abiotic stresses caused by exposure to crude tar from the 2002 oil spill of the Prestige tanker, which contained a complex mixture of hydrocarbons. In this case, the expression profile of *xyl* genes and stress-responding markers were followed over time. These results clearly suggested that adaptation to external insults precedes any significant expression of the catabolic genes (Velazquez et al. 2006). Limited as they are, these results provided two valuable pieces of information: [i] as expected, aromatic hydrocarbons are indeed sensed by *P. putida* mt-2 both as nutrients and stressors and there is competition between the physiological responses to each of them, and [ii], the way cells handle the challenge is by first adapting to the stress and then deploying the ability to metabolize the corresponding chemicals.

As the genomics technologies improved, the same question was revisited in more detail by using a genome-wide DNA chip (Dominguez-Cuevas et al. 2006; Yuste et al. 2006) rather than just a TOL chip and few indicator genes as before. This technique allowed visualization of the entire response of the hydrocarbon-exposed *P. putida* mt-2 strain to either the head substrates of the pathway and some of the metabolic intermediates. Furthermore, the same method revealed how the biodegradative pathway influenced the stress caused by the aromatic substrate. In this way, the interplay between the TOL route versus the background metabolism could be dissected and the activity of stress-responses versus substrate assimilation blown up with great accuracy. To this end, Dominguez-Cuevas et al. (2006) evaluated the genome-wide effects of three aromatic compounds chosen based on their known differential roles as inducers of the TOL degradation pathway. These included toluene (head substrate and primary inducer of the route), *m*-toluate (i.e., 3-methylbenzoate [3MBz] a metabolic intermediate and inducer of the *lower* pathway) and *o*-xylene (a non-metabolizable effector of XylR). Cultures of *P. putida* grown to exponential phase on succinate were exposed to these compounds and whole genome expression profiling was done using dedicated oligonucleotide-

based DNA microarray representing the entire genomic complement of the strain under study. Using as a reference >1.8-fold change in transcript levels, the results indicated that 180, 185, and 64 genes were upregulated in response to toluene, *o*-xylene, and 3MBz, respectively. Out of all these, 36 genes were over expressed in the presence all these aromatics, while 71, 67, and 11 ORFs were differentially transcribed. In contrast, the same compounds downregulated 127, 217, and 69 genes (only 18 common in all three conditions) while 130, 36, and 33 were inhibited in response to *o*-xylene, toluene, and 3MBz exclusively. These observations clearly indicated that there are substantial differential responses to the three aromatics in terms of up and down regulation of genes – and thus they are sensed and physiologically responded to separately. At the same time, the type of genes involved in such a response hint at the way *P. putida* detects these aromatics in the medium and reacts to their presence by eliciting specific response programs – both regarding metabolic and stress-management. The most conspicuous cases are discussed below.

3 Membrane-Associated Functions

The first biological material that aromatics interact with when added to *P. putida* cultures is the cell membrane. The bi-layered bacterial envelope is a barrier to be crossed for making it inside through more or less specific transport, and also a hydrophobic chemical milieu where aromatics easily dissolve – thereby influencing the membranes structure and functionality (Ramos et al. 2015). It thus comes as no surprise that the most conspicuous transcriptional responses to exposure to toluene, *o*-xylene, and 3MBz are detected in genes encoding envelope-related utilities. These include lipid metabolism, transport of nutrients and small molecules, flagellar machinery, and diverse types of pili. Not surprisingly, *o*-xylene had the maximum impact on the regulation of this class of genes, thereby suggesting that cells perceive this non-biodegradable molecule as more toxic than the others. Interestingly, the three compounds cause a complete loss of motility because of its inhibition of genes for assembly of the flagella. This could reflect a defensive strategy of *P. putida* through which a significant amount of ATP is saved by shutting down the high energy-consuming synthesis of the swimming apparatus (8% of total protein) and channeling it instead for dealing with the aromatics. In addition, there was down regulation of a gene associated with cell division (*parA*), while functions associated with efflux pumps become up regulated. The same aromatics also exerted indirect effects on cells because of their harm to membranes. The prime consequence is the down regulation of genes associated with the electron transport chain, concomitantly with an increase in transcription of glutathione and glutathione S-transferases. These observations surely reflect that perturbing membrane composition generates active oxygen species (and ensuing oxidative stress) that need to be counterbalance with production of reductive metabolic currencies.

4 Effects on Metabolism of Aromatic Compounds

Not surprisingly, upon exposure to different aromatic compounds, cells upregulate expression of genes associated with their cognate utilization pathways in a fashion in which the precise metabolic operon(s) and the extent of their transcription varies depending on the specific substrate. In *P. putida* cells bearing the TOL plasmid, toluene, *m*-xylene or *o*-xylene each induces expression of both the *upper* pathway and the *lower* pathway promoters because of the regulatory architecture that rules the corresponding metabolic system (Fig. 1). Benzoate and 3MBz originating from degradation of toluene and *m*-xylene, respectively, further exacerbate expression of the *lower* TOL pathway because of their action on XylS. If one adds directly 3MBz to the medium, the *lower* operon becomes transcribed only to an extent because it lacks the XylS overproduction effect triggered by XylR on *Ps* (Fig. 1). By the same token, the non-metabolizable XylR inducer *o*-xylene brings about only a partial activation of the *lower* pathway because the lack of production of the intermediate 3MBz misses the amplifying cascade effect of having a XylS inducer generated intracellularly. Note that the same cells also carry a chromosomally-encoded *ortho*-cleavage pathway for benzoate degradation with a leading dioxygenase that acts both on benzoate and 3MBz (Fig. 2). This creates a metabolic conflict scenario involving both a competition between enzymes for the same substrate, and also generation of toxic dead-end products that cells need to handle (see below). In any case, exposure to TOL substrates also resulted in down-regulation of genes encoding enzymes of the TCA cycle, perhaps as a way to tune the downstream carbon utilization pathways to the much slower upstream metabolism of aromatics.

5 The Heat Shock Connection

Regardless of the type of transcriptomic technology adopted, one of the most noticeable effects of having cells of *P. putida* exposed to aromatic hydrocarbons is the upregulation of the heat-shock (HS) response, in particular many characteristic genes encoding HS proteins. Their identity and relative expression levels do change, however, depending on the exact compound and its concentration in the medium. The non-substrate *o*-xylene was systematically a better inducer of the HS response as compared to the others. This is likely to be related to the fact that nonmetabolizable aromatics accumulate in the cell envelope because there is no way to get rid of them other than by membrane-associated pumps. In contrast, and depending on the dose, metabolizable hydrocarbons can be drained from the inner membrane and channeled towards the corresponding enzymatic devices for their degradation. Not surprisingly, the weakest inducer of the HS response was 3MBz, as it is more polar and thus less prone to cause membrane damage. It is intriguing that the peak of HS caused by aromatic hydrocarbons coincides with a significant induction of genes associated with amino acid biosynthesis. This may expose a requirement of new proteins

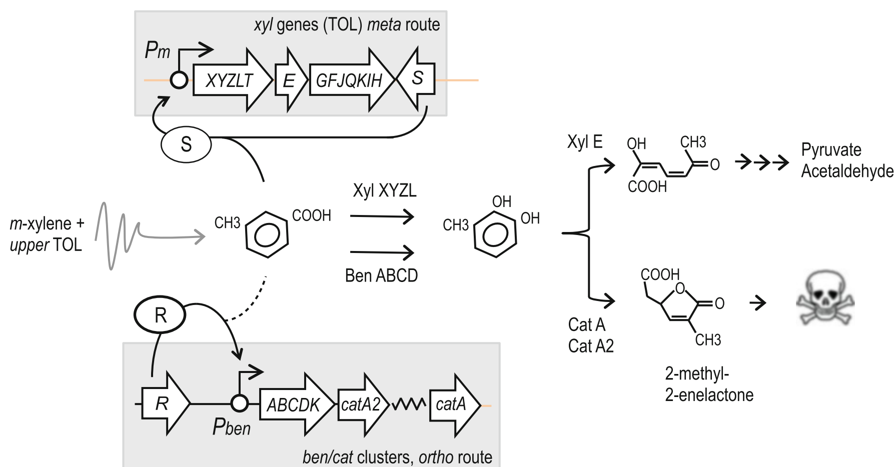


Fig. 2 Genetic, regulatory and biochemical connections of 3-methylbenzoate (3MBz) catabolism in *P. putida* mt-2. 3MBz can be available either exogenously or generated intracellularly through biodegradation of *m*-xylene by the *upper* TOL pathway. The figure sketches both alternative metabolic itineraries involved in the further biodegradation of 3MBz, i.e., the *xyl* genes of the *lower* TOL operon (top), which are expressed from the *Pm* promoter activated by XylS, and the chromosomal ben genes (bottom) that are transcribed from promoter *Pben* upon activation by the XylS homologue BenR. Both the *xyl* and the *ben* routes make 3MBz to converge towards methylcatechol, but then diverge at that point: this compound can be subject to extradiol cleavage by XylE or intradiol fission by CatA and CatA2. The action of the chromosomally encoded benzoate 1,2-dioxygenase on 3-methylbenzoate may produce also a small fraction of 4-methylcatechol, which becomes efficiently converted by CatA/CatA2 into the equally toxic compound 4-methyl-2-enelactone (adapted from Perez-Pantoja et al. 2015)

for mounting the stress related response and – in the case of metabolizable substrates – utilization of the aromatics as carbon sources.

The interplay between HS and degradation of aromatics by the TOL system may involve more than just competition for cellular resources to run either a stress-response program or a metabolic program. There are also imprinted mechanistic details of the regulatory network that controls expression of either system. When cells growing on succinate are exposed to *m*-xylene, it takes as little as 15 min to detect full expression of the *upper* pathway, while it takes 3 h to see a full-fledged *meta* route. However, *xylS* expression is observed also after 15 min of *m*-xylene addition – along with expression of *upper* pathway genes. What is the logic of this apparently incongruous scenario? One intriguing link between aromatic biodegradation and heat shock is that XylS activates works in concert with variants of the RNAP that contain either the stationary phase and general stress response sigma factor σ^S or the HS-specific σ^H, which is by default in charge of expressing functions for coping with heat stress (Marques et al. 1999; Dominguez-Cuevas et al. 2005). At the same time, artificially induced HS conditions (e.g., growth at 42 °C) clearly indicated that heat downregulates both the *upper* and the *meta* pathway. This state of affairs is prone to generate a considerable stochasticity in expression of each of

the pathways, as has indeed been proven experimentally. In addition, the connection of *meta* pathway expression to HS is reflected in other phenomena. For example, the level of expression of the *meta* pathway is different depending on whether the head substrate elicits better or worse HS. Addition of 3-methyl benzyl alcohol bypasses the energy-consuming mono-oxygenase step and thereby accelerates accumulation of 3MBz in a shorter period of time. This results in faster transcription kinetics of the *lower* TOL pathway but – unexpectedly – in inferior expression levels as compared to *m*-xylene. This is because the latter is a better trigger of the HS response and thus of higher levels of σ^H available for transcribing the *lower* operon. Interestingly, direct induction of the pathway upon addition of 3MBz to the medium results in a moderate upregulation of the *meta* pathway compared to *m*-xylene. Although this is due in part to the lack of the cascading effect of XylR on XylS that control expression of the TOL system, one primary reason for this modest induction is that 3MBz is a poor inducer of HS proteins and therefore σ^H is likely to be in short supply. Finally, a yet unexplained phenomenon also related to differential expression of TOL genes is the divergent effect of bona fide substrates like *m*-xylene versus gratuitous, non-metabolizable XylR effectors such as o-xylene. While both of them bring about an approximately similar HS level and activate transcription from the *Pu* promoter equally well, mRNAs become terminated earlier when *o*-xylene is used. While this makes perfect sense from the point of view of the cell's economy, the mechanisms behind the phenomenon remain obscure.

6 Genomic Insights into the Control of Expression of the TOL Plasmid Aromatic Degradation Pathways

Besides the features of the regulation of the TOL catabolic operons discussed thus far and reviewed by Domínguez-Cuevas and Marqués (2017), the paragraphs below expose some new, emerging qualities of the system. They were uncovered by genomic analyses that contribute to highlight the profile of this experimental system as the paradigm of optimal catabolic device in environmental bacteria.

7 Revisiting the Regulation of the TOL System with Deep Transcriptomics

The insights on the responses of *P. putida* to aromatic hydrocarbons addressed above were largely generated with gene fusion and reporter technology and somewhat simple DNA arrays. In 2016, the first article (Kim et al. 2016) was published revisiting the same questions but using two far more powerful methods: DNA tiling arrays and deep RNA sequencing. The combination of the two afforded an unprecedented level of resolution of the *m*-xylene/toluene biodegradation sub-transcriptome. This allowed the dissection of operon structures and also monitoring of the interplay between plasmid and chromosomal functions. These new methodological approaches exposed new characteristics of the system that could not be

grasped by traditional genetics or more primitive genomic technologies. The highlights included the finding that a significant level of basal expression of the *lower* TOL pathway does occur in the absence of inducers. In contrast, virtually no expression of the *upper* route occurred. Furthermore, the overlapping transcription between the 3′ ends of the convergent *xylS* and *xylH* mRNAs did not have regulatory consequences. No role for small RNAs could be identified either. However, the most surprising finding was that benzoate generated from toluene catabolism by the *upper* pathway failed to induce expression of the *ortho* cleavage genes for the same compound encoded in the chromosome. How this can happen mechanistically is uncertain but suggests a degree of channeling that avoids misrouting of the benzoate produced by the *upper* TOL pathway to the *ortho* route – an issue that has been separately examined (Perez-Pantoja et al. 2015).

In *P. putida* mt-2, the regulation of Bz and 3MBz degradation routes relies on transcriptional regulators belonging to AraC-family; the so-called XylS and BenR activators. The pWW0-encoded XylS regulator mediates transcriptional activation of the *Pm* promoter driving the expression of the *meta* pathway in response to 3MBz and Bz as inducers (Fig. 2). Likewise, BenR is able to trigger the activity of *Pben* promoter by recognition of Bz as effector, allowing the expression of the chromosomal *benABCD-catA2* operon. Interestingly, BenR-activation of the *Pm* promoter of the *meta* TOL pathway in response to Bz has been recognized. The opposite may be true as well, and XylS could activate the chromosomal *Pben* promoter. If that were the case, the question is how the corresponding parameters are set to avoid the predicted scenario of metabolic competition between the TOL-encoded enzymes and the genome-encoded counterparts. By adopting a suite of supersensitive promoter fusions to a *luxCDABE* reporter, for transcriptomic analyses of *benA* and *xylX* under diverse conditions and growth tests of strains bearing different regulatory combinations, Perez-Pantoja et al. (2015) showed that expression ranges of XylS under various conditions are insufficient to cause a significant cross-regulation of *Pben* whether cells face endogenous or exogenous aromatics. This seems to stem from the nature of the operators for binding either transcriptional factor, which in the case of the chromosomal *Pben* promoter of *P. putida* mt-2 appear to have evolved for avoiding a strong interaction with XylS. It thus seems that rather than physical channeling, some metabolic clashes are avoided through a mere adjustment of the regulatory parameters that control the interplay between otherwise conflicting metabolic devices.

But this is not the only metabolic problem between the *meta*-cleavage pathway borne by the TOL plasmid and the *ortho*-cleavage route encoded in the chromosome of *P. putida* mt-2. It turns out that this strain has a second chromosomal copy of the 1,2-catechol dioxygenase *catA* gene (named *catA2*) located downstream of the *ben* operon that encodes an additional enzyme with the same activity but different parameters. What is the role of such an enzyme? What is it needed for? (Jimenez et al. 2014) showed the key function of CatA2 as a sort of metabolic *safety valve* for excess catechol when cells are exposed to benzoate and the substrate can be simultaneously processed by the TOL system and the chromosomal *ben/cat* pathways. In this situation, CatA2 activity alleviates the metabolic conflict generated

by simultaneous expression of the *meta* and *ortho* pathways*,* thereby facilitating their coexistence. The high level of paralogy found in the genome of *P. putida* is intriguing, as sequence redundancy is a source of genetic instability. But at least in the case of the two functional *catA* genes just mentioned, it seems that their retention has to do with solving regulatory problems. In reality, counteracting accumulation of toxic intermediates must be a general principle of metabolic evolution (de Lorenzo et al. 2015) which, in this case, has been solved by stitching in an extra gene.

Finally, one derivative of the complex interplay between the meta and the ortho pathways in *P. putida* mt-2 is the possibility of altering the effector specificity of the cognate *Pm* and *Pben* promoters not by mutating the corresponding transcriptional factors, but by modifying their target *cis*-regulatory elements. How can this be achieved? As mentioned above, although BenR and XylR belong to the same AraC family of activators, there is no appreciable cross activation of the Ben pathway by XylS bound to 3MBz. However, *Pben* promoter variants engineered to have and additional TF binding motif upstream of the promoter region significantly enhances the sensitivity of BenR towards 3MBz, which leads to stronger activation of *ben* pathway by this effector compared to wild type sequences (Silva-Rocha and de Lorenzo 2012a). This showed that one can change the specificity of inducer recognition without mutation in TF and by just altering (addition/deletion) of TF binding sites to the corresponding promoters. This is reminiscent of cases where artificially changing the architecture and connectivity of the parts of a regulatory device one can amplify an otherwise marginal responsiveness to a suboptimal inducer (de Las Heras et al. 2012). Having so many plastic regulatory parts at hand, it comes as no surprise that even the most intricate metabolic conflicts between different pathways have been so efficiently solved in a relatively short evolutionary time (Fig. 3).

8 Metabolic Division of Labor? Stochastic Expression of the Catabolic Operons of TOL Plasmid

The basic regulatory blueprint of the TOL system and the very low levels of the principal regulator of the system, XylR, create a scenario prone to strong stochastic effects. When expression dynamics of the catabolic promoters borne by pWW0 was examined in single cells exposed to *m*-xylene, some unexpected results became apparent. While expression of *xylR* behaved in a unimodal fashion, the activation of *Pu* and *Pm* displayed a high degree of bi-modality which resulted in a time-dependent shift in the bacterial population between two induction states (Silva-Rocha and de Lorenzo 2012b). The most striking feature of this stocasticity was observed with activation of the *meta* pathway by XylS. It was demonstrated that 3MBz-bound XylS (XylSa) has a very minor role in activation of the route while it is largely governed by effector-free XylS overproduced through the action of XylR on *Ps.* In addition, when induced with 3MBz alone, much of the population remains silent and does not express the *lower* pathway. This observation clearly indicates alternate induction mechanism of *meta*-pathway by XylS and XylSa (i.e., bound to

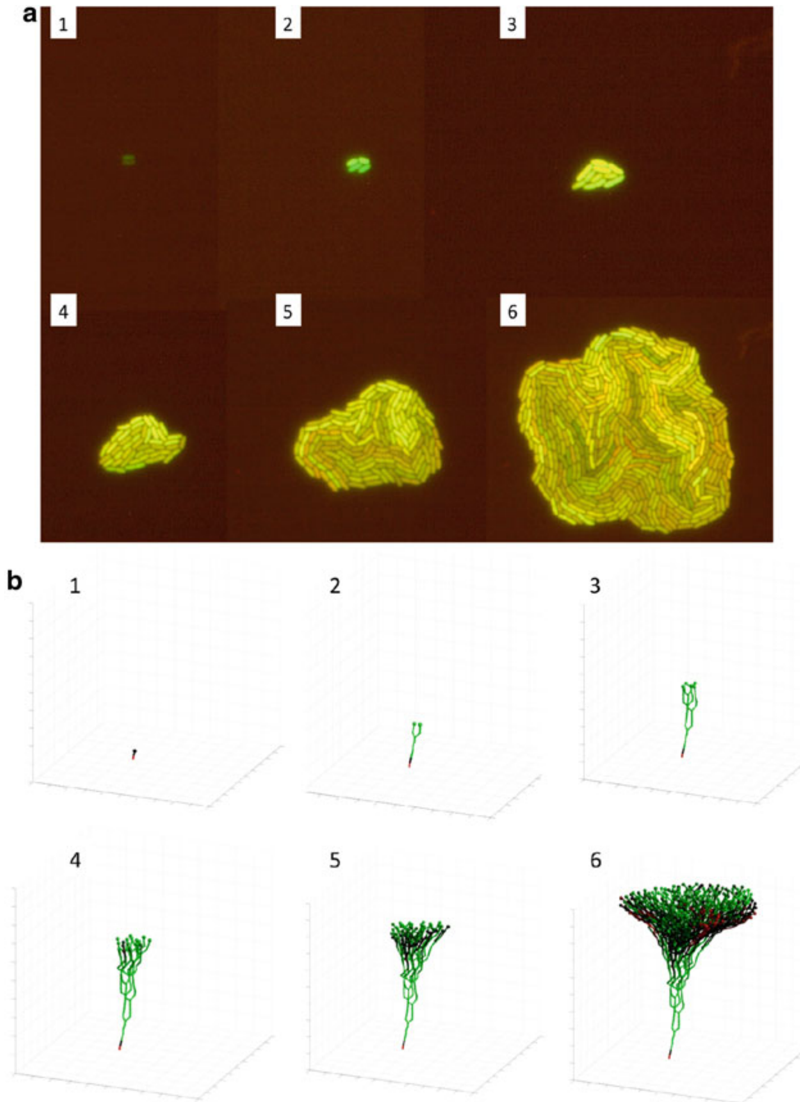


Fig. 3 Stochastic induction of TOL promoters of *P. putida* mt-2 upon exposure to *m*-xylene. Single cells of a strain derivative bearing genomic fusions of the *Pu* promoter to GFP and *Pm* promoter to mCherry reporters were laid on agar surface of an epifluorescence microscope slide and exposed to *m*-xylene vapours. Green and red fluorescence was recorded as cells divided with time. (**a**) direct green an red fluorescence images. (**b**) fate of single cells (example). Note how the population splits in individual cells of different colours along time. Lineage trees – kindly provided by Bastian Voegeli, Simon van Vliet, Colette Bigosch and Martin Ackermann – were generated as described in van Vliet et al. (2018)

3MBz). Yet, higher levels of induction of *meta*-pathway in presence of *m*-xylene corroborates previous observations of synergistic effect of both XylS and XylSa on activation of the *meta*-pathway. These stochastic phenomena disappeared in the

stationary phase. This suggested that when cells face a mixture of succinate and *m*-xylene, only part of the bacteria is actively engaged with degrading *m*-xylene while the rest instead grow on succinate (Silva-Rocha and de Lorenzo 2012b). Such a metabolic bifurcation of the population ends when cells run out of succinate and have only *m*-xylene during the stationary phase. This phenomenon could be a case of *metabolic bet hedging*, meaning that a genetically homogeneous population splits into different metabolic regimes when bacteria confront a mixture of nutrients.

It is likely that such a metabolic specialization is not just a fortuitous occurrence but is mainly an intrinsic feature of the regulatory circuit that has been duly selected as an advantageous trait (Nikel et al. 2014b). Interestingly, when compared to *Pu* and *Pm*-mediated activation of the plasmid-encoded TOL pathways, the trigger of the *Pben*-related chromosomal counterpart (the *ortho* pathway) in a plasmid-free strain follows a unimodal activation (Silva-Rocha and de Lorenzo 2014). The situation is, however, changed when cells acquire the TOL plasmid, as then the activation becomes bimodal. A similar transition was observed with the *cat* pathway for catechol metabolism, which acquires a stochastic expression regime in the presence of the TOL plasmid. This observation demonstrates how recruitment of a new metabolic pathway influences the expression of genes present on the genome and provides some insights on evolution of metabolic pathways.

9 Outlook and Research Needs

The onset of genomic technologies has allowed revisiting of the responses of *Pseudomonas putida* to aromatic hydrocarbons, with a focus on the interplay between the TOL-plasmid bearing strain *P. putida* mt-2 and *m*-xylene. In general, the data confirms the regulatory schemes already derived from genetic experiments, but they also enable a view of global responses to the hydrocarbon and also expose the evolutionary solutions found to what would otherwise be intricate biochemical conflicts. This allows us to put in perspective the apparently exaggerated over-control of the TOL system, with two operons, five promoters, two plasmid-encoded transcription factors, and a large number of host's regulatory components. We believe that the complex regulatory architectures that we often find in environmental biodegradative systems have been shaped by prior biochemical, populational, and community conflicts. Microbes must solve simultaneously many issues, and the architecture of genetic circuits surely encodes a record of *historical* bottlenecks (e.g., biochemical chaos) as well as the evolutionary novelty that has solved them. This resembles what in some proteins has been called *moonlighting*, i.e., the property of a single polypeptide to hold entirely different functions in the same structure (Silva-Rocha et al. 2013). The data obtained with the TOL plasmid seem to show that one regulatory scheme can solve a considerable number of conflicts at the level of single cells, populations, and even multi-strain communities. Therefore it could well happen that regulatory devices encode both the traffic lights for physiological and biochemical issues along with a code of social conduct of environmental bacteria. This is an issue that certainly deserves future investigations.

Acknowledgments Authors are indebeted to Bastian Voegeli, Simon van Vliet, Colette Bigosch, and Martin Ackermann (EAWAG, Switzerland) for sharing the data shown in Fig. 3. Pablo Nikel is gratefully acknowledged for reading and commenting the manuscript. The work in VdL's Laboratory was funded by the HELIOS Project of the Spanish Ministry of Science BIO 2015-66960-C3-2-R (MINECO/FEDER), the ARISYS (ERC-2012-ADG-322797), EmPowerPutida (EU-H2020-BIOTEC-2014-2015-6335536) and MADONNA (H2020-FET-OPEN-RIA-2017-1-766975) Contracts of the European Union, and the InGEMICS-CM (B2017/BMD-3691) contract of the Comunidad de Madrid (FSE, FECER). Authors declare no conflict of interest.

References

Belda E, van Heck RG, Jose Lopez-Sanchez M, Cruveiller S, Barbe V, Fraser C et al (2016) The revisited genome of *Pseudomonas putida* KT2440 enlightens its value as a robust metabolic chassis. Environ Microbiol 18:3403–3424

de Las Heras A, Fraile S, de Lorenzo V (2012) Increasing signal specificity of the TOL network of *Pseudomonas putida* mt-2 by rewiring the connectivity of the master regulator XylR. PLoS Genet 8:e1002963

de Lorenzo V, Loza-Tavera H (2011) Microbial bioremediation of chemical pollutants: how bacteria cope with multi-stress environmental scenarios. In: Bacterial stress responses, 2nd edn. American Society of Microbiology, Washington, DC, pp 481–492

de Lorenzo V, Sekowska A, Danchin A (2015) Chemical reactivity drives spatiotemporal organisation of bacterial metabolism. FEMS Microbiol Rev 39:96–119

Domínguez-Cuevas P, Marqués S (2017) Current view of the mechanisms controlling the transcription of the TOL plasmid aromatic degradation pathways. In: Rojo F (ed) Aerobic utilization of hydrocarbons, oils and lipids. Springer International Publishing, Cham, pp 1–22

Dominguez-Cuevas P, Marin P, Ramos JL, Marques S (2005) RNA polymerase holoenzymes can share a single transcription start site for the pm promoter. Critical nucleotides in the −7 to −18 region are needed to select between RNA polymerase with σ^{38} or σ^{32}. J Biol Chem 280:41315–41323

Dominguez-Cuevas P, Gonzalez-Pastor JE, Marques S, Ramos JL, de Lorenzo V (2006) Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. J Biol Chem 281:11981–11991

dos Santos VA, Heim S, Moore ER, Stratz M, Timmis KN (2004) Insights into the genomic basis of niche specificity of *Pseudomonas putida* KT2440. Environ Microbiol 6:1264–1286

Jimenez JI, Perez-Pantoja D, Chavarria M, Diaz E, de Lorenzo V (2014) A second chromosomal copy of the *catA* gene endows *Pseudomonas putida* mt-2 with an enzymatic safety valve for excess of catechol. Environ Microbiol 16:1767–1778

Kim J, Perez-Pantoja D, Silva-Rocha R, Oliveros JC, de Lorenzo V (2016) High-resolution analysis of the *m*-xylene/toluene biodegradation subtranscriptome of *Pseudomonas putida* mt-2. Environ Microbiol 18:3327–3341

Marques S, Manzanera M, Gonzalez-Perez MM, Gallegos MT, Ramos JL (1999) The XylS-dependent *Pm* promoter is transcribed in vivo by RNA polymerase with sigma 32 or sigma 38 depending on the growth phase. Mol Microbiol 31:1105–1113

Nikel PI, Martinez-Garcia E, de Lorenzo V (2014a) Biotechnological domestication of pseudomonads using synthetic biology. Nat Rev Microbiol 12:368–379

Nikel PI, Silva-Rocha R, Benedetti I, de Lorenzo V (2014b) The private life of environmental bacteria: pollutant biodegradation at the single cell level. Environ Microbiol 16:628–642

Nikel PI, Chavarria M, Fuhrer T, Sauer U, de Lorenzo V (2015) *Pseudomonas putida* KT2440 strain metabolizes glucose through a cycle formed by enzymes of the Entner-Doudoroff, Embden-Meyerhof-Parnas, and pentose phosphate pathways. J Biol Chem 290:25920–25932

Perez-Pantoja D, Kim J, Silva-Rocha R, de Lorenzo V (2015) The differential response of the *Pben* promoter of *Pseudomonas putida* mt-2 to BenR and XylS prevents metabolic conflicts in m-xylene biodegradation. Environ Microbiol 17:64–75

Ramos JL, Sol Cuenca M, Molina-Santiago C, Segura A, Duque E, Gomez-Garcia MR et al (2015) Mechanisms of solvent resistance mediated by interplay of cellular factors in *Pseudomonas putida*. FEMS Microbiol Rev 39:555–566

Reva ON, Weinel C, Weinel M, Bohm K, Stjepandic D, Hoheisel JD, Tummler B (2006) Functional genomics of stress response in *Pseudomonas putida* KT2440. J Bacteriol 188:4079–4092

Silva-Rocha R, de Lorenzo V (2012a) Broadening the signal specificity of prokaryotic promoters by modifying *cis*-regulatory elements associated with a single transcription factor. Mol BioSyst 8:1950–1957

Silva-Rocha R, de Lorenzo V (2012b) Stochasticity of TOL plasmid catabolic promoters sets a bimodal expression regime in *Pseudomonas putida* mt-2 exposed to m-xylene. Mol Microbiol 86:199–211

Silva-Rocha R, de Lorenzo V (2014) The pWW0 plasmid imposes a stochastic expression regime to the chromosomal *ortho* pathway for benzoate metabolism in *Pseudomonas putida*. FEMS Microbiol Lett 356:176–183

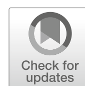
Silva-Rocha R, Perez-Pantoja D, de Lorenzo V (2013) Decoding the genetic networks of environmental bacteria: regulatory moonlighting of the TOL system of *Pseudomonas putida* mt-2. ISME J 7:229–232

van Vliet S, Dal Co A, Winkler AR, Spriewald S, Stecher B, Ackermann M (2018) Spatially correlated gene expression in bacterial groups: the role of lineage history, spatial gradients, and cell-cell interactions. Cell Syst 6:496–507.e6

Velazquez F, Parro V, de Lorenzo V (2005) Inferring the genetic network of *m*-xylene metabolism through expression profiling of the *xyl* genes of *Pseudomonas putida* mt-2. Mol Microbiol 57:1557–1569

Velazquez F, de Lorenzo V, Valls M (2006) The m-xylene biodegradation capacity of *Pseudomonas putida* mt-2 is submitted to adaptation to abiotic stresses: evidence from expression profiling of *xyl* genes. Environ Microbiol 8:591–602

Yuste L, Hervas AB, Canosa I, Tobes R, Jimenez JI, Nogales J et al (2006) Growth phase-dependent expression of the *Pseudomonas putida* KT2440 transcriptional machinery analysed with a genome-wide DNA microarray. Environ Microbiol 8:165–177



Biodegradation of Ether Pollutants

17

Amie McElroy and Michael Hyman

Contents

1 Introduction 304
2 Alkyl Ethers 305
  2.1 DME Metabolism 305
  2.2 DME Cometabolism 307
  2.3 Metabolism of Other Alkyl Ethers 307
  2.4 Cometabolism of Other Alkyl Ethers 308
3 Chloroalkyl Ethers 308
  3.1 BCEE Metabolism 309
  3.2 BCEE Cometabolism 310
  3.3 Metabolism of Other Chloroalkyl Ethers 310
  3.4 Cometabolism of Other Chloroalkyl Ethers 311
4 Branched Alkyl Ethers 312
  4.1 MTBE Metabolism 312
  4.2 MTBE Cometabolism 315
  4.3 Metabolism of Other Branched Alkyl Ethers 317
  4.4 Cometabolism of Other Branched Alkyl Ethers 318
5 Cyclic Ethers 319
  5.1 14D Metabolism 319
  5.2 14D Cometabolism 321
  5.3 Metabolism of Other Cyclic Ethers 323
  5.4 Cometabolism of Other Cyclic Ethers 323
6 Future Research 324
References 324

A. McElroy
Department of Civil, Construction, and Environmental Engineering, North Carolina State University, Raleigh, NC, USA
e-mail: acmcelro@ncsu.edu

M. Hyman (✉)
Department of Plant and Microbial Biology, North Carolina State University, Raleigh, NC, USA
e-mail: mrhyman@ncsu.edu

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_27

Abstract
Ether-bonded compounds are often poorly biodegradable and can become important environmental pollutants. In this chapter, we summarize the microorganisms and key enzymes associated with the aerobic biodegradation of some of the most environmentally significant alkyl, chlorinated, branched, and cyclic ethers. Particular emphasis is placed on representative compounds from each of these groups including dimethyl ether (DME), bis(2-chloroethyl) ether (BCEE), methyl *tertiary* butyl ether (MTBE), and 1,4-dioxane (14D). The chapter emphasizes differences between growth-related and cometabolic ether biodegradation and the important roles that diverse oxygenase enzymes play in aerobic ether biodegradation.

1 Introduction

Ethers are defined as organic chemicals that contain an oxygen atom bonded to two alkyl or aryl groups (R–O–R′). Ethers are relatively stable and tend not to react with acids, bases, metals, or oxidizing/reducing agents although some classes of ethers (e.g., chloroalkyl ethers) can hydrolyze rapidly. Ethers lack strongly polarized O–H bonds and therefore do not hydrogen bond with themselves. Consequently, they tend to be volatile and have low boiling points. Conversely, the ether O atom possesses two lone electron pairs that enable ethers to form hydrogen bonds with water. This property makes smaller ethers highly water soluble. These lone electron pairs also make ethers good solvents for organic compounds. In combination, these properties make ethers a versatile class of organic compounds that are used in a wide variety of industrial processes.

In addition to their chemical stability, it has long been recognized that ether-bonded compounds are also often resistant to biodegradation and can therefore become recalcitrant environmental pollutants (White et al. 1996). From a historical perspective, the different groups of ethers examined in this chapter have been the focus of sequential waves of research as examples of these compounds either have posed important physiological and biochemical questions or have risen at different times to prominence as important pollutants. For example, this chapter starts with a discussion of the degradation of the simplest alkyl ether, dimethyl ether (DME). The role of DME as a potential growth-supporting substrate for aerobic methane-oxidizing bacteria and as a potential intermediate in the methane-oxidation pathway perplexed researchers for several years in the 1970s. Early studies of DME biodegradation therefore played a role in developing our understanding of both the basic physiology and enzymology of methane-oxidizing bacteria. These studies also helped highlight that some organics can act as growth-supporting substrates for microorganisms while others are only susceptible to fortuitous cometabolic degradation processes. This issue of growth-related (metabolic) and non-growth related (cometabolic) degradation is reiterated throughout this chapter. Studies with DME also highlighted the important role of nonspecific oxygenase enzymes

in aerobic ether degradation processes. This theme is also continued throughout this chapter.

Later sections of the chapter have focused on compounds such as methyl *tertiary* butyl ether (MTBE) and 1,4-dioxane (14D). At the turn of the millennium, MTBE became one of the most highly produced industrial chemicals due to its global use as a gasoline oxygenate. Biodegradation of this compound is particularly challenging as it contains both an ether linkage and a branched structure. Aerobic biodegradation processes for this compound subsequently became important as they offered inexpensive and reliable remediation approach for this compound in gasoline-impacted groundwater. Most recently, much effort has focused on understanding the aerobic biodegradation of 14D. As the most recent area of aerobic ether biodegradation research, our understanding of aerobic 14D-degrading microorganisms has been strongly impacted by modern "omic" approaches. An important driver of much of the research with compounds such as 14D is the identification of microbial processes that can be used to control and remediate environmental contamination by these chemicals. Like MTBE which has taste and odor thresholds in the low μg L^{-1} range (ITRC 2005), the challenge with aerobic 14D biodegradation is that biological systems need to be effective in the low to sub μg L^{-1} range to decrease the threat of this compound to human health. As growth-related microbial metabolism of ethers such as MTBE and 14D is challenging at these low concentrations, our understanding of cometabolic systems is particularly important for the development of effective biological treatment systems.

2 Alkyl Ethers

As indicated in the Introduction, ethers have many properties that make then useful as aprotic solvents. Alkyl ethers, and particularly diethyl ether (DEE), are therefore common reagents in organic chemistry. The simplest dialkyl ether, DME, is used as an aerosol propellant, a solvent, a fuel additive, a refrigerant, and as a substitute for liquefied petroleum gas (Semelsberger et al. 2006). DME is also a potential replacement diesel fuel as it has a high cetane number (55), generates low amounts of particulates, NO_x and CO, and can be used with only minor modifications to existing diesel engines (Semelsberger et al. 2006). DME is water-soluble (~46 g/L [~1 M]) (Hine and Mookerjee 1975) but does not hydrolyze under normal environmental conditions.

2.1 DME Metabolism

Historically, studies of the aerobic microbial metabolism of DME focused on methane-oxidizing bacteria (methanotrophs). The pathway of aerobic methane oxidation is now known to involve the initial hydroxylation of methane to methanol by one of two forms of methane monooxygenase (MMO). Methanol is then

sequentially oxidized to formaldehyde, formate, and CO_2 (Fig. 1). Both the particulate (pMMO) and soluble (sMMO) forms of MMO are nonspecific and can oxidize a wide variety of compounds that do not support the growth of methanotrophs (Higgins et al. 1979; Colby et al. 1977). In some of the earliest studies of methanotrophs, it was reported that some strains could grow on DME (Wilkinson 1971), and a pathway for methane oxidation in which DME was an integral intermediate was proposed (Wilkinson 1975). Degradation of DME without a direct role in methane oxidation was subsequently suggested to involve an initial hydroxylation of DME to form a hemiacetal (methoxymethanol) (Stirling and Dalton 1980). This hemiacetal could then either be oxidized to methylformate by alcohol dehydrogenases or could abiotically decompose to methanol and formaldehyde. Both abiotic and enzyme-catalyzed hydrolysis of methylformate would also be expected to generate methanol and formate (Fig. 2).

Although effects of DME on methanotrophs were investigated by others (Hazeu 1975; Ribbons 1975; Patel et al. 1976), the confusion surrounding actual growth of methanotrophs on DME and its role as an MMO substrate was only resolved when it was recognized that commercial sources of DME typically contain significant amounts of impurities (Stirling and Dalton 1980; Meyers 1982) including both methane (De Bont and Mulder 1974) and methanol (Stirling and Dalton 1980). With the development of purified sMMO preparations, it became clear that DME is an sMMO substrate that is oxidized to methanol and formaldehyde (Colby et al. 1977; Stirling and Dalton 1980). It was also established that methylformate but not DME can support the growth

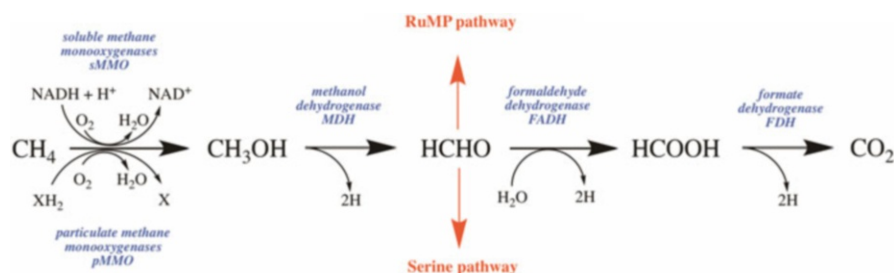


Fig. 1 Pathway of methane oxidation in methanotrophs

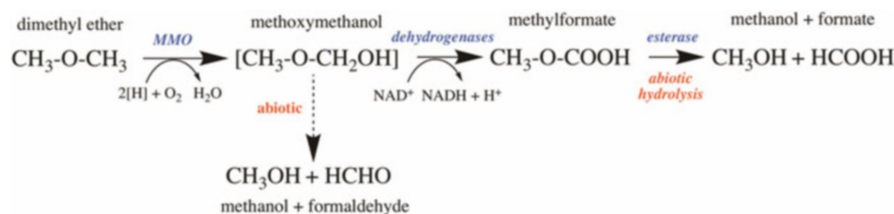


Fig. 2 *Biotransformations of DME by methanotrophs.* Solid lines represent enzyme-catalyzed reactions. Dashed lines represent abiotic reactions. The type of enzyme involved in each reaction is shown in italics. Undetected intermediates are shown in brackets

the model methanotroph *Methylococcus capsulatus* (Bath) and that methyformate can also be oxidized by sMMO (Stirling and Dalton 1980).

2.2 DME Cometabolism

Even though purified DME does not support the growth of methanotrophs, its high aqueous solubility and its effects as a substrate for MMO led to its use as an inhibitor of methane oxidation by free-living (Oremland and Culbertson 1992) and symbiotic methanotrophs (Fisher et al. 1987). DME is also an effective inhibitor of NH_3-dependent N_2O production by autotrophic nitrifying bacteria and has been used to discriminate between nitrifier- and denitrifier-dependent N_2O-production (Miller et al. 1993). These effects arise because DME is also a substrate for ammonia monooxygenase (AMO) in aerobic ammonia-oxidizing bacteria. This membrane-bound enzyme is closely related to pMMO and in the canonical ammonia-oxidizing bacterium *Nitrosomonas europaea*, AMO oxidizes DME to equimolar amounts of methanol and formaldehyde (Hyman et al. 1994).

2.3 Metabolism of Other Alkyl Ethers

Unlike DME, aerobic, growth-associated metabolism of diethyl ether (DEE) has been reported for a wide range of microorganisms including a hydrocarbon-oxidizing filamentous fungus, *Graphium* sp., (Hardison et al. 1997) and diverse bacteria including *Rhodococcus* (Bock et al. 1996; Kim and Engesser 2004; Kim et al. 2007, 2008; Moreno-Horn et al. 2005; Tajima et al. 2012), *Gordonia* (Kim et al. 2007), *Pseudonocardia* (Parales et al. 1994; Kohlweyer et al. 2000; Vainberg et al. 2006), and *Burkholderia* (Hur et al. 1997) strains. The toluene-oxidizing bacterium *B. cepacia* (*vietnamensis*) G4 expresses toluene-2-monooxygenase (T2MO) when grown on toluene (Newman and Wackett 1995). A mutant strain (PR1) that constitutively expresses T2MO grows well on DEE (Shields and Reagin 1992; Hur et al. 1997). Purified T2MO generates equimolar amounts of ethanol and acetaldehyde from DEE oxidation and this reaction is proposed to involve the subterminal oxidation of DEE to form an unstable hemiacetal (Hur et al. 1997). As strain G4 can grow on both ethanol and acetaldehyde but not DEE, the ability of strain PR1 to grow on DEE suggests DEE fails to induce expression of T2MO in the wild-type strain G4.

A DEE-metabolizing strain, *Rhodococcus* sp. DEE5151, grows on the homologous series of *n*-alkyl ethers up to *n*-heptyl ether (Kim and Engesser 2004). Like the oxidation of DEE by T2MO discussed above, it has been proposed that all of these ethers are also initially oxidized by monooxygenase-catalyzed reactions that target the subterminal carbons that form the ether bond. The enzyme responsible for this activity has not been identified at the molecular level but in vivo it is inhibited by glutaraldehyde and ethyl vinyl ether (Kim and Engesser 2005) and it can also O-dealkylate anisole and phenetole (Kim et al. 2008). A physiological and molecular

study of a wide variety of alkyl ether-degrading strains enriched from activated sludge suggests that alkyl ether-metabolizing activity is widely distributed but also often lost following cultivation in the absence of ethers, presumably due to the loss of catabolic plasmids (Kim et al. 2007). This study also suggests that the ability of strains to grow on DEE is also closely associated with the ability to grow on tetrahydrofuran (THF).

2.4 Cometabolism of Other Alkyl Ethers

Several microorganisms are known to cometabolically degrade DEE. For example, methanotrophs expressing sMMO can oxidize diethyl ether (DEE) to ethanol and acetaldehyde (Colby et al. 1977) and the same activity is also seen with *N. europaea* due to the activity of AMO (Hyman 2009). The oxidation of ethers such as *n*-propyl ether by AMO also gives rise to hydroxylated ethers rather than mixtures of alcohols and aldehydes (Hyman 2009).

3 Chloroalkyl Ethers

In this section, we examine several chloroalkyl ethers including bis(2-chloroethyl) ether (BCEE), 2-chloroethyl vinyl ether (2CEVE), bis(2-chloroisopropyl) ether (BCIP) (Fig. 3) that are included on the United State Environmental Protection Agency Priority Pollutant List (USEPA 2014). Our major focus is on the biodegradation of bis(2-chloroethyl)ether (BCEE) which is classified by USEPA as a probable human carcinogen (B2) (USEPA 2002). This ether is primarily used as an intermediate for pesticide synthesis, but it has also been used as a solvent for fats, waxes, greases, textiles, paints and varnishes (ATSDR 2017). BCEE is a semivolatile organic compound (vapor pressure 0.71 mm Hg at 20 °C). It is reasonably soluble in water (~10.4 g/L [0.073 M]) (Bednar et al. 2009) but undergoes a slow sequential hydrolysis to 2-(2-chloroethoxy) ethanol (2CEE) and diethylene glycol (DEG ($t_{1/2}$ = ~3 years at pH 7 and 25 °C)) (Payne and Collette 1989).

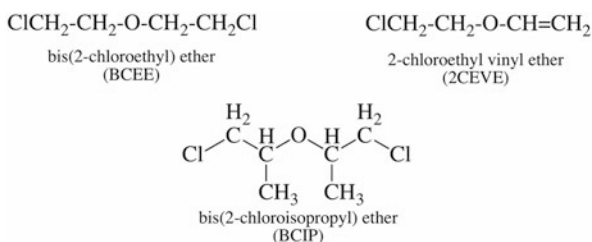


Fig. 3 Structures of representative chloralkyl ethers

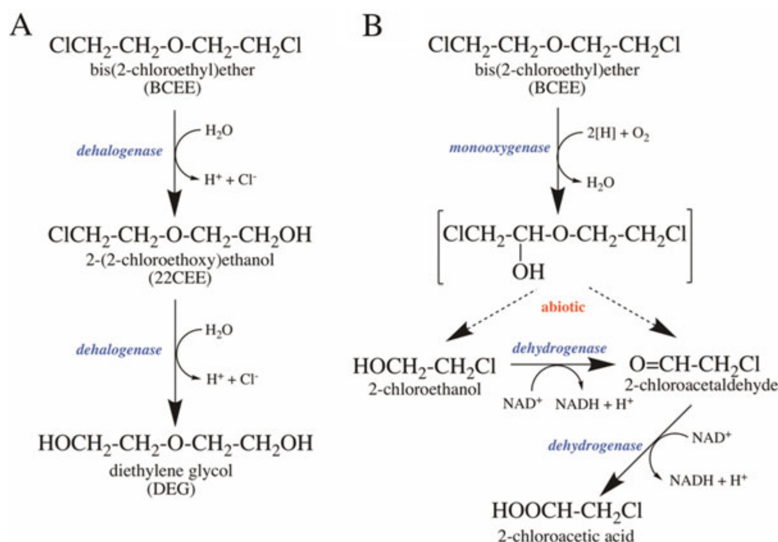


Fig. 4 *Initial reactions in the aerobic degradation of bis(2-chloroethyl)ether (BCEE). Panel A:* Initial reactions in metabolism of BCEE. *Panel B:* Initial reactions in cometabolic degradation of BCEE. Solid lines represent enzyme-catalyzed reactions. Dashed lines represent abiotic reactions. The type of enzyme involved in each reaction is shown in italics. Undetected intermediates are shown in brackets

3.1 BCEE Metabolism

The most detailed study of aerobic BCEE biodegradation to date involves *Xanthobacter* sp. ENV481. This aerobic bacterium was isolated by enrichment culture using BCEE as a sole source of carbon and energy and it can grow on both BCEE and 2CEVE (McClay et al. 2007). The stoichiometry of chloride (Cl^-) production and detected organic metabolites, as well as growth on putative intermediates, suggest the pathway of BCEE degradation involves two initial dehalogenation reactions that generate 2-(2-chloroethoxy) ethanol (22CEE) and diethylene glycol (DEG) as sequential intermediates (Fig. 4A). Degradation of BCEE to 22CEE and Cl^- also occurs in the absence of oxygen, indicating that oxygenases are not involved in this initial reaction. Haloalkane dehalogenases from other microorganisms are known to dechlorinate both BCEE and 2CEVE (Janssen et al. 1988; van den Wijngaard et al. 1993). It is likely that a similar enzyme is responsible for initiating the growth-supporting metabolism of both of these ethers in strain ENV481. Two *Ancylobacter aquaticus* strains (AD25 and AD27) have been described that can both grow on 2CEVE (van den Wijngaard et al. 1993). Like the degradation of BCEE by strain ENV481, the degradation pathway for 2CEVE in these strains is initiated by hydrolytic dehalogenases and does not appear to involve oxygenases.

3.2 BCEE Cometabolism

Unlike *Xanthobacter* sp. ENV481, oxygenase activity is clearly involved in BCEE degradation by *Pseudonocardia* sp. ENV478 (McClay et al. 2007). Strain ENV478 does not grow on BCEE but can rapidly degrade this ether after growth on propane. It also more slowly degrades BCEE after growth on THF (Vainberg et al. 2006). The enzyme(s) responsible for propane-oxidation in strain ENV478 have not been identified. However, growth of strain ENV478 on THF has been linked to THF monooxygenase (THFMO) through transcriptional studies of the gene encoding a hydroxylase component of this enzyme (*thmB*) and through the inhibition of growth on THF in cells expressing an antisense *thmB* transcript (Masuda et al. 2012). Degradation of BCEE by THF-grown cells of strain ENV478 leads to the stoichiometric accumulation of 2-chloroethanol and smaller amounts of 2-chloroacetic acid. Based on these products, BCEE degradation by THF-grown strain ENV478 likely involves an initial THFMO-catalyzed reaction that generates an unstable hemiacetal that subsequently decomposes to 2-chloroethanol and 2-chloroacetaldehyde. The further dehydrogenase-catalyzed oxidation of both of these compounds can then led to production of 2-chloracetic acid (Fig. 4B). The same metabolites are also observed during the cometabolic degradation of BCEE by *Rhodococcus* sp. DTB after growth on DEE (Moreno-Horn et al. 2005) (see below).

3.3 Metabolism of Other Chloroalkyl Ethers

Most of our understanding of the microbial metabolism of BCIP is derived from studies of *Rhodococcus* sp. DTB. This strain was isolated from a mixed culture developed to treat BCIP in a fixed bed reactor (Hauck et al. 2001). Strain DTB generates both 1-chloro-2-propanol and chloroacetone during BCIP degradation. The kinetics of production of these metabolites and the delayed but stoichiometric release of Cl^{-} suggests that cleavage of the ether bond is the initial step in a pathway that eventually fully mineralizes BCIP (Moreno-Horn et al. 2003). Degradation of BCIP is strongly inhibited by methimazole which suggests a role for a flavin-dependent monooxygenase in this initial reaction. Degradation of BCIP also generates a third product identified as 1′-chloro-2′-propyl-3-chloro-2-prop-1-enyl-ether; a dichloro vinyl ether (DVE) (Garbe et al. 2004). This metabolite is also degraded to 1-chloro-2-propanol and chloroacetone by living, but not heat-killed cells. Together, these results suggest that in addition to a monooxygenase-catalyzed oxidative ether cleavage reaction, BCIP degradation by strain DTB can also involve an initial monooxygenase-catalyzed desaturation reaction to form the DVE. This is followed by a second enzyme-catalyzed hydrolytic reaction to form an unstable hemiketal that subsequently decomposes to release 1-chloro-2-propanol and chloroacetone (Fig. 5). The relative contributions of the pathways involving oxidative scission of the ether bond and the formation of the DVE to the overall degradation of BCIP are not known. Strain DTB also catalyzes other interesting reactions with chloroalkyl ethers.

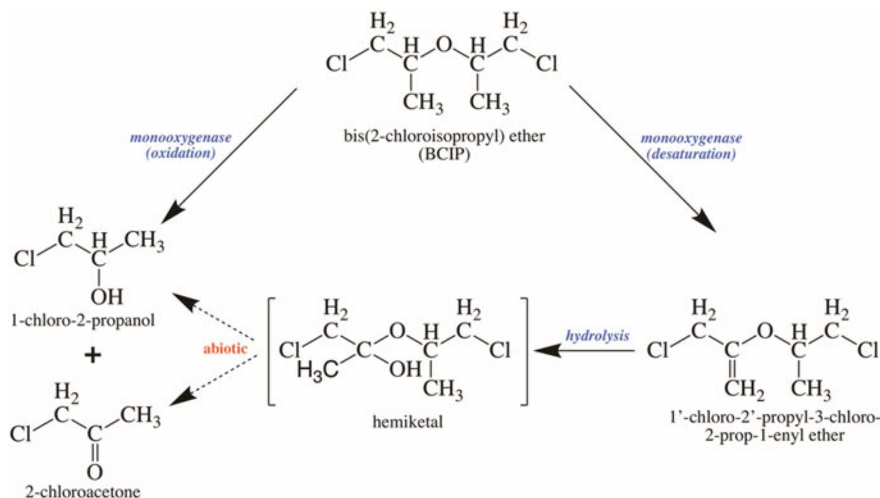


Fig. 5 *Oxidation and desaturation reactions in the biodegradation of bis(2-chloroisopropyl) ether (BCIP) biodegradation.* Solid lines represent enzyme-catalyzed reactions. Dashed lines represent abiotic reactions. The type of enzyme involved in each reaction is shown in italics. Undetected intermediates are shown in brackets

For example, the degradation of BCIP and its DVE metabolite are both enantioselective and the bacterium preferentially degrades (*S*)-configured ether-bonded carbons in these compounds (Garbe et al. 2006). It is likely that this enantioselectivity is conferred by the monooxygenase responsible for initiating BCIP degradation.

3.4 Cometabolism of Other Chloroalkyl Ethers

Strain DTB discussed above also grows on DEE and DEE-grown cells can then degrade several chlorinated and non-chlorinated ethers that otherwise do not support growth of this strain (Moreno-Horn et al. 2005). These include BCEE, bis(4-chloro-*n*-butyl) ether (BCBE), 1,2-dichloroethyl-ethyl ether, 2-chloroethyl-ethyl ether, 1,3-dichloro-2-propy-1′-chlor-2′-propyl ether, THF, and phenetole. γ-Butyrolactone is a product of both THF and BCBE degradation by strain DTB. In the case of THF oxidation, THF can potentially be initially oxidized by a monooxygenase to form 2-hydroxytetrahydrofuran and γ-butyrolactone can then be formed by the further dehydrogenase-catalyzed oxidation of this intermediate. Conversely, during BCBE degradation, it is thought an initial monooxygenase-catalyzed scission generates mixtures of 4-chlorobutanol and 4-chlorobutyraldehyde. The hydrolytic dehalogenation of these intermediates and their further oxidation by dehydrogenases generates 4-hydroxybutyrate which can then lactonize to form γ-butyrolactone (Moreno-Horn et al. 2005).

4 Branched Alkyl Ethers

The branched alkyl ethers considered in this section have all be used to one extent or another as gasoline oxygenates. In the United States, ether oxygenates were initially added to gasoline in the 1980s to compensate for the loss of octane rating following the removal of tetraethyl lead during the transition to lead-free gasoline (ITRC 2005). Branched ethers and alcohols were subsequently used to meet Federal requirements for oxygen that aimed to improve air quality by decreasing exhaust emission of partially oxidized hydrocarbons and CO (ITRC 2005). The main focus of this section is on the biodegradation of methyl *tertiary* butyl ether (MTBE) but also extends to the structurally- and functionally-related compounds, ethyl *tertiary* butyl ether (ETBE), *tertiary* amyl ether (TAME), and diisopropyl ether (DIPE) (Fig. 6). The aerobic biodegradation of MTBE and ETBE generates *tertiary* butyl alcohol (TBA) as an intermediate while biodegradation of TAME generates *tertiary* amyl alcohol (TAA). The biodegradation of these alcohols is also briefly addressed in this section.

4.1 MTBE Metabolism

Our understanding of the aerobic microbial growth-related metabolism of MTBE is based primarily on studies with three bacteria: *Hydrogenophaga flava* ENV735, *Methylibium petroleiphilum* PM1, and *Aquincola tertiaricarbonis* L108. *H. flava* ENV735 is a H_2-oxidizing bacterium that grows slowly and inefficiently (0.4 g/g) on MTBE (Hatzinger et al. 2001). The bacterium also generates TBA from MTBE and

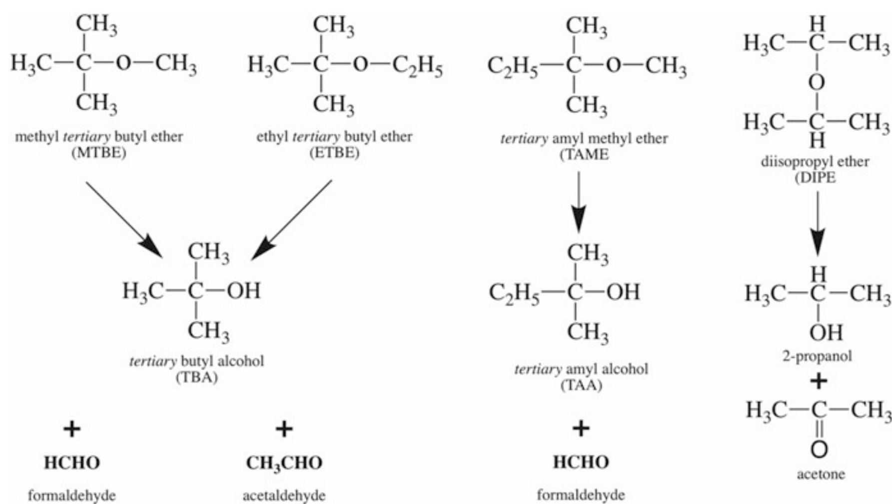


Fig. 6 Structures of the four main branched ether oxygenates and their corresponding major metabolites

can grow on TBA alone. MTBE and TBA are apparently oxidized by two different enzymes as MTBE-oxidizing activity is constitutive and sensitive to the cytochrome P450 inhibitor, aminobenzotriazole, whereas TBA oxidation is inducible and insensitive to aminobenzotriazole. Strain ENV735 also generates 2-hydroxyisobutyric acid (2HIBA) during MTBE oxidation and can grow on this compound as a sole source of carbon and energy (Hatzinger et al. 2001). The enzymes thought to be responsible for MTBE and TBA degradation in this strain have not been identified but both are reasonably anticipated to be oxygenases.

Like *H. flava* ENV735, *M. petroleiphilum* PM1 also grows slowly and inefficiently (<0.2 g/g) on MTBE (Hanson et al. 1999). This strain can also grow on methanol, ethanol, toluene, benzene, and ethylbenzene (Nakatsu et al. 2006). The genome of *M. petroleiphilum* PM1 consists of a ~4 Mb circular chromosome and a large (~600 kb) circular plasmid (pPM1) (Kane et al. 2007). Many of the genes encoding enzymes proposed to be involved in the MTBE oxidation pathway are located on pPM1. These include *mdpA*, *mdpE,* and *mdpJK* which encode an MTBE-oxidizing alkane hydroxylase-like monooxygenase, a hemiacetal-oxidizing dehydrogenase, and a TBA-oxidizing oxygenase, respectively. Although *tertiary* butyl formate (TBF) has not been observed as an intermediate in MTBE oxidation by *M. petroleiphilum* PM1, it has been proposed that the first enzyme (MpdA) oxidize MTBE to a hemiacetal. This hemiacetal then rapidly abiotically decomposes to form TBA and formaldehyde or is enzymatically oxidized to TBF by MpdE. The hydrolysis of TBF then releases TBA and formate. The TBA is then further oxidized to 2-methyl-1,2-propanediol (MPD) by MpdJK (Hristova et al. 2007) (Fig. 7).

The MTBE-oxidizing monooxygenase (MpdA) is an AlkB-like alkane hydroxylase. Genes encoding other enzymes typically associated with AlkB-like alkane hydroxylases such as an AlkG-like rubredoxin, an AlkT-like rubredoxin reductase, and a putative AlkS-like transcriptional regulator (MdpC) do not occur as a discrete operon but candidate genes all reside within a 10 kb locus on pPM1 (Schmidt et al. 2008). Another MTBE-degrading *Methylibium* isolate, strain R8, also contains *mdpA*. A ^{13}C-protein stable isotope probing analysis of a mixed MTBE-degrading culture that contained PM1-like bacteria characterized 23 ^{13}C-labeled proteins including MpdA, MdpJ, and MdpK (Bastida et al. 2010). This analysis also detected several other as yet uncharacterized proteins (MpeB0532-MpeB0535) whose genes are strongly up regulated in MTBE-grown cells of strain PM1 (Hristova et al. 2007).

A. tertiaricarbonis L108 grows on MTBE, TBA, and 2HIBA (Lechner et al. 2007; Müller et al. 2008). Among these, MTBE supports the lowest maximal growth rate (0.045 h^{-1}), and growth yield (~0.5 g/g) (Müller et al. 2007). This bacterium must sustain high rates of MTBE utilization to overcome the high minimum substrate concentration and maintenance energy barrier required for growth on this ether (Müller et al. 2007). The initial oxidation of MTBE in strain L108 is catalyzed by a 43 kDa cytochrome P450 (EthB) that was first identified in *Rhodococcus ruber* IFP 2001 (Chauvaux et al. 2001). The *eth* operon in strain L108 lacks the transcriptional regulator, *ethR* found in the *ethRABCD* operon in strain IFP 2001 (Chauvaux et al. 2001). This leads constitutive expression of the *eth*-gene-encoded cytochrome P450

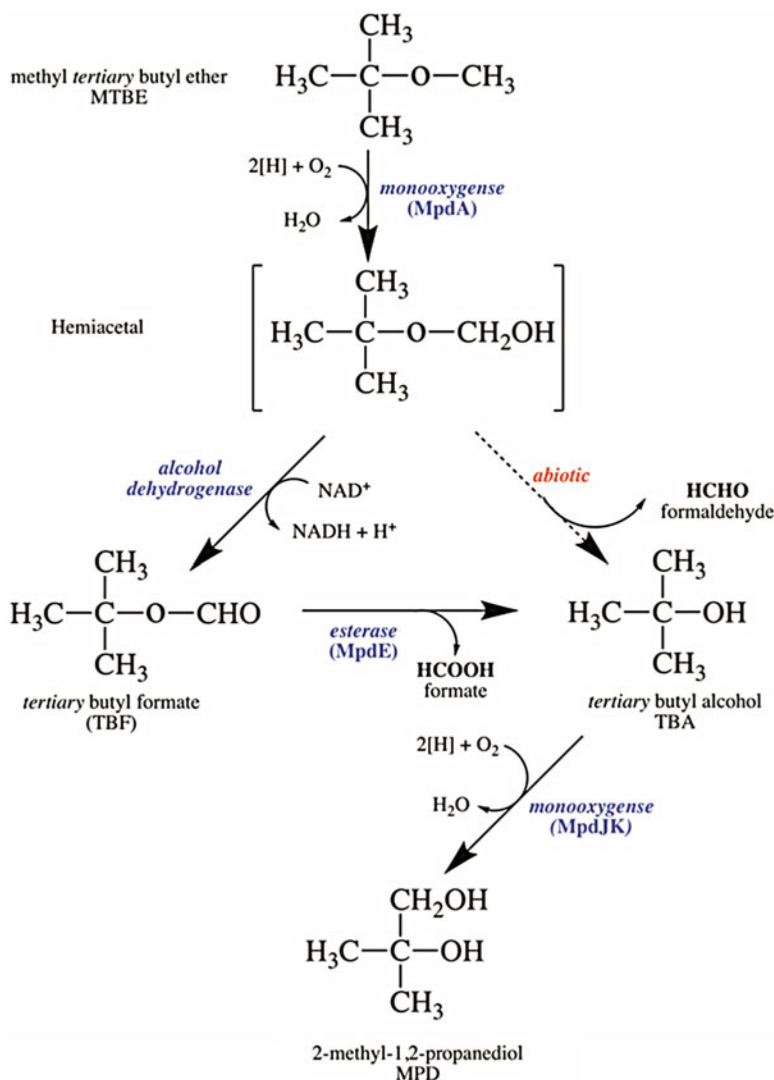


Fig. 7 *Initial reactions in the metabolism and cometabolism of methyl tertiary butyl ether.* Solid lines represent enzyme-catalyzed reactions. Dashed lines represent abiotic reactions. The type of enzyme involved in each reaction is shown in italics. Undetected intermediates are shown in brackets

system and MTBE-degrading activity in strain L108 (Schuster et al. 2013; Lechner et al. 2007). The other genes in the *eth* operon encode a ferredoxin reductase (EthA [~45 kDa]), a [2Fe-2S]-like ferredoxin (EthC [~12 kDa]), and a ~10 kDa protein of unknown function (EthD). The further oxidation of TBA to 2MPD by both strains L108 and PM1 is catalyzed by Rieske nonheme mononuclear iron oxygenase that consists of a 55 kDa hydroxylase (MdpJ) and a 38 kDa reductase (MdpK).

4.2 MTBE Cometabolism

The aerobic cometabolic degradation of MTBE has also been extensively studied and this activity occurs in diverse bacteria including *Pseudomonas*, *Pseudonocardia*, *Rhodococcus*, and *Mycobacterium* strains, as well as some fungi. In all cases, the initial step in MTBE oxidation appears to involve nonspecific oxygenases and leads to the production of TBA. For example, *Pseudomonas* strains that can grow on short chain ($<C_{10}$) *n*-alkanes have frequently been shown to oxidize MTBE (Garnier et al. 1999, 2000; Smith et al. 2003b; Morales et al. 2009; Smith and Hyman 2010; Salazar et al. 2012; Li et al. 2016). There are multiple lines of evidence that indicate this activity is catalyzed by AlkB-containing alkane hydroxylases. For instance, in *P. putida* GPo1, MTBE-oxidizing activity is observed in *n*-octane-grown cells and a gratuitous inducer, dicyclopropylketone (DCPK), also promotes MTBE-oxidizing activity in cells grown on alkane-free media (Smith and Hyman 2004). A similar effect of DCPK has been observed with other *Pseudomonas* strains (Bravo et al. 2015). Oxidation of MTBE in DCPK-induced cells of strain GPo1 is also inhibited by *n*-alkane substrates for alkane hydroxylase (C_3–C_{10}) and 1,7-octadiyne, a mechanism-based inactivator of alkane hydroxylase in this strain (Smith and Hyman 2004; Katapodis et al. 1984). The form of alkane hydroxylase in strain GPo1 is typically found in *Pseudomonas* strains that grow on shorter chain ($<C_{10}$) *n*-alkanes (Vomberg and Klinner 2000; Belhaj et al. 2002) and several *Pseudomonas* strains that contain genes that are identical to *alkB* in strain GPo1 all oxidize MTBE but not TBA (Smith and Hyman 2010). Conversely, MTBE-oxidizing activity is not associated with the more common forms of alkane hydroxylase that oxidize longer chain *n*-alkanes ($>C_{10}$) (Smith and Hyman 2010).

Pseudonocardia sp. ENV478 and *P. tetrahydrofuranoxidans* K1 both oxidize MTBE after growth on THF, and both strains generate TBA which is not further oxidized (Vainberg et al. 2006; McKelvie et al. 2009). In both cases, MTBE oxidation has been attributed to the activity of THFMO. In the case of strain ENV478, this is the same enzyme implicated in the degradation of BCEE. This monooxygenase is encoded by the *thm* operon (*thmADBC*) and the holoenzyme consists of two hydroxylase components (α- [ThmA, 60 kDa] and β- [ThmB, 38 kDa]), a small coupling protein (ThmC, 13 kDa) and a flavin-containing reductase (ThmD, 40 kDa) (Thiemer et al. 2003).

Several bacteria including *Rhodococcus ruber* ENV425, *Mycobacterium* sp. ENV421, and *Mycobacterium vaccae* JOB5 oxidize MTBE after growth on propane (Steffan et al. 1997; Smith et al. 2003a). These strains can be distinguished from microorganisms such as *P. putida* GPo1, *Pseudonocardia* ENV478, and *P. tetrahydrofuranoxidans* K1 discussed above as they do further oxidize TBA. The MTBE-degrading activity of strain ENV425 is inhibited by the cytochrome P450 inhibitors, CO and aminobenzotriazole. However, a subsequent molecular study did not find a role for a cytochrome P450 in propane-induced activities in strain ENV425 (Fournier et al. 2009). A recent genome-enabled proteomic analysis indicates propane-grown cells of strains ENV425 express two different soluble diiron monooxygenases (SDIMOs) (Tupa and Masuda 2018b, c). One of these is a Group 6

SDIMO, while the other is a Group 5 SDIMO. In contrast, propane-grown cells of ENV421 express a single Group 6 SDIMO, a membrane-bound, pMMO-like, copper-containing monooxygenase (CuMMO), and a cytochrome P450 (Tupa and Masuda 2018a, c). The role of these individual enzymes in propane, MTBE, and TBA oxidation by these strains has not been established. Similar Group 6 SDIMOs have recently been detected in other gaseous hydrocarbon-oxidizing strains such as *Rhodococcus* sp. strain BCP1 (Cappelletti et al. 2015) and have also recently been implicated in 14D degradation (He et al. 2017a, b; Deng et al. 2018a; Bennett et al. 2018).

The enzyme(s) responsible for MTBE oxidation in strain JOB5 have not been determined although MTBE- and TBA-oxidizing activity is observed in cells previously grown on a wide range of alkanes ($<C_{10}$) (House and Hyman 2010). In propane-grown cells of strain JOB5, TBF is an initial product of MTBE oxidation. Production of TBF has been proposed to involve the further alcohol-dehydrogenase-catalyzed oxidation of a hemiacetal generated from the initial monooxygenase-catalyzed oxidation of a C–H bond in the methoxy group of MTBE (Smith et al. 2003a). Mechanistically, this is the same pathway proposed for MTBE oxidation by strain PM1 (Fig. 7). However, unlike strain PM1, kinetic and inhibition studies using alkanes and acetylene suggest the same monooxygenase is responsible for the oxidation of both MTBE and TBA in strain JOB5 (Smith et al. 2003a). This enzyme is also thought to be responsible for initiating the oxidation of propane and other short chain alkanes ($<C_8$).

Similar effects of acetylene on both MTBE and TBA oxidation and similar kinetics and patterns of metabolite excretion and degradation have been observed in *Mycobacterium* strains that have been reported to grow on MTBE and TBA as a sole source of carbon and energy (François et al. 2002, 2003; Ferreira et al. 2006). However, these studies have typically used high initial MTBE and TBA concentrations and very high initial inoculum levels and have not shown an unequivocal relationship between MTBE removal and increases in culture density or any other potential measurement of growth. Although more compelling evidence is available for the growth of these strains on TBA, it has been suggested that the enzyme responsible for initiating both MTBE and TBA oxidation in these strains and strain JOB5 is an AlkB-like alkane hydroxylase (Ferreira et al. 2007). This seems unlikely as the MTBE-oxidizing AlkB-alkane hydroxylases characterized to date do not further oxidize TBA at any physiologically relevant rate. Molecular studies of AlkB-like alkane hydroxylase expression in strain JOB5 also indicate these enzymes are not expressed in propane-grown cells (Sharp et al. 2010). In contrast to the high MTBE and TBA concentrations used in the studies described above, *n*-butane-grown cells of an *Arthrobacter* strain (ATCC 27778) and propane-grown cells of strain ENV425 have been shown to oxidize low concentrations of MTBE (100–800 μg L^{-1}) (Liu et al. 2001). Similar to strain JOB5, MTBE oxidation by *Arthrobacter* sp. ATCC 27778 is sensitive to inhibition by alkanes (e.g., *n*-butane), TBA, and acetylene (Liu et al. 2001). The enzyme(s) responsible for the oxidation of MTBE in ATCC 27778 have not been identified.

The filamentous fungus, *Graphium* sp., grows on gaseous *n*-alkanes (Curry et al. 1996), DEE (Hardison et al. 1997), and THF (Skinner et al. 2009) and mycelia grown on propane, *n*-butane, or DEE all slowly degrade MTBE (Hardison et al. 1997). With *n*-alkane-grown mycelia, TBF can initially represent as much as 95% of the MTBE oxidized and its hydrolysis product, TBA, is not further oxidized at significant rates (Hardison et al. 1997; Skinner et al. 2008). Evidence that TBF production from MTBE involves rapid enzymatic oxidation of an initial monooxygenase-generated hemiacetal is supported by the observation that propane- and THF-grown mycelia can rapidly generate methylformate from concentrated mixtures of formaldehyde and methanol (Skinner et al. 2009). RNAi silencing of posttranscriptional translation of a single cytochrome P450-encoding gene severely inhibits growth of this fungus on *n*-alkanes and THF and suggests this gene encodes an enzyme with a broad hydrocarbon- and ether-oxidizing capabilities (Trippe et al. 2014). Another fungus, *Agrocybe aegerita*, excretes an extracellular heme-containing peroxygenase that can also oxidize MTBE (Kinne et al. 2009). A similar enzyme is also produced by a variety of other fungi (Hofrichter and Ullrich 2014). The well-characterized camphor-oxidizing cytochrome $P450_{cam}$ in *P. putida* CAM (ATCC 17453) can also oxidize MTBE to TBA but does not further oxidize TBA but ether-oxidizing activity does not appear to be widespread activity among these heme-containing enzymes (Steffan et al. 1997).

4.3 Metabolism of Other Branched Alkyl Ethers

Many of the bacteria described above that metabolize MTBE also metabolize other branched alkyl ethers. For example, *M. petroleiphilum* PM1 can grow on both MTBE and TAME, but not ETBE (Schuster et al. 2012). As both MTBE and ETBE oxidation are expected to generate TBA as a key metabolite, this growth substrate range suggests the ether substrate range of MdpA, like other AlkB-containing alkane hydroxylases, is limited to methyl ethers (van Beilen et al. 1994; Katapodis et al. 1984). Similarly, in addition to growth on MTBE, TBA, and 2HIBA, *A. tertiaricarbonis* L108 can also grow on ETBE, TAME, and TAA (Lechner et al. 2007; Müller et al. 2008). The initial multicarbon metabolites generated from ETBE and TAME by the EthB cytochrome P450 are assumed or known to be TBA plus acetaldehyde, and TAA, respectively (Müller et al. 2008; Schuster et al. 2013). It is further assumed that these products are derived from unstable monooxygenase-generated hemiacetal intermediates. The further oxidation of TBA to 2MPD involved in both MTBE and ETBE metabolism by strain L108 and PM1 involves an MpdJK-catalyzed monooxygenation reaction. The analogous products of TAA oxidation would be expected to be various diols. However, the immediate product of TAA degradation by these strains is 2-methyl-3-buten-2-ol which indicates the further degradation of TAA involves a desaturase rather than a monooxygenase activity of MdpJK (Schuster et al. 2012).

Although some alkyl ether-metabolizing bacteria have broad growth substrate ranges, other strains are more restricted. For example, some of the earliest studies of ether oxygenate degradation described the isolation and characterization of *Rhodococcus ruber* IFP 2001 and *R. zopfii* IFP 2002 (Fayolle et al. 1998). Strain IFP 2001 generates stoichiometric amounts of TBA during growth on ETBE suggesting that it grows on the 2-carbon fragment released during ETBE oxidation, rather than TBA. Although strain IFP 2001 does not grow on MTBE or TAME, resting ETBE-grown cells readily oxidize both compounds without a lag phase and generate near stoichiometric amounts of TBA and TAA, respectively (Hernandez-Perez et al. 2001). As indicated earlier, molecular studies of the ETBE-oxidizing system in strain IFP 2001 led to the identification of the *eth* operon and the role of *ethB*-encoded cytochrome P450 in ether oxygenate biodegradation. Transcriptional analyses have demonstrated that ETBE but not MTBE or TAME induces expression of the *eth* genes in strain IFP 2001 (Malandain et al. 2010). It is therefore not clear whether the principal restriction on the ability of strain IFP to grow on methyl ether oxygenates (MTBE and TAME) is a lack of *eth* gene induction or an inability to utilize the C_1 fragments generated from their oxidation.

Several bacterial strains have been reported to grow aerobically on DIPE. These include *Rhodococcus ruber* 219 (Bock et al. 1996) and *Pseudonocardia* sp. ENV 478 (Vainberg et al. 2006). The intermediates generated from DIPE oxidation and the oxygenase enzymes potentially involved in their biodegradation are currently not known. Although *A. tertiaricarbonis* L108 has not been described to grow on DIPE, it can constitutively oxidize this ether and acetone has been reported as a major metabolite (Schuster et al. 2013). Based on other oxygenase-catalyzed reactions, it is likely that DIPE is initially oxidized to a hemiacetal that then decomposes to form 2-propanol and acetone. In the case of strain L108, it is likely that 2-propanol is further oxidized to acetone which accumulates due to lack of a pathway for its further metabolism.

4.4 Cometabolism of Other Branched Alkyl Ethers

The AlkB-containing alkane hydroxylases found in *P. putida* GPo1 (Smith and Hyman 2004) *P. mendocina* KR1 (Smith et al. 2003b) and other closely related Pseudomonads (Smith and Hyman 2010) appear to be restricted to methyl ethers as cells expressing this enzyme can oxidize MTBE and TAME but are largely unreactive towards ETBE and the *tertiary* alcohols, TBA and TAA. A mixed culture containing *Pseudomonas* strains has been described that can mineralizes MTBE, ETBE, TAME, TBA, and TAA in the presence of C_3–C_7 *n*-alkanes (Morales et al. 2009). A *P. citronellolis* strain isolated from this culture expresses a very similar AlkB-containing alkane hydroxylase to the enzyme in *P. putida* GPo1 (Bravo et al. 2015) and it is likely that this mixed culture contains separate TBA-metabolizing strains rather than Pseudomonads with full MTBE-mineralizing capabilities. In addition to MTBE, other strains including THF-grown *P. tetrahydrofuranoxidans* K1 (McKelvie et al. 2009) and propane-grown *R. ruber*

ENV425 (Steffan et al. 1997) can also oxidize ETBE and TAME. Unlike strain K1 which does not appear to oxidize *tertiary* alcohols at any significant rate (McKelvie et al. 2009), propane-grown strain ENV425 can also oxidize both TBA and TAA (Steffan et al. 1997). It is likely both of these alcohols are further oxidized to diols through monooxygenase activity.

5 Cyclic Ethers

Cyclic ethers are oxygen-containing heterocyclic compounds. The simplest cyclic ether is ethylene oxide (1,2-epoxyethane). This highly reactive gas is widely used as a chemical feedstock and as a sterilizing agent. The model cyclic ether considered in this section is 14D. This compound was extensively used as a stabilizing agent in commercial formulations of chlorinated solvents such as 1,1,1-trichloroethane (Mohr et al. 2010). It is also found in cosmetics, detergents, and shampoos as a byproduct of manufacturing processes involving ethylene oxide (USEPA 2016). 14D is a likely human carcinogen with an increased one in one million cancer risk associated with lifetime consumption of drinking water at a concentration of 0.35 μg/L (USEPA 2017). The other cyclic ethers considered in this section include THF, tetrahydropyran (THP), 1,3-dioxane (13D), and 1,3-dioxalane (13DO) (Fig. 8).

5.1 14D Metabolism

A wide variety of microorganisms are known to grow on 14D as a sole source of carbon and energy. These include fungi such as *Cordyceps sineies* (Nakamiya et al. 2005) and representatives of the following bacterial genera; *Acinetobacter* (Huang et al. 2014; Zhou et al. 2016), *Afipia* (Sei et al. 2013a), *Mycobacterium* (Kim et al. 2009; Sei et al. 2013a), *Rhodanobacter* (Pugazhendi et al. 2015), *Rhodococcus* (He et al. 2014; Inoue et al. 2016, 2018), *Pseudonocardia* (Parales et al. 1994; Mahendra

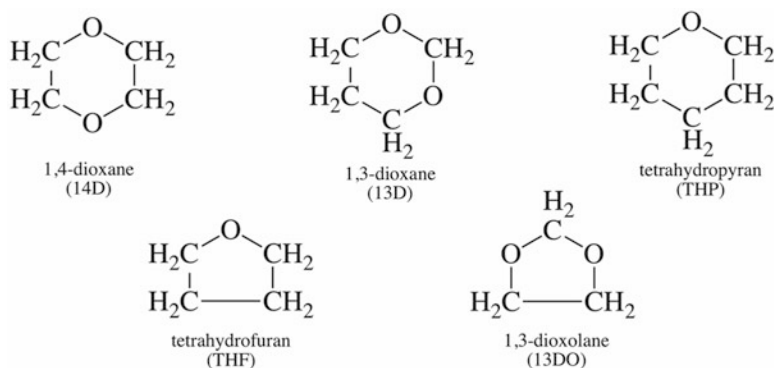


Fig. 8 Structures of representative cyclic ethers

and Alvarez-Cohen 2005; Matsui et al. 2016; Inoue et al. 2016; Yamamoto et al. 2018), and *Xanthobacter* (Jin et al. 2012; Chen et al. 2016).

The best-characterized 14D-metabolizing bacterial strains at the molecular level are *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanitrophicus* PH-06. Strain CB1190 (ATCC 55486) was isolated a mixed culture enriched from a 14D-contaminanted sludge. The culture was initially grown on THF and slowly adapted to 14D (Parales et al. 1994). This bacterium is most likely a THF-metabolizing strain that acquired a stable mutation affecting a regulatory, transport, or enzymatic feature that now allows it to grow, albeit slowly (30 h generation time) (Parales et al. 1994) and inefficiently (0.09 g/g) (Mahendra and Alvarez-Cohen 2005), on 14D. The genome of strain CB1190 consists of a circular 7.1 Mb chromosome with three plasmids (pPSED01 (192 kb), pPSED02 (137 kb), and pPSED03 (15 kb)) (Sales et al. 2011). Among several other multicomponent monooxygenases encoded in the genome, a complete operon encoding THFMO is present on pPSED02. This operon is nearly identical to the operon encoding this enzyme in other strains including *Rhodococcus* sp. YLL (He et al. 2014; Yao et al. 2013), *Pseudonocardia* ENV478 (Masuda et al. 2012), and *P. tetrahydrofuranoxidans* K1 (Bennett et al. 2018) that can all cometabolically degrade 14D after growth on THF but do not grow on 14D.

The role of THFMO in initially oxidizing 14D and THF by strain CB1190 and *P. tetrahydrofuranoxidans* K1 has been confirmed by heterologous expression of their respective THFMO gene clusters (*thmADBC*) in *Rhodococcus jostii* RHA1 (Sales et al. 2013). It has also been determined that THFMO does not participate in the further oxidation of a key 14D-derived metabolite, 2-hydroxyethoxyacetic acid (2HEAA) (Sales et al. 2013). Transcription-based studies of *thm* gene expression in response to 14D, THF, and inhibition of THFMO activity by acetylene also support a role for THFMO in 14D metabolism by strain CB1190 (Gedalanga et al. 2014). Genes encoding THFMO hydroxylase subunits have been suggested to be useful biomarkers for detecting the potential for 14D biodegradation in field samples and in these studies THFMO in strain CB1190 has been referred to as DXMO (Li et al. 2013; Gedalanga et al. 2016). This nomenclature may have been introduced to suggest that THFMO expressed by CB1190 is somehow different from other THFMOs as it is associated with an organism that can grow on 14D. If so, this is misleading because all THFMOs currently known are structurally and catalytically almost indistinguishable.

Mycobacterium PH-06 was originally isolated from a 14D-contaminated river sediment by enrichment culture using 14D as the enrichment substrate (Kim et al. 2009). The genome of the bacterium consists of a circular chromosome (7.6 Mb), with four plasmids (156, 153, 106, and 70 kb). The genome encodes several monooxygenases including a group 6 SDIMO and a copper-containing monooxygenase (CuMMO) that are closely located on plasmid 3. RNAseq analyses indicate that expression of all components of both of these enzymes is greatly increased in cells grown on 14D as compared to glucose-grown cells (He et al. 2017b). This suggests both enzymes may have a role in 14D metabolism. The Group 6 SDIMO has been further characterized and transcriptional analyses have shown

that all four genes encoding this enzyme (*prmABCD*) are expressed as a single polycistronic transcript when cells are exposed 14D, THF or propane (Deng et al. 2018a). This SDIMO has also been heterologously expressed in *M. smegmatis* mc^2-155 and shown to oxidize 14D, THF, and propane (Deng et al. 2018a). In contrast, the CuMMO that is co-expressed with the SDIMO has not been further characterized so it is currently not clear whether the propane and cyclic ether-degrading activities of strain PH-06 can be attributed to just one or multiple monooxygenases. A role of the CuMMO in propane oxidation is likely as similar enzymes have been detected in other gaseous alkane-oxidizing bacteria including *Mycobacterium* sp. NBB4 (Coleman et al. 2012), *Nocardiodes* CF8 (Sayavedra-Soto et al. 2011), and several marine bacteria identified by stable isotope probing (Redmond et al. 2010)

The SDIMO detected in strain PH-06 has been called a propane monooxygenase, presumably based on its ability to oxidize propane (Deng et al. 2018a). Like the use of DXMO to describe THFMO discussed above, this, nomenclature is likely to cause confusion as a group 5 SDIMO that is found in strains with typically weak and narrow gaseous alkane-oxidizing activities such as *R. jostii* RHA1 (Sharp et al. 2007), *Gordonia* TY-5 (Kotani et al. 2003), and others is also called propane monooxygenase. A Group 5 SDIMO propane monooxygenase is also co-expressed with THFMO during 14D degradation by strain CB1190 but is not thought to be directly involved in cyclic ether biodegradation by this bacterium (Gedalanga et al. 2014).

The immediate product of 14D oxidation by the different monooxygenase associated with 14D oxidation in both strains PH-06 and CB1190 is thought to be 1,4-dioxane-2-ol (Kim et al. 2009; Mahendra et al. 2007). This metabolite may undergo ring chain tautomerism to 2-hydroxyethoxyacetaldehyde or may be further oxidized to a lactone (1,4-dioxanone) that can then undergo reversible hydrolysis and lactonization to generate 2-hydroxyethoxyacetic acid (2HEAA) (Fig. 9). This metabolite has been detected in several studies of microbial 14D degradation (Vainberg et al. 2006; Mahendra et al. 2007; Sales et al. 2013; Deng et al. 2018a) and it has been suggested that another monooxygenase-catalyzed reaction is required to further oxidize 2HEAA (Mahendra et al. 2007). Heterologous expression studies have indicated that if needed, this reaction is not catalyzed by THFMO (Sales et al. 2013). Although 1,4-dioxane-2-ol is also thought to be an initial product of 14D degradation by *Acinetobacter baumannii* DD1, 1,4-dioxene has been detected as a minor metabolite (Huang et al. 2014). 1,4-Dioxene has also been observed in studies of 14D degradation by *Xanthobacter flavus* DT8 and has been suggested to be a dehydration product of 1,4-dioxane-2-ol (Chen et al. 2016). 2HEAA has not been detected as a metabolite of 14D degradation by strain DT8 which suggests that production of 1,4-dioxene may be indicative of a 2HEAA-independent pathway of 14D degradation.

5.2 14D Cometabolism

Given the structural similarities between 14D and THF, it is not surprising that many THF-metabolizing strains can cometabolically degrade 14D. Bacterial strains that

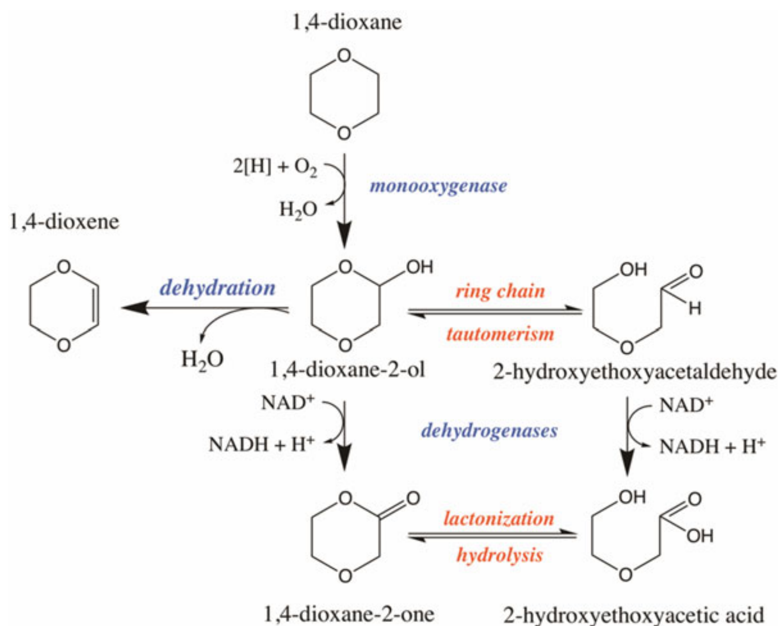


Fig. 9 *Initial reactions in the metabolism and cometabolism of 1,4-dioxane.* Solid lines represent enzyme-catalyzed reactions. Dashed lines represent abiotic reactions. The type of enzyme involved in each reaction is shown in italics. Undetected intermediates are shown in brackets

cometabolically degrade 14D after growth on THF include *Rhodococcus ruber* strains 219 (Bock et al. 1996), T1, and T5 (Sei et al. 2013b), and *Pseudonocardia* isolates including strains ENV478 (Vainberg et al. 2006), K1 (Mahendra and Alvarez-Cohen 2006; Bennett et al. 2018), and others (Inoue et al. 2016). The filamentous fungus *Graphium* sp. also slowly oxidizes 14D after growth on THF (Skinner et al. 2009) through the activity of a cytochrome P450 (Trippe et al. 2014).

Due to its own human toxicity, THF is not a useful substrate for stimulating microbial processes in the environment. Consequently, there is considerable current interest in other substrates that can stimulate cometabolic degradation of 14D. Three model toluene-oxidizing strains expressing either toluene-2-, -3-, or -4-monooxygenase can all oxidize high ($\leq$500 mg L^{-1}) initial concentrations of 14D at faster initial rates than cells of strain CB1190 grown on 14D (Mahendra and Alvarez-Cohen 2006). This activity has also been confirmed with cloned and heterologously expressed forms of these enzymes. *Methylosinus trichosporium* OB3b expressing sMMO has also been reported to degrade 14D although this activity was not sustainable (Mahendra and Alvarez-Cohen 2006). A subsequent study using purified sMMO has not confirmed 14D-degrading activity for this enzyme (Hatzinger et al. 2017). In the same study, an ethane-metabolizing strain (*Mycobacterium* ENV482) was isolated from a site where ethane had been demonstrated to stimulate cometabolic degradation of low concentrations of ethylene dibromide. This bacterium can also oxidize 14D after growth on ethane. *M. vaccae* JOB5 has also been reported to oxidize 14D after growth on propane (Burback and

Perry 1993; Mahendra and Alvarez-Cohen 2006; Lan et al. 2013) and other alkanes including *n*-butane, *n*-pentane, isobutane, and isopentane (Lan et al. 2013). The enzyme(s) responsible for the oxidation of 14D and these alkanes has not been identified but cells grown on these substrates are also able to oxidize both MTBE and TBA (House and Hyman 2010). The most recent report of a pure culture capable of propane-dependent cometabolic degradation of 14D describes an *Azoarcus* strain, DD4 (Deng et al. 2018b). When spiked into groundwater, this strain was able to oxidize 14D to very low concentrations (<0.4 μg L^{-1}). Although the genome of this bacterium has not been described and transcriptional studies have not been conducted, the 14D-degrading activity has been tentatively attributed to a Group 5 SDIMO, propane monooxygenase. This bacterium was also shown to concurrently degrade 1,1-dichloroethene which is a common co-contaminant often encountered in 14D-contaminanted groundwater.

5.3 Metabolism of Other Cyclic Ethers

In addition to 14D, many of currently known 14D-metabolizing strains also grow on other ethers. For example, strain CB1190 can also grow on both cyclic (THF, THP, 13D, 2-methyl-1,3-dioxane) and alkyl ethers (DEE and butyl methyl ether) (Parales et al. 1994). Among the cyclic ethers, many 14D-metabolizing microorganisms can grow on the 14D isomer, 13D (Nakamiya et al. 2005; Huang et al. 2014, Sei et al. 2013a; Kim et al. 2009) and the structurally similar compound, THP (Nakamiya et al. 2005; Sei et al. 2013a). The enzymes responsible for initiating the degradation of these ethers has not be unequivocally identified in most cases although it is assumed that the enzymes responsible for initiating the metabolism of these compounds are the same as the enzymes used to initiate 14D metabolism.

5.4 Cometabolism of Other Cyclic Ethers

Overall, little attention has been paid to the cometabolic biodegradation of cyclic ethers other than 14D. *M. vaccae* JOB5 has been shown to oxidize trimethylene oxide, THF, THP, 13D, and 13DO after growth on propane, *n*-butane, *n*-pentane, isobutane, and isopentane (Lan et al. 2013). During the degradation of THF by propane-grown cells, near stoichiometric amounts of γ-butyrolactone accumulate and are subsequently consumed. This is thought to reflect the initial oxidation of THF to 2-hydroxytetrahydrofuran followed by the rapid further oxidation of this intermediate to γ-butyrolactone by alcohol dehydrogenases that are abundant in alkane-grown cells. When cells were grown on *n*-pentane in the presence of THF, γ-butyrolactone is generated and consumed and, based on substantial increases in culture density, appears to enhance the growth of the bacterium compared to cells grown on *n*-pentane alone (Lan et al. 2013). Although 14D is extensively consumed, a growth stimulating effect is not observed for cells grown on *n*-pentane in the presence of 14D. This suggests that metabolites generated from 14D oxidation are not further degraded or are degraded at rates too slow to impact growth.

6 Future Research

As this chapter has hopefully demonstrated, there is strong and consistent evidence that a wide variety of simple ether-bonded compounds can be biodegraded by aerobic microbial processes. In many cases, these biodegradation processes involve the activities of non-specific oxygenase enzymes that catalyze oxidative ether cleavage reactions. However, as studies with some compounds such as BCIP (Garbe et al. 2004) and TAA (Schuster et al. 2012) have demonstrated, these may also involve desaturation reactions. The possibility that nonoxidative oxygenase-catalyzed reactions are involved in other ether degradation reactions could reveal interesting results.

Another theme that has emerged from recent research into aerobic ether biodegradation is the concentration of ethers that can be biodegraded by organisms that can grow on ethers and those that can only cometabolically transform these compounds. For example, there is a wealth of information about strains such as *P. dioxanivorans* CB1190 and *Mycobacterium dioxanitrophicus* PH-06 that can grow on 14D. However, the K_s values for the oxidation 14D by these strains have recently been determined to be 145 ± 17 mg L^{-1} and 78 ± 10 mg L^{-1}, respectively (He et al. 2018), and are both far above the μg L^{-1} concentrations of 14D that are frequently encountered in 14D-contaminated environments. A recent kinetic and modeling study has suggested that organisms such as strain CB1190 are unlikely to be effective at biodegrading 14D at concentrations below ~400 μg L^{-1}, whereas cometabolic processes are effective below this concentration (Barajas-Rodriguez and Freedman 2018). Recent studies with pure cultures (Deng et al. 2018b) and field studies (Lippincott et al. 2015; Chu et al. 2018) have also confirmed that propanotrophic bacteria are very effective at degrading 14D to low μg L^{-1} concentrations and have the added capability of simultaneously degrading chlorinated solvent co-contaminants that are frequently detected with 14D in groundwater. There is also growing interest in the role of cometabolism in the natural attenuation of ethers such as 14D. To fully understand this, additional research is needed to define the kinetics of ether biodegradation reactions at low concentrations and physiological studies are needed to determine whether ethers such as 14D have their own independent regulatory effects on the expression of these oxygenases that can biodegrade this compound. Additional research is also needed to determine whether 14D-derived metabolites can contribute to the carbon nutrition of microorganisms that can otherwise not grow on this compound and the interspecies interactions that can be established through metabolite exchange between bacteria that can cometabolically degrade 14D and those that can grow on excreted metabolites generated from this process.

References

Agency for Toxic Substances and Disease Registry (ATSDR) (2017) Toxicological profile for bis(2-chloroethyl)ether (BCEE). https://www.atsdr.cdc.gov/toxprofiles/tp127.pdf

Barajas-Rodriguez FJ, Freedman DL (2018) Aerobic biodegradation kinetics for 1,4-dioxane under metabolic and cometabolic conditions. J Hazard Mater 350:180–188

Bastida F, Rosell M, Franchini AG, Seifert J, Finsterbusch S, Jehmlich N, Jechalke S, von Bergen M, Richnow HH (2010) Elucidating MTBE degradation in a mixed consortium using a multidisciplinary approach. FEMS Microbiol Ecol 73:370–384

Bednar AJ, Kirgan RA, Karn RA, Donovan B, Mohn MF, Sirkis DM (2009) Mobility and sorption of bis-2-chloroethyl ether in an aquifer material. J Hazard Mater 168:1041–1046

Belhaj A, Desnoues N, Elmerich C (2002) Alkane biodegradation in *Pseudomonas aeruginosa* strains isolated from a polluted zone: identification of *alkB* and *alkB*-related genes. Res Microbiol 153:339–344

Bennett P, Hyman M, Smith C, El Mugammar H, Chu M-Y, Nicklelsen M, Aravena R (2018) Enrichment with carbon-13 and deuterium during monooxygenase-mediated biodegradation of 1,4-dioxane. Environ Sci Technol Lett 5:148–153

Bock C, Kroppenstedt RM, Diekmann H (1996) Degradation and bioconversion of aliphatic and aromatic hydrocarbons by *Rhodococcus ruber* 219. Appl Microbiol Biotechnol 45:408–410

Bravo AL, Sigala JC, Borgne SL, Morales M (2015) Expression of an alkane hydroxylase (*alkb*) gene and methyl-*tert*-butyl ether co-metabolic oxidation in *Pseudomonas citronellolis*. Biotechnol Lett 37:807–814

Burback BL, Perry JJ (1993) Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. Appl Environ Microbiol 59:1025–1029

Cappelletti M, Presentato A, Milazzo G, Turner RJ, Fedi S, Frascari D, Zannoni D (2015) Growth of *Rhodococcus* sp. strain BCP1 on gaseous *n*-alkanes: new metabolic insights and transcriptional analysis of two soluble di-iron monooxygenase genes. Front Microbiol 6. https://doi.org/10.3389/fmicb.2015.00393

Chauvaux S, Chevalier F, Le Dantec C, Fayolle F, Miras I, Kunst F, Béguin P (2001) Cloning of a genetically unstable cytochrome P-450 gene cluster involved in degradation of the pollutant ethyl *tert*-butyl ether by *Rhodococcus ruber*. J Bacteriol 183:6551–6557

Chen D-Z, Jin X-J, Chen J, Ye J-X, Jiang N-X, Chen J-M (2016) Intermediates and substrate interaction of 1,4-dioxane degradation by effective metabolizer *Xanthobacter flavus* DT8. Int Biodeterior Biodegrad 106:133–140

Chu M-YJ, Bennett PJ, Dolan ME, Hyman MR, Peacock AD, Bodour A, Andersen RH, Mackay DM, Goltz MN (2018) Concurrent treatment of 1,4-dioxane and chlorinated aliphatics in a groundwater recirculation system via aerobic cometabolism. Groundwater Monit Remediat 38:53–64

Colby J, Stirling DI, Dalton H (1977) The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. Biochem J 165:395–402

Coleman NV, Le NB, Ly MA, Ogawa HE, McCarl V, Wilson NL, Holmes AJ (2012) Hydrocarbon monooxygenase in *Mycobacterium*: recombinant expression of a member of the ammonia monooxygenase superfamily. ISME J 6:171–182

Curry S, Ciuffetti L, Hyman M (1996) Inhibition of growth of a *Graphium* sp. on gaseous *n*-alkanes by gaseous *n*-alkynes and *n*-alkenes. Appl Environ Microbiol 62:2198–2200

de Bont JAM, Mulder EG (1974) Nitrogen fixation and co-oxidation of ethylene by a methane-utilizing bacterium. J Gen Microbiol 83:113–121

Deng D, Li F, Li M (2018a) A novel propane monooxygenase initiating degradation of 1,4-dioxane by *Mycobacterium dioxanotrophicus* PH-06. Environ Sci Technol Lett 5:86–91

Deng D, Li F, Wu C, Li M (2018b) Synchronic biotransformation of 1,4-dioxane and 1,1-dicloroethylene by a Gram-negative propanotroph *Azoarcus* sp. DD4. Environ Sci Technol Lett 5:526–532

Fayolle F, Hernandez G, Roux FL, Vandecasteele J-P (1998) Isolation of two aerobic bacterial strains that degrade efficiently ethyl *t*-butyl ether (ETBE). Biotechnol Lett 20:283–286

Ferreira NL, Maciel H, Mathis H, Monot F, Fayolle-Guichard F, Greer CW (2006) Isolation and characterization of a new *Mycobacterium austroafricanum* strain, IFP 2015, growing on MTBE. Appl Microbiol Biotechnol 70:358–365

Ferreira NL, Mathis H, Labbé D, Monot F, Greer CW, Fayolle-Guichard F (2007) *N*-alkane assimilation and *tert*-butyl alcohol (TBA) oxidation capacity in *Mycobacterium austroafricanum* strains. Appl Microbiol Biotechnol 75:909–919

Fisher CR, Childress JJ, Oremland RS, Bidigare RR (1987) The importance of methane and thiosulfate in the metabolism of the bacterial symbionts of two deep-sea mussels. Mar Biol 96:59–71

Fournier D, Hawari J, Halasz A, Streger SH, McClay KR, Masuda H, Hatzinger PB (2009) Aerobic biodegradation of *N*-nitrosodimethylamine by the propanotroph *Rhodococcus ruber* ENV425. Appl Environ Microbiol 75:5088–5093

François A, Mathis H, Godefroy D, Piveteau P, Fayolle F, Monot F (2002) Biodegradation of methyl *tert*-butyl ether and other fuel oxygenates by a new strain, *Mycobacterium austroafricanum* IFP 2012. Appl Environ Microbiol 68:2754–2762

François A, Garnier L, Mathis H, Fayolle F, Monot F (2003) Roles of *tert*-butyl formate, *tert*-butyl alcohol and acetone in the regulation of methyl *tert*-butyl ether degradation by *Mycobacterium austroafricanum* IFP 2012. Appl Microbiol Biotechnol 62:256–262

Garbe LA, Moreno-Horn M, Rewicki D, Tressl R, Görisch H (2004) Microbial desaturation of bis (1-chloro-2-propyl) ether into a dichloro vinyl ether. Chembiochem 5:876–878

Garbe LA, Moreno-Horn M, Tressl R, Görsich H (2006) Preferential attack of the (S)-configured ether-linked carbons in bis-(1-chloro-2-propyl) ether by *Rhodococcus* sp. strain DTB. FEMS Microbiol Ecol 55:113–121

Garnier PM, Auria R, Augur C, Revah S (1999) Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane. Appl Microbiol Biotechnol 51:498–503

Garnier PM, Auria R, Augur C, Revah S (2000) Cometabolic biodegradation of methyl *tert*-butyl ether by a soil consortium: effect of components present in gasoline. J Gen Appl Microbiol 46:79–84

Gedalanga PB, Pornwongthing P, Mora R, Chiang S-YD, Baldwin B, Ogles D, Mahendra S (2014) Identification of biomarker genes to predict biodegradation of 1,4-dioxane. Appl Environ Microbiol 80:3209–3218

Gedalanga P, Madison A, Miao YR, Richards T, Hatton J, DiGuiseppi WH, Wilson J, Mahendra S (2016) A multiple lines of evidence framework to evaluate intrinsic biodegradation of 1,4-dioxane. Remediat J 27:93–114

Hanson JR, Ackerman CE, Scow KM (1999) Biodegradation of methyl *tert*-butyl ether by a bacterial pure culture. Appl Environ Microbiol 65:4788–4792

Hardison LK, Curry SS, Ciuffetti LM, Hyman MR (1997) Metabolism of diethyl ether and cometabolism of methyl *tert*-butyl ether by a filamentous fungus, a *Graphium* sp. Appl Environ Microbiol 63:3059–3067

Hatzinger PB, McClay K, Vainberg S, Tugusheva M, Condee CW, Steffan RJ (2001) Biodegradation of methyl *tert*-butyl ether by a pure bacterial isolate. Appl Environ Microbiol 67:5601–5607

Hatzinger PB, Banerjee R, Rezes R, Streger SH, McClay K, Schaefer CE (2017) Potential for cometabolic biodegradation of 1,4-dioxane in aquifers with methane or ethane as primary substrates. Biodegradation 28:453–468

Hauck R, Adrian L, Wendelr P, Amidjojo M, Hegemann W, Görisch H (2001) Transformation of 2,2′-dichlorodiisopropyl ether in mixed and pure culture. Appl Microbiol Biotechnol 56:491–495

Hazeu W (1975) Some cultural and physiological aspects of methane-utilizing bacteria. Antonie Van Leeuwenhoek 41:121–134

He Z, YaoY LZ, Ye Y (2014) Dynamic metabolic and transcriptional profiling of *Rhodococcus* sp. strain YYL during the degradation of tetrahydrofuran. Appl Environ Microbiol 80:2656–2664

He Y, Wei K, Si K, Mathieu J, Li M, Alvarez PJJ (2017a) Whole genome sequence of the 1,4-dioxane-degrading bacterium *Mycobacterium dioxanotrophicus* PH-06. Genome Announc 5: e00625-17

He Y, Mathieu J, Yang Y, Yu P, Da Silva MLB, Alvarez PJJ (2017b) 1,4-Dioxane biodegradation by *Mycobacterium dioxanotrophicus* PH-06 is associated with a group 6 soluble di-iron monooxygenase. Environ Sci Technol Lett 4:494–499

He Y, Mathieu J, da Silva MLB, Li M, Alvarez PJJ (2018) 1,4-Dioxane-degrading consortia can be enriched from uncontaminated soils: prevalence of *Mycobacterium* and soluble di-iron monooxygenase genes. Microb Biotechnol 11:189–198

Hernandez-Perez G, Fayolle F, Vandecasteele J-P (2001) Biodegradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE) and t-amyl ether (TAME) by *Gordonia terrae*. Appl Microbiol Biotechnol 55:117–121

Higgins IJ, Hammond RC, Sariaslani FS, Best D, Davies MM, Tryhorn SE, Taylor F (1979) Biotransformation of hydrocarbons and related compounds by whole organism suspensions of methane-grown *Methylosinus trichosporium* OB3b. Biochem Biophys Res Commun 89:671–677

Hine J, Mookerjee PK (1975) The intrinsic hydrophilic character of organic compounds. Correlations in terms of structural contributions. J Org Chem 40:292–298

Hofrichter M, Ullrich R (2014) Oxidations catalyzed by fungal peroxygenases. Curr Opin Chem Biol 19:116–125

House AJ, Hyman MR (2010) Effects of gasoline components on MTBE and TBA cometabolism by *Mycobacterium austroafricanum* JOB5. Biodegradation 21:525–541

Hristova KR, Schmidt R, Chakicherla AY, Legler TC, Wu J, Chain PS, Scow KM, Kane SR (2007) Comparative transcriptome analysis of *Methylibium petroleiphilum* PM1 exposed to the fuel oxygenates methyl *tert*-butyl ether and ethanol. Appl Environ Microbiol 73:7347–7357

Huang H, Shen D, Li N, Shan D, Shentu J, Zhou Y (2014) Biodegradation of 1,4-dioxane by a novel strain and its biodegradation pathway. Water Air Soil Pollut 225:2135–2146

Hur H-G, Newman LM, Wackett LP, Sadowsky MJ (1997) Toluene-2-monooxygenase-dependent growth of *Burkholderia cepacia* G4/PR1 on diethyl ether. Appl Environ Microbiol 63:1606–1609

Hyman MR (2009) Aerobic cometabolism of ether-bonded compounds. Final report. United State Environmental Protection Agency grant # R823426. https://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/673/report/F

Hyman MR, Page CL, Arp DJ (1994) Oxidation of methyl fluoride and dimethyl ether by ammonia monooxygenase in *Nitrosomonas europaea*. Appl Environ Microbiol 60:3033–3035

Inoue D, Tsunoda T, Sawada K, Yamamoto N, Saito Y, Sei K, Ike M (2016) 1,4-Dioxane degradation potential of members of the genera *Pseudonocardia* and *Rhodococcus*. Biodegradation 27:277–286

Inoue D, Tsunoda T, Yamamoto N, Ike M, Sei K (2018) 1,4-Dioxane degradation characteristics of *Rhodococcus aetherivorans* JCM 14343. Biodegradation 29:301–310

Interstate Technology & Regulatory Council (ITRC) (2005) Overview of groundwater remediation technologies for MTBE and TBA. MTBE-1. Interstate Technology & Regulatory Council, MTBE and Other Fuel Oxygenates Team, Washington, DC

Janssen DB, Gerritse J, Brackman J, Kalk C, Jager D, Witholt B (1988) Purification and characterization of a bacterial dehalogenase with activity toward halogenated alkanes, alcohols, and ethers. Eur J Biochem 171:67–72

Jin X-J, Chen D-Z, Zhu RY, Chen J, Chen J-M (2012) Characteristics of 1,4-dioxane degradation by *Xanthobacter flavus* DT8. Environ Sci 33:1657–1662

Kane SR, Chakicherla AY, Chain PSG, Schmidt R, Shin MW, Legler TC, Scow KM, Larimer FW, Lucas SM, Richardson PM, Hristova KR (2007) Whole-genome analysis of the methyl-*tert*-butyl ether-degrading beta-proteobacterium *Methylibium petroleiphilum* PM1. J Bacteriol 189:1931–1945

Katapodis AG, Wimalesena K, Lee J, May SW (1984) Mechanistic studies on non-heme iron monooxygenase catalysis: epoxidation, aldehyde formation, and demethylation by the ω-hydroxylation system of *Pseudomonas oleovorans*. J Am Chem Soc 110:7928–7935

Kim Y-H, Engesser K-H (2004) Degradation of alkyl ethers, aralkyl ethers, and dibenzyl ether by *Rhodococcus* sp. Strain DEE5151, isolated from diethyl ether-containing enrichment cultures. Appl Environ Microbiol 70:4398–4401

Kim Y-H, Engesser K-H (2005) Inhibition of diethyl ether degradation by *Rhodococcus* sp. strain DEE5151 by glutaraldehyde and ethyl vinyl ether. FEMS Microbiol Lett 243:317–322

Kim Y-H, Engesser K-H, Kim S-J (2007) Physiological, numerical and molecular characterization of alkyl ether-utilizing rhodococci. Environ Microbiol 9:1497–1510

Kim Y-H, Cha C-J, Engesser K-H, Kim S-J (2008) Degradation of various alkyl ethers by alkyl ether-degrading *Actinobacteria* isolated from activated sludge of a mixed wastewater treatment. Chemosphere 73:1442–1447

Kim Y-M, Jeon J-R, Murugesan K, Kim E-J, Chang Y-S (2009) Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp. PH-06. Biodegradation 20:511–519

Kinne M, Poraj-Kobielska M, Ralph SA, Ullrich R, Hofrichter M, Hammel KE (2009) Oxidative cleavage of diverse ethers by an extracellular fungal peroxygenase. J Biol Chem 284:29343–29349

Kohlweyer U, Thiemer B, Schräder T, Andreesen JR (2000) Tetrahydrofuran degradation by a newly isolated culture of *Pseudonocardia* sp. strain K1. FEMS Microbiol Lett 186:301–306

Kotani T, Yamamoto T, Yurimoto H, Sakai Y, Kato N (2003) Propane monooxygenase and NAD^+-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. J Bacteriol 185:7120–7128

Lan RS, Smith CA, Hyman MR (2013) Oxidation of cyclic ethers by alkane-grown *Mycobacterium vaccae* JOB5. Remediat J 23:23–42

Lechner U, Brodkorb D, Geyer R, Hause G, Härtig C, Auling G, Fayolle-Guichard F, Piveteau P, Müller RH, Rohwerder T (2007) *Aquincola tertiaricarbonis* gen. nov., sp. nov., a *tertiary* butyl moiety-degrading bacterium. Int J Syst Evol Microbiol 57:1295–1303

Li M, Mathieu J, Liu Y, van Orden ET, Yang Y, Fiorenza S, Alvarez PJJ (2013) The abundance of tetrahydrofuran/dioxane monooxygenase genes (*thmA*/*dxmA*) and 1,4-dioxane degradation activity are significantly correlated at various impacted sites. Environ Sci Technol Lett 1:122–127

Li S, Wang S, Yan W (2016) Biodegradation of methyl *tert*-butyl ether by co-metabolism with a *Pseudomonas* sp. strain. Int J Environ Res Public Health 13:883

Lippincott D, Streger SH, Schafer CE, Hinkle J, Stormo J, Steffan RJ (2015) Bioaugmentation and propane biosparging for *in situ* biodegradation of 1,4-dioxane. Groundwater Monit Remediat 35:81–92

Liu CY, Speitel GE Jr, Georgiou G (2001) Kinetics of methyl *t*-butyl ether cometabolism at low concentrations by pure cultures of butane-degrading bacteria. Appl Environ Microbiol 67:2197–2201

Mahendra S, Alvarez-Cohen L (2005) *Pseudonocardia dioxanivorans* sp. nov., a novel actinomycete that grows on 1,4-dioxane. Int J Syst Evol Microbiol 55:593–598

Mahendra S, Alvarez-Cohen L (2006) Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. Environ Sci Technol 40:5435–5442

Mahendra S, Petzold CJ, Baidoo EE, Keasling JD, Alvarez-Cohen L (2007) Identification of the intermediates of *in vivo* oxidation of 1,4-dioxane by monooxygenase-containing bacteria. Environ Sci Technol 41:7330–7336

Malandain C, Fayolle-Guichard F, Vogel TM (2010) Cytochrome P450-mediated degradation of fuel oxygenates by environmental isolates. FEMS Microbiol Ecol 72:289–296

Masuda H, McClay K, Steffan RJ, Zylstra GJ (2012) Biodegradation of tetrahydrofuran and 1,4-dioxane by soluble diiron monooxygenase in *Pseudonocardia* sp. strain ENV478. J Mol Microbiol Biotechnol 22:312–316

Matsui R, Takagi K, Sakakibara F, Abe T, Shiiba K (2016) Identification and characterization of 1,4-dioxane-degrading microbe separated from surface water by the seawater-charcoal perfusion apparatus. Biodegradation 27:155–163

McClay K, Schaefer CE, Vainberg S, Steffan RJ (2007) Biodegradation of bis(2-chloroethyl) ether by *Xanthobacter* sp. strain ENV481. Appl Environ Microbiol 73:6870–6875

McKelvie JR, Hyman MR, Elsner M, Smith C, Aslett DM, Lacrampe-Couloume G, Sherwood-Lollar B (2009) Isotopic fractionation of methyl *tert*-butyl ether suggests different initial reaction mechanisms during aerobic biodegradation. Environ Sci Technol 43:2793–2799

Meyers AJ (1982) Obligate methylotrophy: evaluation of dimethyl ether as C_1 compound. J Bacteriol 150:966–968

Miller LE, Coutlakis MD, Oremland RS, Ward BB (1993) Selective inhibition of ammonium oxidation and nitrification-linked N_2O formation by methyl fluoride and dimethyl ether. Appl Environ Microbiol 59:2457–2464

Mohr T, Stickney J, Diguiseppi B (2010) Environmental investigation and remediation: 1,4-dioxane and other solvent stabilizers. CRC Press, Boca Raton

Morales M, Nava V, Velásquez E, Razo-Flores E, Revah S (2009) Mineralization of methyl *tert*-butyl ether and other gasoline oxygenates by Pseudomonads using short chain *n*-alkanes as growth source. Biodegradation 20:271–280

Moreno-Horn M, Garbe L-A, Tressl R, Adrian L, Görisch H (2003) Biodegradation of bis(1-chloro-2-propyl) ether via initial ether scission and subsequent dehalogenation by *Rhodococcus* sp. strain DTB. Arch Microbiol 179:234–241

Moreno-Horn M, Garbe L-A, Tressl R, Görisch H (2005) Transient accumulation of γ-butyrolactone during degradation of bis(4-chloro-*n*-butyl) ether by diethyl ether-grown *Rhodococcus* sp. strain DTB. Appl Microbiol Biotechnol 69:335–340

Müller RH, Rohwerder T, Harms H (2007) Carbon conversion efficiency and limits or productive degradation of methyl *tert*-butyl ether and related compounds. Appl Environ Microbiol 73:1783–1791

Müller RH, Rohwerder T, Harms H (2008) Degradation of fuel oxygenates and their main intermediates by *Aquincola tertiaricarbonis* L108. Microbiology 154:1414–1421

Nakamiya K, Hashimoto S, Ito H, Edmonds JS, Morita M (2005) Degradation of 1,4-dioxane and cyclic ethers by an isolated fungus. Appl Environ Microbiol 71:1254–1258

Nakatsu CH, Hristova K, Hanada S, Meng X-Y, Hanson JR, Scow KM, Kamagata Y (2006) *Methylibium petroleiphilum* gen nov., sp. nov., a novel methyl *tert*-butyl ether-degrading methylotroph of the Betaproteobacteria. Int J Syst Evol Microbiol 56:983–989

Newman LM, Wackett LP (1995) Purification and characterization of toluene-2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry 34:14066–14076

Oremland RS, Culbertson CW (1992) Evaluation of methyl fluoride and dimethyl ether as inhibitors of aerobic methane oxidation. Appl Environ Microbiol 58:2983–2992

Parales RE, Adamus JE, White N, May HD (1994) Degradation of 1,4-dioxane by an actinomycete in pure culture. Appl Environ Microbiol 60:4527–4530

Patel RN, Hou CT, Felix A (1976) Inhibition of dimethyl ether and methane oxidation in *Methylococcus capsulatus* and *Methylosinus trichosporium*. J Bacteriol 126:1017–1019

Payne WD, Collette TW (1989) Identification of bis(2-chloroethyl) ether hydrolysis products by direct aqueous injection GC/FT-IR. J High Resolut Chromatogr 12:693–696

Pugazhendi A, Banu JR, Dhavamani J, Yeom IT (2015) Biodegradation of 1,4-dioxane by *Rhodanobacter* AYS5 and the role of additional substrates. Ann Microbiol 65:2210–2208

Redmond MC, Valentine DL, Sessions LA (2010) Identification of novel methane-ethane-, and propane-oxidizing bacteria at marine hydrocarbon seeps by stable isotope probing. Appl Environ Microbiol 76:6412–6422

Ribbons DW (1975) Oxidation of C_1-compounds by particulate fractions from *Methylococcus capsulatus*: distribution and properties of methane-dependent reduced nicotinamide adenine dinucleotide oxidase (methane hydroxylase). J Bacteriol 122:1351–1363

Salazar M, Morales M, Revah S (2012) Biodegradation of methyl tert-butyl ether by cometabolism with hexane in biofilters inoculated with *Pseudomonas aeruginosa*. J Environ Sci Health A Tox Hazard Subst Environ Eng 47:1017–1026

Sales CM, Mahendra S, Grostern A, Parales R, Goodwin LA, Woyke T, Nolan M, Lapidus A, Chertkov O, Ovchinnikova G, Sczyrba A, Alvarez-Cohen L (2011) Genome sequence of the 1,4-dioxane-degrading *Pseudonocardia dioxanivorans* strain CB1190. J Bacteriol 193:4549–4550

Sales CM, Grostern A, Parales JV, Parales RE, Alvarez-Cohen L (2013) Oxidation of cyclic ethers 1,4-dioxane and tetrahydrofuran by a monooxygenase in two *Pseudonocardia* species. Appl Environ Microbiol 72:7702–7708

Sayavedra-Soto LA, Hamamura N, Liu CW, Kimbrel JA, Chang JH, Arp DJ (2011) The membrane-associated monooxygenase in the butane-oxidizing Gram-positive bacterium *Nocardiodes* sp. strain CF8 is a novel member of the AMO/PMO family. Environ Microbiol Rep 3:390–396

Schmidt R, Battaglia V, Scow K, Kane S, Hristova KR (2008) Involvement of a novel enzyme, MdpA, in methyl-*tert*-butyl ether degradation in *Methylibium petroleiphilum* PM1. Appl Environ Microbiol 74:6631–6638

Schuster J, Schäfer F, Hübler N, Brandt A, Rosell M, Härtig C, Harms H, Müller RH, Rohwerder T (2012) Bacterial degradation of *tert*-amyl alcohol proceeds *via* hemiterpene 2-methyl-3-buten-2-ol by employing the *tertiary* alcohol desaturase function of the Rieske nonheme mononuclear iron oxygenase MdpJ. J Bacteriol 194:972–981

Schuster J, Purswani J, Breuer U, Pozo C, Harms H, Müller RH, Rohwerder T (2013) Constitutive expression of the cytochrome P450 *ethABCD* monooxygenase system enables degradation of synthetic dialkyl ethers in *Aquincola tertiaricarbonis* L108. Appl Environ Microbiol 79:2321–2327

Sei K, Miyagaki K, Kakinoki T, Fukugasako K, Inoue D, Ike M (2013a) Isolation and characterization of bacterial strains that have high ability to degrade 1,4-dioxane as a sole carbon and energy source. Biodegradation 24:665–674

Sei K, Oyama M, Kakinoki T, Inoue D, Ike M (2013b) Isolation and characterization of tetrahydrofuran-degrading bacteria for 1,4-dioxane-containing wastewater treatment by cometabolic degradation. J Water Environ Technol 11:11–19

Semelsberger TA, Borup RL, Greene HL (2006) Dimethyl ether (DME) as an alternative fuel. J Power Sources 156:497–511

Sharp JO, Sales CM, LeBlanc JC, Liu J, Wood TK, Eltis LD, Mohn WW, Alvarez-Cohen L (2007) An inducible propane monooxygenase is responsible for *n*-nitrosodimethylamione degradation by *Rhodococcus* sp. strain RHA1. Appl Environ Microbiol 73:6930–6938

Sharp JO, Sales CM, Alvarez-Cohen L (2010) Functional characterization of propane-enhanced *N*-nitrosodimethylamine degradation by two actinomycetales. Biotechnol Bioeng 107:924–932

Shields MS, Reagin MJ (1992) Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl Environ Microbiol 58:3977–3983

Skinner KM, Martinez-Prado A, Hyman MR, Williamson KJ, Ciuffetti LM (2008) Pathway, inhibition and regulation of methyl *tertiary* butyl ether oxidation in a filamentous fungus, *Graphium* sp. Appl Microbiol Biotechnol 77:1359–1365

Skinner K, Cuifetti L, Hyman M (2009) Metabolism and cometabolism of cyclic ethers by a filamentous fungus, a *Graphium* sp. Appl Environ Microbiol 75:5514–5522

Smith CA, Hyman MR (2004) Oxidation of methyl *tert*-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and n-octane-grown *Pseudomonas putida* GPo1. Appl Environ Microbiol 70:4544–4550

Smith CA, Hyman MR (2010) Oxidation of gasoline oxygenates by closely related non-haem-iron alkane hydroxylases in *Pseudomonas mendocina* KR1 and other n-octane-utilizing *Pseudomonas* strains. Environ Microbiol Rep 2:426–432

Smith CA, O'Reilly KT, Hyman MR (2003a) Characterization of the initial reactions during the cometabolic oxidation of methyl *tert*-butyl ether by propane-grown *Mycobacterium vaccae* JOB5. Appl Environ Microbiol 69:796–804

Smith CA, O'Reilly KT, Hyman MR (2003b) Cometabolism of methyl *tertiary* butyl ether and gaseous *n*-alkanes by *Pseudomonas mendocina* KR-1 grown on C5-C8 *n*-alkanes. Appl Environ Microbiol 69:7385–7394

Steffan RJ, McClay K, Vainberg S, Condee CW, Zhang D (1997) Biodegradation of the gasoline oxygenates methyl *tert*-butyl ether, ethyl *tert*-butyl ether, and *tert*-amyl methyl ether by propane-oxidizing bacteria. Appl Environ Microbiol 63:4216–4222

Stirling DI, Dalton H (1980) Oxidation of dimethyl ether, methylformate and bromomethane by *Methylococcus capsulatus* (Bath). J Gen Microbiol 116:277–283

Tajima T, Hayashida N, Matsumura R, Omura A, Nakashimada Y, Kato J (2012) Isolation and characterization of tetrahydrofuran-degrading *Rhodococcus aetherivorans* strain M8. Process Biochem 47:1665–1669

Thiemer B, Andressen JR, Schräder T (2003) Cloning and characterization of a gene cluster involved in tetrahydrofuran degradation in *Pseudonocardia* sp. strain K1. Arch Microbiol 179:266–277

Trippe KM, Wolpert TJ, Hyman MR, Ciuffetti LM (2014) RNAi silencing of a cytochrome P450 monooxygenase disrupts the ability of the filamentous fungus, *Graphium* sp., to grow on short-chain gaseous alkanes and ethers. Biodegradation 25:137–151

Tupa PR, Masuda H (2018a) Genomic analysis of propane metabolism in methyl *tert*-butyl ether-degrading *Mycobacterium* sp. strain ENV421. J Genomics 6:24–29

Tupa PR, Masuda H (2018b) Draft genome sequence of a propanotroph, *Rhodococcus* sp. strain ENV425, capable of degrading methyl *tert*-butyl ether and *N*-nitrosodimethylamine. Genome Announc 6:1–2

Tupa PR, Masuda H (2018c) Comparative proteomic analysis of propane metabolism in *Mycobacterium* sp. strain ENV421 and *Rhodococcus* sp. strain ENV425. J Mol Microbiol Biotechnol 28:107–115

U.S. Environmental Protection Agency (2002) Bis(chloroethyl)ether (BCEE); CASRN 111-44-4. Integrated Risk Information System

U.S. Environmental Protection Agency (2016) 1,4-Dioxane (CASRN 123–91-1) – Report on carcinogens, 14th edn

U.S. Environmental Protection Agency (2017) Technical fact sheet – 1,4- dioxane (EPA 505-F-17-011)

U.S. Environmental Protection Agency (2014) Priority Pollutant list. https://www.epa.gov/sites/production/files/2015-09/documents/priority-pollutant-list-epa.pdf

Vainberg S, McClay K, Masuda H, Root D, Condee C, Zylstra GJ, Steffan RJ (2006) Biodegradation of ether pollutants by *Pseudonocardia* sp. ENV478. Appl Environ Microbiol 72:5218–5224

van Beilen JB, Kingma J, Witholt B (1994) Substrate specificity of the alkane hydroxylase system in *Pseudomonas oleovorans* GPo1. Enzym Microb Technol 16:904–911

van den Wijngaard AJ, Prins J, Smal AJAC, Janssen D (1993) Degradation of 2-chloroethyl vinyl ether by *Ancyclobacter aquaticus* AD25 and AD27. Appl Environ Microbiol 59:2777–2783

Vomberg A, Klinner U (2000) Distribution of *alkB* genes within *n*-alkane-degrading bacteria. J Appl Microbiol 89:339–348

White G, Russell N, Tidswell E (1996) Bacterial scission of ether bonds. Microbiol Rev 60:216–232

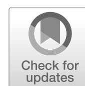
Wilkinson JF (1971) Hydrocarbons as a source of single cell protein. Symp Soc Gen Microbiol 21:15–46

Wilkinson JF (1975) Physiological studies of bacteria grown on methane. In: Microbial growth on C_1 compounds. The Society of Fermentation Technology, Tokyo, pp 45–58

Yao Y, Lu Z, Zhu F, Min H (2013) Evidence for horizontal gene transfer of a tetrahydrofuran monooxygenase: cloning and analysis of a gene cluster for tetrahydrofuran degradation in *Rhodococcus* sp. YYL. Afr J Microbiol Res 7:1809–1818

Yamamoto N, Saito Y, Inoue D Sei K, Ike M (2018) Characterization of a newly isolated Pseudonocardia sp. N23 with high 1,4-dioxane-degrading ability. J Biosci Bioeng 125:552–558

Zhou Y, Huang H, Shen D (2016) Multi-substrate biodegradation interaction of 1,4-dioxane and BTEX mixtures by *Acinetobacter baumannii* DD1. Biodegradation 27:37–46



Plastic Biodegradation: Challenges and Opportunities

18

Nick Wierckx, Tanja Narancic, Christian Eberlein, Ren Wei, Oliver Drzyzga, Audrey Magnin, Hendrik Ballerstedt, Shane T. Kenny, Eric Pollet, Luc Avérous, Kevin E. O'Connor, Wolfgang Zimmermann, Hermann J. Heipieper, Auxiliadora Prieto, José Jiménez, and Lars M. Blank

Contents

1 Introduction 334
2 Natural Fates of Plastics 337
 2.1 Biodegradable Plastics 337
 2.2 Non-biodegradable Plastics 340
3 Plastic-Degrading Enzymes 342
 3.1 Cutinases 344
 3.2 Lipases 346

N. Wierckx (✉) · H. Ballerstedt · L. M. Blank
Institute of Applied Microbiology (iAMB), RWTH Aachen University, Aachen, Germany
e-mail: nick.wierckx@rwth-aachen.de; hendrik.ballerstedt@rwth-aachen.de; Lars.Blank@rwth-aachen.de

T. Narancic
UCD Earth Institute and School of Biomolecular and Biomedical Science, University College Dublin, Belfield, Dublin, Ireland
e-mail: Tanja.narancic@ucd.ie

C. Eberlein · H. J. Heipieper
Department of Environmental Biotechnology, Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany
e-mail: christian.eberlein@ufz.de; hermann.heipieper@ufz.de

R. Wei · W. Zimmermann
Department of Microbiology and Bioprocess Technology, Institute of Biochemistry, Leipzig University, Leipzig, Germany
e-mail: wei@uni-leipzig.de; wolfgang.zimmermann@uni-leipzig.de

O. Drzyzga · A. Prieto
Biological Research Center (CIB-CSIC), Madrid, Spain
e-mail: drzyzga@cib.csic.es; auxi@cib.csic.es

A. Magnin · E. Pollet · L. Avérous
BioTeam/ICPEES-ECPM, UMR CNRS 7515, Université de Strasbourg, Strasbourg, France
e-mail: audrey.magnin@etu.unistra.fr; eric.pollet@unistra.fr; luc.averous@unistra.fr

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_23

3.3 Carboxylesterases 346
3.4 Proteases 347
3.5 Lignin-Modifying Enzymes 347
4 Thermochemical Plastic Depolymerization 347
4.1 Integrating Thermochemical Degradation of Plastic Waste into Biological Upcycling Processes 348
4.2 Biological Conversion Processes of Syngas from Pyrolyzed Plastic Waste 349
5 Research Needs 350
References 352

Abstract

Plastics are extremely useful materials that have transformed our society in a myriad of ways. However, the widespread use of these materials has led to a staggering amount of plastic pollution in man-made and natural environments. The biodegradation of plastics is a key factor to reduce the impact of this plastic pollution. On the one hand, organisms are emerging that can degrade relatively recalcitrant plastics. On the other hand, biodegradable plastics are being developed that are intrinsically more amenable to microbial attack. In this chapter we provide an overview of the natural fates of these two types of plastics, the molecular bonds that occur in them, and the enzymatic activities associated with their degradation. Finally, an outlook is provided for the biotechnological utilization of plastics waste as a substrate, either using these enzymes or through thermochemical pretreatment.

1 Introduction

To date 8.3 billion metric tons (MT) of virgin plastics have been produced (Geyer et al. 2017), of which 6.3 billion MT became waste. Of this waste about 9% has been recycled, while 12% was incinerated. The other 79% of plastic waste is stored in landfills or was directly released into the environment (Geyer et al. 2017). Worryingly, according to the report "The New Plastic Economy: Rethinking the future of plastics" by the Ellen MacArthur Foundation, approximately 40% of the remaining plastic

S. T. Kenny
Bioplastech Ltd., Nova UCD, Belfield Innovation Park, University College Dublin, Belfield, Dublin, Ireland
e-mail: skenny@bioplastech.eu

K. E. O'Connor
UCD Earth Institute and School of Biomolecular and Biomedical Science, University College Dublin, Belfield, Dublin, Ireland

BEACON – Bioeconomy Research Centre, University College Dublin, Belfield, Dublin, Ireland
e-mail: kevin.oconnor@ucd.ie

J. Jiménez
Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK
e-mail: j.jimenez@surrey.ac.uk

waste is landfilled with a potentially staggering 32% directly released to the environment (Ellen MacArthur Foundation et al. 2016). The plastic pollution arising from mismanagement of waste has been a source of concern since the 1970s. Since then, plastic production has greatly increased and so has the pollution of marine environments (Derraik 2002). Plastic polymers are found in all oceanic niches ranging from beaches and the surfaces to shallow seabed and abysses (Barnes et al. 2009; Ryan et al. 2009). Plastic materials are dangerous to marine species due to entanglement and ingestion of litter (Derraik 2002) and also due to toxicological effects of the microplastic particles resulting from the degradation of larger polymers. These particles tend to facilitate the aggregation of other organic materials and contaminants changing their bioavailability, which results in the loss of fitness of marine species through decreased feeding, fecundity, and growth (Galloway et al. 2017).

The sources of marine pollution vary, and although initial reports pointed to fishing and merchant fleets (Horsman 1982), recreational boats (UNESCO 1994), and beachgoers (Pruter 1987) as the largest polluters, it is unlikely that the combination of these sources accounts for the amount of plastic disposed of in the ocean. Recent efforts modeling global inputs of plastics into the ocean estimate that the contribution due to mismanagement of waste in coastal countries was 12.7 million MT (Jambeck et al. 2015) in 2010. Fresh waters are also heavily contaminated by plastics (Dris et al. 2015), and rivers contribute an estimated 1.4–2.4 million MT to ocean plastic pollution per annum (Lebreton et al. 2017).

The recycling rates of different kinds of plastics also give an overview of the magnitude of the problem. When considering plastic used for packaging, polyethylene terephthalate (PET) has the highest rate of recovery (70% in Europe), although only 7% of it is recycled bottle to bottle (World Economic Forum et al. 2015). In 2015 the USA only recycled 30% of PET bottles, whereas bottles made of high-density polyethylene (HDPE) had a slightly higher recycling rate of 34.4%. The recycling rates of polypropylene (PP), low-density polyethylene (LDPE), and polyvinyl chloride (PVC) are much lower (18%, 4.1%, and 3.3%, respectively) (ACC and APR 2016). These figures are far from the recycling rates of other materials in the USA such as lead acid batteries (98.9%), steel cans (70.7%), and aluminum cans (55.1%) (US EPA 2016) and well below those of some European countries such as Germany where in 2015 thanks in part to the German "pfandpflichtige PET-Verpackungen" (PET mandatory deposit scheme) over 92% of PET bottles were recycled and 26% of the recycled "R-PET" has been used for new PET bottles (GVM 2016).

Recycling rates vary greatly depending on the country, the management policies, and the nature of the plastic item (Fig. 1). Taking Europe as an example, countries such as Malta, Cyprus, and Greece achieve recycling rates of waste plastic below 20% with no documented incineration, resulting in extremely high rates of plastics ending in landfill or environmental release. In stark contrast, nine countries in Europe have banned landfill of plastic between 1996 and 2006, and by 2014 all of these countries had achieved recycling and incineration rates over 95% (PlasticsEurope 2016). For example, Sweden has a total recycling fraction of about 30% of the postconsumer plastic recovered, while the rest is incinerated (PlasticsEurope 2016). The change in consumer awareness and political agendas

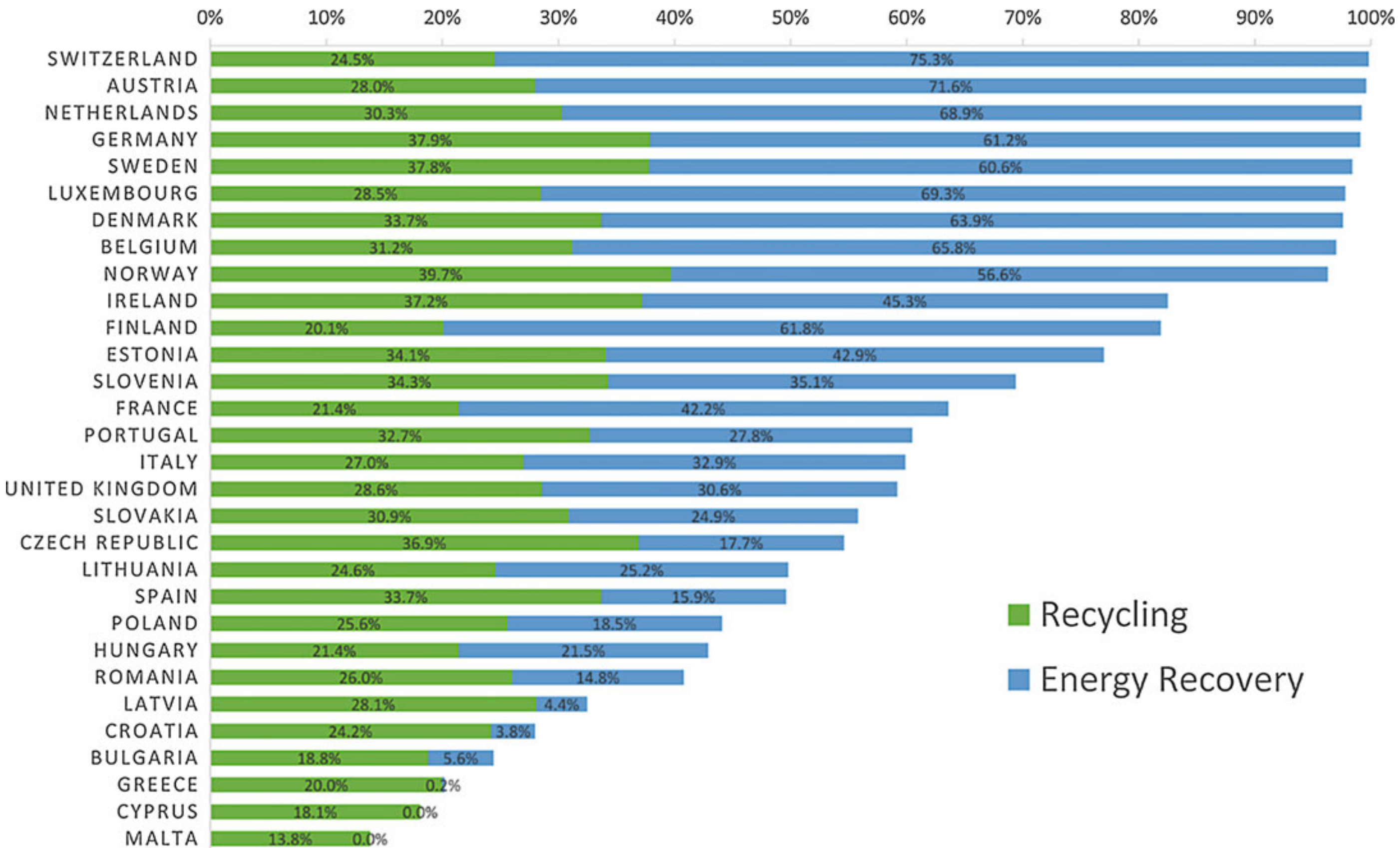


Fig. 1 Recovery of postconsumer plastic waste from the EU28 + 2 countries in 2014. Numbers are based on (PlasticsEurope 2016)

in the EU is ongoing, which can be seen in a 64% and 46% increase in recycling and incineration, respectively, alongside a 38% reduction in landfill across the EU between 2006 and 2014 (PlasticsEurope 2016). While these figures show that with proper legislation and regulation significant strides can be made in improving plastic recycling rates, they and the trends they show are highly specific to consumer packaging polymers and overlook many plastics that do not have existing recycling and collection technologies, such as polyurethanes (PU). However, only about 30% of waste plastic is recycled at best according to European figures, with all the major improvements in landfill and environmental release statistics being driven by increased rates of incineration. An obvious but important observation about plastic waste incineration is that these materials are limited in nature and costly to produce and to recover. This means that once they are incinerated, they leave the value chain, for good.

We hence require new ways of lowering the impact of waste plastic on the environment. In this chapter we discuss new value chains for plastic waste use, including ever more advanced chemical and biotechnological recycling or even upcycling routes. An alternative strategy that relies on a different material property is the use of biodegradable plastics. Biodegradable plastics can, similarly to their oil-derived counterparts, be recycled, be incinerated, or, when released to the environment, be rapidly degraded by microbial enzymes.

2 Natural Fates of Plastics

2.1 Biodegradable Plastics

Biodegradable plastics generate a lot of interest due to the ever-increasing levels of plastic waste accumulated in various environments. By definition, biodegradable plastic is decomposed by the action of living organisms. Similar to petrochemical nondegradable counterparts, biodegradable plastic is not a single material, rather a family of different materials with various properties and applications. This diversity is reflected in different biodegradation routes and rates. Polylactic acid (PLA), polyhydroxyalkanoates (PHA), and cellulose are biobased and biodegradable materials perceived as a potential solution to plastic littering. The advantage of these materials, in addition to their environmental degradation, is that they can be produced from renewable and sustainable resources (Cerrone et al. 2015; Walsh et al. 2015; Succinity 2016; Müller et al. 2017) and can also be degraded in the environment. It is worth noting that there are plastic polymers derived from oil such as polycaprolactone (PCL) and poly(butylene adipate-co-terephthalate) (PBAT), which are biodegradable. Others, like polybutylene succinate (PBS), can be both bio- and petroleum based (Succinity 2016), whereas polyethylene (PE) and polyethylene terephthalate (PET), which represented 47.2% of global bioplastic production in 2014 (European Bioplastics 2016), can be total or partially biobased but are not biodegradable. Therefore, the origin of a polymer does not determine its biodegradability characteristics.

2.1.1 Polylactic Acid

Biodegradable plastics offer new end-of-life management options creating new possibilities for a sustainable society. One of the management options that has been extensively researched is industrial composting (Emadian et al. 2017). While some polymers such as polyhydroxybutyrate (PHB) are home compostable (Greene 2014), polymers such as PLA require higher temperatures used in industrial composting (around 60 °C) to allow complete biodegradation in a reasonable time (Farah et al. 2016). The microorganisms involved in the biodegradation of bioplastics are widely distributed and can be found among aerobic and anaerobic bacteria, archaea, and eukaryotic organisms (Tokiwa and Suzuki 1981; Suyama et al. 1998; Emadian et al. 2017).

Out of the different bioplastics, PLA is a polyester widely used for packaging, and some commercial variants, such as NatureWorks' polymer Ingeo™, are certified for industrial composting (NatureWorks 2017). There are many identified enzymes active toward PLA, i.e., serine proteases from *Amycolatopsis* specific to poly(L-lactic acid) and thermophilic lipases from thermophilic *Bacillus* strains specific for poly(D-lactic acid) (Kawai 2010). However, nonenzymatic hydrolysis is still the main route of PLA degradation (Tsuji 2002), and similar biotic and abiotic PLA degradation rates demonstrated that PLA degradation is not enhanced by the presence of microorganisms (Agarwal et al. 1998). Microbial degradation of PLA is challenging for different reasons. For instance, temperatures above the glass transition temperature of the plastic (T_g = 55–62 °C) are usually required for the onset of PLA hydrolysis making its degradation difficult at ambient temperatures. Additionally, degradation rates of PLA depend on the molecular weight of the polymer, and, while high-molecular-weight PLA is mechanically stronger, it is also less susceptible to biodegradation (Ho et al. 1999).

2.1.2 Polyhydroxyalkanoates

PHAs are polyesters produced by a range of bacteria. Commercial PHAs represented less than 2% of the global bioplastic market in 2014 (European Bioplastics 2016). With over 150 known PHA monomers, (*R*)-3-hydroxyalkanoic acids, PHAs have highly diverse material properties. Their crystallinity ranges from 30% to 70%, and melting temperature ranges from 50 °C to 180 °C (Madison and Huisman 1999). They are generally divided into short-chain-length (scl) polymers, containing (*R*)-3-hydroxyalkanoic acids with four or five carbon atoms, and medium-chain-length (mcl) polymers of (*R*)-3-hydroxyalkanoic acids containing 6–12 carbon atoms (Sudesh et al. 2000). The key enzymes involved in microbial intracellular and extracellular degradation of PHA are depolymerases (Jendrossek and Handrick 2002), which can also be employed in the in vitro enzymatic degradation of PHAs (Jendrossek and Handrick 2002; Calabia and Tokiwa 2006; Rhee et al. 2006). The environmental fate of some members of the PHA family, such as PHB, has been studied in detail. For instance, polymers mainly composed of PHB or copolymers of PHB and other compounds (3-hydroxyvalerate, 4-hydroxybutyrate, or 3-hydroxyhexanoate) can be degraded to completion in soil and sludges (Wang et al. 2004; Sridewi et al. 2006; Volova et al. 2017). Other PHA polymers, such as

medium-chain-length PHA (mcl-PHA[1]) can be degraded as well although at a lower extent (3–17% weight loss) in different soils after 112 days (Lim et al. 2005).

2.1.3 Cellulose

Cellulose-based bioplastics are gaining attention due to their excellent mechanical properties and the sustainability of their life cycle (Wang et al. 2016). Cellulose is the most common biopolymer in nature accounting for 1.5 trillion tons of the annual biomass production (Klemm et al. 2002). Applications of cellulose are limited by its low solubility. However, it is possible to produce cellulose plastics using solvents such as N-methylmorpholine N-oxide or ionic liquids (Lindman et al. 2010), followed by shaping the resulting dissolved cellulose into fibers or films. Even though cellulose is ubiquitously present, it made only 1.6% of the total bioplastic production in 2014 (European Bioplastics 2016). While cellulose forms insoluble crystalline microfibrils, which are quite resistant to enzymatic hydrolysis (Beguin and Aubert 1994), regenerated cellulose was shown to completely degrade in soil in 2 months (Zhang et al. 1996) due to the action of microorganisms producing a battery of enzymes collectively named cellulases with different specificities (Glass et al. 2013).

2.1.4 Polybutylene Succinate

Polybutylene succinate (PBS) used to be produced exclusively from fossil resources. However, it is now possible to obtain a fully biobased PBS polymers by polycondensation reactions of succinic acid and 1,4-butanediol and PBS copolymers, respectively, by adding a third monomer, e.g., sebacic or adipic acid, all produced from renewable feedstocks (Bechthold et al. 2008; Babu et al. 2013). The melting temperature of PBS is similar to PE and PP, and it is less brittle than PLA (Tokiwa and Pranamuda 2002; Succinity 2016). In addition, PBS is certified as compostable under industrial conditions (Succinity 2016), and it is biodegradable by microbial lipases and cutinases mainly produced by *Actinomycetes* and fungi (Tokiwa and Pranamuda 2002; Abe et al. 2010).

2.1.5 Polycaprolactone

The petroleum-based PCL is a hydrophobic, semicrystalline polymer of good solubility, low melting temperature (59–64 °C), and good blend compatibility and as such is a very attractive candidate for applications in the biomedical field, particularly tissue engineering (Woodruff and Hutmacher 2010). There is a wide diversity of PCL-degrading microorganisms (Chen et al. 2000), which are distributed in various environments (Nishida and Tokiwa 1993; Suyama et al. 1998), and as with PBS, PCL is degraded through the enzymatic activity of lipases and cutinases.

[1]mcl-PHA monomer composition: C6:C8:C10:C12:C14:C16 = 6.9:58.4:26.7:6.5:1:0.5.

2.1.6 Polybutylene Adipate Terephthalate

Polybutylene adipate terephthalate (PBAT) is a well-known petroleum-based bioplastic. Along with PCL and PBS, it makes up 13% of bioplastics manufactured in 2014 globally (European Bioplastics 2016). This polymer is flexible and hydrophilic and can be processed easily, with typical application in packaging and the biomedical field. PBAT biodegradation was demonstrated by a thermophilic strain, *Thermomonospora fusca* (Witt et al. 2001), and one of the PBAT-containing materials, Ecoflex® used for packaging, is certified as compostable (BASF 2017).

2.2 Non-biodegradable Plastics

Many plastics are designed to be durable, i.e., to resist abiotic and microbial degradation. However, once the materials are released into the environment, their longevity turns into a problem. Generally, plastics consisting of C–C chains only or containing ether bonds connecting the building blocks are considered not easily amenable to microbial degradation. Prominent examples of such non-hydrolyzable plastics are polystyrene (PS), PP, PE, PVC, and PU. In addition to those chemical structures resisting enzymatic attacks, the low bioavailability of plastics made of synthetic polymers also hampers the initiation of biodegradation. However, their bioavailability can be increased by the action of abiotic factors like UV light and high temperatures facilitating biodegradation (Gilan et al. 2004; Hakkarainen and Albertsson 2004; Hadad et al. 2005; Krueger et al. 2017). In this sense, it is possible to enhance biodegradation by the use of material blends of combined polymers (e.g., PE and starch) (Cacciari et al. 1993), but here we will focus on the degradation of neat plastic polymers.

2.2.1 Polystyrene

The versatile polymer PS can be degraded to a certain extent. Reports on bacterial (Sielicki et al. 1978; Mor and Sivan 2008) or fungal (Kaplan et al. 1979; Krueger et al. 2017) degradation of PS revealed only minor effects on the building blocks or the polymer, respectively. In contrast to this, it was reported recently that mealworm gut bacteria metabolized almost half of the provided PS to CO_2 within 16 days (Yang et al. 2015a). However, in bacterial pure cultures of *Exiguobacterium* sp. strain YT2 isolated from mealworm gut, the ability to degrade PS pieces within 60 days dropped down to about 7% (Yang et al. 2015b). Recently, oxidative biodegradation of polystyrene sulfonate (PSS) by brown-rot basidiomycetes obtained up to 50% reduction of molecular mass number-average within 20 days. The PSS depolymerization was shown to be carried out by an extracellular hydroquinone-driven Fenton reaction (Krueger et al. 2015b). On the contrary, the very poor bioavailability of polystyrene and its inert basic structure did not allow significant degradation of PS via biologically driven Fenton chemistry (Krueger et al. 2017).

2.2.2 Polyethylene

The different reports on PE degradation should sometimes be treated with caution because often the material is weathered to facilitate the biotic process of degradation. In addition, in many investigations, LDPE is used that is more branched and of lower crystallinity than the more commonly used HDPE. The higher number of branched molecules of LDPE can improve biodegradation, although this effect is not as important as the aforementioned weathering.

Some studies reported bacterial biodegradation of PE material but treated the polymer with acid (Rajandas et al. 2012) or subjected it to photooxidation or high temperatures beforehand (Albertsson et al. 1995; Koutny et al. 2006; Fontanella et al. 2010). In addition to this, fungal biodegradation of PE films was reported mostly on pretreated material (Pometto et al. 1992; Yamada-Onodera et al. 2001; Volke-Sepulveda et al. 2002). One study on the biodegradation of PE by the thermophilic bacterium *Brevibacillus borstelensis* strain 707 revealed that within 30 days at 50 °C, weight loss of the PE material was about 11% and could almost be increased threefold when the photooxidized polymer was used (Hadad et al. 2005). The following studies all utilized untreated PE and therefore relied only on biotic degradation. For example, the strain *Rhodococcus ruber* C20 caused an 8.8% weight loss on a branched LDPE film (0.2 mm thick) within 30 days of incubation (Gilan et al. 2004; Santo et al. 2013). In the same time range, the assessed weight loss of a HDPE material was nearly 12% for *Arthrobacter* sp. and 15% for *Pseudomonas* sp. (Balasubramanian et al. 2010). Another *Pseudomonas* sp. strain mineralized up to 28.6% of the carbon of a low-molecular-weight PE material within 80 days at 37 °C (Yoon et al. 2012). As shown with PS, a PE-degrading *Bacillus* sp. strain was isolated from waxworm gut. A 60-day incubation of this strain with a PE film resulted in a loss of 10% weight of PE, and soluble putative degradation products were found in the media (Yang et al. 2014). Recently, it was found that larvae of the wax moth can also feed on bags made of PE, but a potential underlying microbial activity was not investigated (Bombelli et al. 2017).

2.2.3 Polypropylene

PP is also difficult to degrade as reflected by the low weight losses of the polymer after treatments reported by most studies. For instance, after 1 year of incubation in soil or seawater, the observed gravimetric loss was below 1% (Artham et al. 2009). Weight loss was increased to approximately 10% when PP was pretreated at high temperatures. A similar pattern was observed by the same research group after incubation of PP with *Bacillus* and *Pseudomonas* strains. After 1 year, the pretreated material lost about 2.5% in weight, whereas there was no notable difference with the untreated one (Arkatkar et al. 2010). Considerably higher degradation of pretreated PP resulting in a 10–19% weight loss after 1 year could be achieved using fungi (Jeyakumar et al. 2013). However, values dropped to 5% when untreated material was supplied.

2.2.4 Polyvinyl Chloride

So far, no successful biodegradation of polyvinyl chloride (PVC) has been reported. An impressive example of this high recalcitrance was observed when a sample of PVC was buried in soil for 32 years and no significant degradation was detected (Otake et al. 1995). Consistent with these negative results is the observation that neither an incubation in soil lasting for months (Santana et al. 2012) nor the addition to liquid fungal cultures caused significant degradation of PVC films (Ali et al. 2014).

2.2.5 Polyurethane

With a market of around 18 million tons a year, PUs are mainly used for long-term applications, including foams for furniture and insulation (soft or rigid foams based on open or closed cells, respectively). Coating, elastomers (TPU), adhesives, and sealants also use PUs. PU is mainly obtained by reactions between polyols (typically polyesters and polyethers) and polyisocyanates (aromatic and aliphatic) and is characterized by the synthesis of urethane or carbamate linkages. By varying these compounds, an infinite variety of PU architectures can be obtained. Most PUs present a complex formulation with several ingredients. PUs contain soft and hard segments, consisting of long polyols and isocyanate, respectively. According to the polyester-polyols structures, some biodegradability properties can be obtained (Krasowska et al. 2012). Numerous studies report the biodegradation of PU based on polyester-polyols by either enzymes, bacteria, or fungi (Wang et al. 1997; Russell et al. 2011; Shah et al. 2013). Polyether-polyols are, on the contrary, more resistant to biodegradation, although recent works describe the biodegradation of polyether-polyol-based PU foams by filamentous fungi from the *Cladosporium* (Álvarez-Barragán et al. 2016) or *Alternaria* genus (Matsumiya et al. 2010) using mechanisms not clearly elucidated.

3 Plastic-Degrading Enzymes

Depending on the type of chemical bonds present in the polymer, plastics such as PET, PU, and PE can be modified or even completely degraded by enzymes (Wei and Zimmermann 2017a). Their enzymatic degradability greatly depends on the type of molecular bonds present in the polymer (Table 1).

Plastics containing hydrolyzable bonds in their backbones, e.g., ester or urethane bonds, are depolymerized by polyester hydrolases, lipases, and proteases. Aromatic moieties in the backbones of, e.g., PET and PU, result in a higher resistance to biodegradation compared to their analogues containing aliphatic building blocks (Marten et al. 2005; Wei and Zimmermann 2017a,b). Synthetic polymers such as PE containing only carbon-carbon bonds in their backbones are recalcitrant to biological attack (Wei and Zimmermann 2017a), and, as explained before, their degradation in the environment has mainly been observed as the result of a combination of abiotic and biotic effects (Lucas et al. 2008). Among the biotic factors, several

Table 1 Chemical structures of typical plastics and putative degrading enzymes

Polymer name (abbreviation)	Structure	Putative enzymes
Vinyl polymers (polyolefins, styrenics, acrylates. . .)	R	
Poly(ethylene) (PE)		Laccase (EC 1.10.3.2): Santo et al. (2013); Manganese peroxidase (MnP, EC 1.11.1.13): Iiyoshi et al. (1998);
Poly(propylene) (PP)		Laccase (EC 1.10.3.2): Jeyakumar et al. (2013)
Poly(vinyl chloride) (PVC)	Cl	
Poly(styrene) (PS)		Hydroquinone peroxidase (EC 1.11.1.7): Nakamiya et al. (1997)
Poly(methyl methacrylate) (PMMA)	O O	
Polyesters	O O	
Poly(ethylene terephthalate) (PET)	O O O O	Cutinase (EC 3.1.1.74): Ronkvist et al. (2009); Wei et al. (2014a); Yoshida et al. (2016); Wei et al. (2016)
		Lipase (EC 3.1.13): Eberl et al. (2009); Carboxylesterase (EC 3.1.1.1): Billig et al. (2010)
Poly(caprolactone) (PCL)	O O	Cutinase (EC 3.1.1.74): Murphy et al. (1996); Wei et al. (2014b)
Poly(lactic acid) (PLA)	O O	Lipase, cutinase, carboxylesterase, alkaline protease: Hajighasemi et al. (2016)
Poly(hydroxyl-alkanoate) (PHA)	R' O O x	PHA depolymerase: Kim et al. (2007)
Poly(butylene succinate co-adipate) (PBSA)	O O O O 2 O 4 O 4	Cutinase (EC 3.1.1.74): Hu et al. (2016); Shinozaki et al. (2013)
		Lipase (EC 3.1.3): Thirunavukarasu et al. (2016)
Polyethers	O	
Poly(ethylene glycol) (PEG)	O	Dehydrogenases: White et al. (1996); Kawai (2002)
Poly(tetramethylene glycol) (PTMEG)	O	Dehydrogenases: White et al. (1996); Kawai (2002)
Polyamides	O N H	

(*continued*)

Table 1 (continued)

Polymer name (abbreviation)	Structure	Putative enzymes
Polyamide 6.6 (PA6.6)		Manganese peroxidase (EC 1.11.1.13): Deguchi et al. (1998), Friedrich et al. (2007); Laccase (EC 1.10.3.2): Fujisawa et al. (2001)
		Nylon hydrolase (EC 3.5.1.117): Negoro et al.(2012)
Polyamide 11 (PA11)		
Polyurethanes		
Polyester-polyol polyurethane		Cutinase (EC 3.1.1.74): Schmidt et al. (2017); Esterase (EC 3.1.1.1): Akutsu et al. (1998); Aryl acylamidase (EC 3.5.1.13): Akutsu-Shigeno et al. (2006); Elastase (EC 3.4.21.36): Labow et al. (1996); Urethanase (EC 3.5.1.75): Ruiz et al. (1999), Matsumiya et al. (2010)
Polyether-polyol polyurethane		Aryl acylamidase (EC 3.5.1.13): Akutsu-Shigeno et al. (2006) Elastase (EC3.4.21.36): Labow et al. (1996); Urethanase (EC 3.5.1.75): Ruiz et al. (1999), Matsumiya et al. (2010)
Polycarbonates		
Poly(bisphenol A carbonate) (PBPA)		
Poly(trimethylene carbonate) (PTMC)		Lipase: Matsumura et al. (2001), Suyama and Tokiwa (1997)

oxidoreductases have been shown to be involved in the biodegradation of PE (Sivan 2011; Restrepo-Flórez et al. 2014).

3.1 Cutinases

Cutinases (EC 3.1.1.74) catalyze the hydrolysis of cutin, an aliphatic polyester found in the plant cuticle (Chen et al. 2013). This group of polyester hydrolases are part of the superfamily of α/β hydrolases and show activity against several polyester plastics (Fig. 2) (Wei et al. 2014c; Wei and Zimmermann 2017a,b). Similar to lipases, cutinases display a catalytic triad composed of Ser-His-Asp. Unlike other enzymes of the family, the active site of cutinases is exposed to the solvent because the enzyme lacks the typical lid structure of lipases (Chen et al. 2013; Wei et al. 2014c). Depending on their origin, homology, and structure, plastic-degrading cutinases can

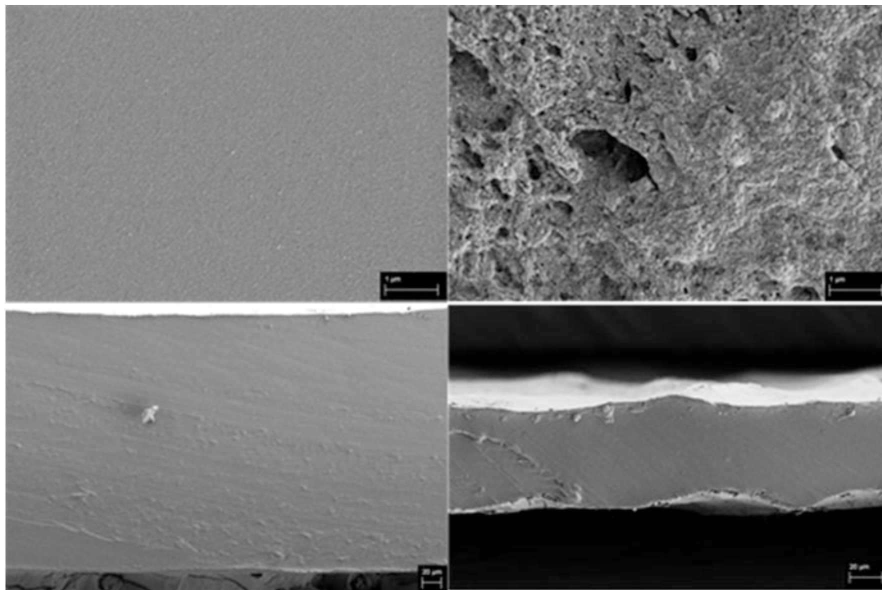


Fig. 2 SEM images of amorphous PET films exposed to the enzymatic hydrolysis catalyzed by TfCut2 from *Thermobifida fusca* KW3 (Wei et al. 2016) resulting a weight loss of 87% (**b**, **d**) as well as the negative control sample in the absence of TfCut2 (**a**, **c**). The comparison of the upper images indicates the progress of the enzymatic surface erosion. The lower images of cross sections show that the films become significantly thinner as a result of the enzymatic hydrolysis. Image kindly provided by Dr. Daniel Breite and Dr. Agnes Schulze, Leibniz Institute of Surface Modification, Permoserstr. 15, 04318 Leipzig, Germany

be divided into fungal (Ronkvist et al. 2009; Baker et al. 2012) and bacterial (Wei et al. 2014c). Fungal cutinases are mainly used in the hydrolysis and surface modifications of PET films and fibers (Vertommen et al. 2005; Araujo et al. 2007; Nimchua et al. 2007; Ronkvist et al. 2009; Zimmermann and Billig 2011). Among these enzymes, the cutinase from *Thermomyces insolens* showed the highest activity against low crystalline PET films due to its outstanding activity and thermal stability at 70 °C near the glass transition temperature of PET (Ronkvist et al. 2009). Bacterial cutinases and their homologues with PET-hydrolyzing activity have been isolated from various *Thermobifida* species (Zimmermann and Billig 2011; Wei et al. 2014c; Then et al. 2015), *Thermomonospora curvata* (Wei et al. 2014b), *Saccharomonospora viridis* (Kawai et al. 2014), *Ideonella sakaiensis* (Yoshida et al. 2016), as well as the metagenome isolated from plant compost (Sulaiman et al. 2012).

As mentioned above, the efficient hydrolysis of PET and PU by polyester hydrolases requires of high reaction temperatures of up to 70 °C (Then et al. 2016; Schmidt et al. 2017). At this temperature, the chains in the amorphous regions of the polymers become more flexible and prone to be accessed by the enzyme (Wei and Zimmermann 2017b). This process can be facilitated if the thermal stability of the enzymes is increased by adding bivalent metal ions (Then et al. 2015, 2016) and

phosphate anions (Schmidt et al. 2016) to the reaction medium, as well as by genetically engineering the thermolabile amino acid residues of the enzyme (Then et al. 2015, 2016). Moreover, the hydrolysis of PET plastics by polyester hydrolases could be strongly improved by the micronization of the plastic material in a pretreatment step to increase the accessible surface area of the substrate (Wei et al. 2014a; Gamerith et al. 2017). The accumulation of the low-molecular-weight products resulting from the hydrolysis reaction can lead to the inhibition of the enzymes (Barth et al. 2015a,b; Gross et al. 2017). This limitation can be overcome by the addition of a second enzyme hydrolyzing the products of the first reaction (Barth et al. 2016; Carniel et al. 2017) or by their continuous removal in an ultrafiltration membrane reactor (Barth et al. 2015b). It is also possible to obtain genetically modified polyester hydrolases insusceptible to product inhibition in order to increase the efficiency of PET hydrolysis (Wei et al. 2016).

3.2 Lipases

Lipases (EC 3.1.1.3) are also part of the superfamily of α/β hydrolases and, like cutinases, display a Ser-His-Asp catalytic triad (Brady et al. 1990). There are multiple examples of microbial lipases capable of hydrolyzing aliphatic polyesters or aliphatic-aromatic co-polyesters (Tokiwa and Suzuki 1981; Marten et al. 2003; Marten et al. 2005; Herzog et al. 2006). For instance, lipases from *Thermomyces lanuginosus* have been shown to degrade PET and poly(trimethylene terephthalate) (Vertommen et al. 2005; Eberl et al. 2009; Ronkvist et al. 2009). Compared to cutinases, lipases exhibit lower hydrolytic activity against PET, possibly due to their lid structure covering the buried hydrophobic catalytic center, which restricts the access of aromatic polymeric substrates to the active site of the enzyme (Guebitz and Cavaco-Paulo 2008; Eberl et al. 2009; Zimmermann and Billig 2011). Lipases from *T. lanuginosus* (Eberl et al. 2009) and *Candida antarctica* (Carniel et al. 2017) also degrade low-molecular-weight degradation products of PET. The latter enzyme has been applied together with the cutinase from *T. insolens* for an improved production of terephthalic acid resulting from the hydrolysis of PET (Carniel et al. 2017).

3.3 Carboxylesterases

Bacterial carboxylesterases (EC 3.1.1.1) from *Bacillus licheniformis*, *Bacillus subtilis*, and *Thermobifida fusca* are known to hydrolyze PET oligomers and their structural analogues (Billig et al. 2010; Oeser et al. 2010; Ribitsch et al. 2011; Lülsdorf et al. 2015; Barth et al. 2016). For example, the carboxylesterase TfCa from *T. fusca* is capable of releasing water-soluble products from high-crystalline PET fibers (Billig et al. 2010; Zimmermann and Billig 2011). These serine esterases also belong to the superfamily of α/β hydrolases, and, compared to the polyester hydrolases, they are significantly larger in size and display a much more buried hydrophobic substrate-binding pocket (Billig et al. 2010). Due to their high activity against PET oligomers,

they have been employed for the removal of inhibitory low-molecular-weight degradation products of PET in combination with polyester hydrolases (Billig et al. 2010; Oeser et al. 2010; Ribitsch et al. 2011; Lülsdorf et al. 2015; Barth et al. 2016).

3.4 Proteases

Several proteases, for example, from *Pseudomonas chlororaphis* and *Pseudomonas fluorescens*, have been reported to degrade polyester PU (Labow et al. 1996; Ruiz et al. 1999; Matsumiya et al. 2010). In addition to the microbial enzymes, other proteases are known to be active against PU such as papain (EC 3.4.22.2), a cysteine protease from papaya, which can hydrolyze amide and urethane bonds (Phua et al. 1987). The porcine pancreatic elastase (EC 3.4.21.36) has been shown to release degradation products from both polyester and polyether PU as a result of the cleavage of hydrolyzable ester, urethane, and urea bonds in the soft segment domains of the polymer (Labow et al. 1996).

3.5 Lignin-Modifying Enzymes

Laccases (EC 1.10.3.2), manganese peroxidases (MnP, EC 1.11.1.13), and lignin peroxidases (LiP, EC 1.11.1.14) are involved in the degradation of lignin, a complex cross-linked aromatic polymer composed of phenylpropanoid units (Ruiz-Dueñas and Martinez 2009). Several of these oxidoreductases are known to be involved in the biodegradation of PE (Restrepo-Flórez et al. 2014; Krueger et al. 2015a). In the presence of copper ions, a thermostable laccase from *Rhodococcus ruber* C208 degraded, both in culture supernatants and in cell-free extracts, UV-irradiated PE films (Santo et al. 2013). This resulted in an increased amount of carbonyl groups and a reduction of the molecular weight within the amorphous part of the PE films. Another laccase isolated from *Trametes versicolor* degraded a high-molecular-weight PE (PE-HMW) membrane in the presence of 1-hydroxybenzotriazole, which mediated the oxidation of non-phenolic substrates by the enzyme (Fujisawa et al. 2001). In other examples, PE-HMW was also degraded by a combination of MnP from the white-rot fungi *Phanerochaete chrysosporium* ME-446 and MnP from isolate IZU-154 (Iiyoshi et al. 1998; Ehara et al. 2000), or cell-free supernatant of a *Phanerochaete chrysosporium* MTCC-787 culture containing both extracellular LiP and MnP, respectively. In this latter case, the combination of enzymes allowed to degrade 70% of a pre-oxidized PE-HMW sample within 15 days of reaction (Mukherjee and Kundu 2014).

4 Thermochemical Plastic Depolymerization

Advances in waste management technologies are promoting the growth of a worldwide industry developing waste-to-bioproduct processes. As mentioned before, petroleum-based plastic wastes contain carbon fractions that are very resistant to

biodegradation. In this context, thermochemical conversion techniques, other than incineration (combustion), such as gasification and pyrolysis are becoming widely accepted as suitable alternatives (Arena 2012; Messenger 2012; Tanigaki et al. 2013). Pyrolysis and gasification are thermal processes that, similar to incineration, use high temperatures to break down wastes, but consuming less oxygen than traditional mass-burn incineration. Whereas in the conventional pyrolysis process waste is thermally degraded in an almost complete absence of air, gasification is a process in which materials are exposed to some oxygen, but not enough to allow combustion.

4.1 Integrating Thermochemical Degradation of Plastic Waste into Biological Upcycling Processes

Aerobic microbial approaches can be applied to further upgrade the products of thermochemical plastic waste treatment to more value-added chemicals and biopolymers. In particular, bacteria such as the metabolically versatile *Pseudomonas* are able to use the thermochemical by-products as substrates for production of biodegradable polymers such as PHA. In one example, a two-step chemo-biotechnological process was used for converting PS to biodegradable mcl-PHA (Ward et al. 2006). In this approach, pyrolysis of PS was carried out at 520 °C to form styrene oil which was then converted by *Pseudomonas putida* CA-3 to PHA. A yield of 62.5 mg of PHA per gram of styrene oil was obtained. Another study investigated the conversion of the plastic PET using pyrolysis at 450 °C (Table 2) (Kenny et al. 2008). Following this step, the solid fraction containing terephthalic acid (>50%) was supplied as a substrate for the production of PHA using soil bacteria. Similarly, PE can also be used as a substrate for pyrolysis-mediated remediation as recently demonstrated with the conversion of

Table 2 Fraction yields (wt%) of mixed plastic waste (MPW)[a] and PET waste (PETW)[b] and composition of the syngas fraction (vol%) with conventional pyrolysis (CP) and microwave-induced pyrolysis (MIP) at different temperatures

Product composition	MPW CP 400 °C	MPW MIP 400 °C	PETW CP 450 °C	MPW CP 800 °C	MPW MIP 800 °C
Solids	79.8	47.9	77	11.2	35.2
Oil	9.2	19.9	6.3	36.8	20.3
Gases	11	32.2	18	53	44.5
Syngas (% of the gas fraction)	35.5	32.7	16.7	30.1	46.2
With H_2 (%)	0	9.1	0.2	9.2	26.6
With CO (%)	7.3	8.8	3.5	9.6	11.5
With CO_2 (%)	28.2	14.8	13	11.3	8.1

[a]Data selection taken from (Beneroso et al. 2015)
[b]Data taken from (Kenny et al. 2008)

postconsumer PE waste into mcl-PHA (Guzik et al. 2014). In this work, pyrolysis of PE in the absence of air produced a set of low-molecular-weight C8-C32 paraffin compounds. In a following step, the products of pyrolysis were emulsified in the presence of biosurfactants (rhamnolipids) and used as feedstock for *Pseudomonas aeruginosa* PAO-1, which accumulated up to 25% of the dry cell weight in the form of PHA.

4.2 Biological Conversion Processes of Syngas from Pyrolyzed Plastic Waste

An important product of gasification and pyrolysis of plastic waste is syngas (aka synthesis gas). Syngas contains mainly CO, H_2, and to a lesser extent CO_2 and other gases. As recently reviewed by (Drzyzga et al. 2015), syngas has been used as a feedstock for the production of bulk chemicals such as acetic acid, butyric acid, or biofuels (e.g., ethanol and butanol) either by chemical catalytic conversion (e.g., Fischer-Tropsch (FT) synthesis) or biological conversion processes (e.g., syngas fermentation).

There are other emerging thermal waste conversion technologies such as microwave-induced pyrolysis (MIP). MIP is one of the most attractive alternatives due to the higher waste conversion and product yields and, at the same time, much lower energy consumption of the process (Fernandez et al. 2011; Byun et al. 2012; Zhang et al. 2012). MIP not only overcomes the disadvantages of CP, such as slow heating and necessity of feedstock shredding, but also improves the quality of the final pyrolysis products. Table 2 shows a comparison between the syngas produced by MIP or CP of mixed plastic waste and waste PET at different temperatures. The data generally demonstrate that MIP is more efficient than CP in many pyrolytic processes of plastic waste, as shown by the higher total amount of syngas produced, which also contains a higher proportion of CO and H_2. In addition, MIP significantly saves processing time and energy compared with CP and often produces lower amounts of unwanted compounds such as CH_4 or hydrocarbons with two or more C atoms (including aromatics) at the highest temperatures (Beneroso et al. 2015).

Regardless of the method used for the production of syngas, in *syngas fermentation*, microorganisms are responsible for the conversion of syngas (the C1 and H_2 fractions) to a diversity of chemical building block compounds (Munasinghe and Khanal 2010; Bengelsdorf et al. 2013; Latif et al. 2014; Drzyzga et al. 2015). Chemicals produced from syngas fermentation include a wide set of compounds such as H_2, CH_4, carboxylic acids (e.g., acetic acid, butyric acid), as well as alcohols and diols (e.g., ethanol, butanol, butanediol) (Köpke et al. 2011a,b; Dürre 2016). Syngas fermentation can produce monomer compounds useful for biopolymer synthesis, such as C4 compounds (e.g., butanediol, succinate, hydroxybutyrate), or it can directly produce biopolymers, such as PHAs, polyhydroxybutyrate (PHB), or poly(hydroxybutyrate-*co*-hydroxyvalerate (Do et al. 2007; Choi et al. 2010). Several anaerobic bacteria are able to use C1 gases such as CO and CO_2 by fermentation, converting them to chemicals, usually acetate, through the acetyl-CoA pathway

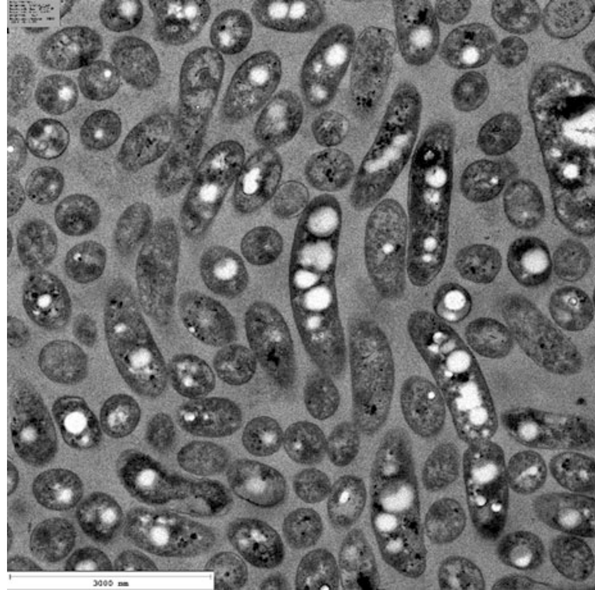


Fig. 3 PHA-producing strain of *Rhodospirillum rubrum* grown with syngas plus acetate as carbon and energy sources

(Müller 2003). These bacteria are named acetogens and include (among others) species such as *Acetobacterium woodii*, *Alkalibaculum bacchi*, *Butyribacterium methylotrophicum*, and many species of the genus *Clostridium* (e.g., *C. ljungdahlii*, *C. aceticum*, *C. thermoaceticum*, *C. autoethanogenum*, *C. ragsdalei*, *C. carboxidivorans*) (Diender et al. 2015; Dürre and Eikmanns 2015).

Besides acetogenic bacteria, *Rhodospirillum rubrum* appears particularly well suited for growth with CO-/CO_2-containing syngas. *R. rubrum*, the type strain of the *Rhodospirillaceae* family, is capable of growth under the broad variety of conditions containing syngas (Do et al. 2007). *R. rubrum* can utilize CO under anaerobic conditions as the sole carbon and energy source. Part of the CO_2 produced is assimilated into cell material (including the stored PHA; Revelles et al. 2016a,b), and the remaining CO_2, along with H_2, is released from the cells. The enrichment of H_2 during syngas fermentation has made *R. rubrum* a particularly attractive organism for the bioconversion of syngas into value-added products, and the feasibility of these processes has been demonstrated with the production of PHA (Fig. 3) in continuous stirred tank reactors fed with syngas (Do et al. 2007; Choi et al. 2010).

5 Research Needs

Recent studies have shown that plastics that are traditionally considered non-biodegradable, such as PET and PE, can be degraded and metabolized by microbes. Several enzymes capable of hydrolyzing the ester-containing PET and other

polyester plastics such as PU have been identified (Wei and Zimmermann 2017b), and the monomers resulting from the reaction can be used as a carbon source by different microorganisms. This constitutes a paradigm shift in what can be considered biodegradable plastic. Notwithstanding these developments, it is clear that environmental plastic pollution is a major issue. Even if new plastic-eating organisms are now emerging in this man-made niche through evolution, these plastics remain highly recalcitrant due to their molecular inaccessibility, macroscopic structure, and the low observed rates of degradation. The rate of accumulation thus far greatly exceeds the rate of biodegradation, and we should not assume that nature will take care of the problem for us.

The problem of plastic waste pollution is primarily one of waste management, which currently is of low priority in many countries. This is caused by the low value of plastic waste, which is comparable to the price of virgin plastics. One solution to this problem is to add value to plastic waste, by using it as a carbon source in biotechnological processes. Such upcycling of plastic waste creates an opportunity to improve the efficiency of resource usage and contribute to a circular economy (European Commission 2017), likely resulting in a transformative technology with an outstanding potential to deliver social and economic benefits. The feasibility of this approach has already been demonstrated in lab-scale processes for the chemo-biotechnological conversion of PS (Goff et al. 2007), PET (Kenny et al. 2008, 2012), and PE (Guzik et al. 2014). In these two-step technologies, the oil obtained by plastic waste pyrolysis is used as a feedstock for microbial production of PHA. The aforementioned plastic-modifying enzymes are key to achieving a complete biological process for plastic waste upcycling into biodegradable plastic, but, in addition, suitable microorganisms capable of assimilating the hydrolysis products are required. The combination of these two elements would give rise to a customizable microbial platform capable of converting plastic waste. It is worth noting, however, that the metabolism of these monomers is not trivial, since plastics are generally made up of molecules that are not common in nature and thus are not always readily metabolized by microbes.

Alternatively, thermochemical depolymerization methods such as pyrolysis would lead to completely different substrates, with strong focus on syngas. With the recent developments of efficient and inexpensive methods to sequence complete microbial genomes, the genetics of many syngas-fermenting microorganisms is becoming better understood. This knowledge in combination with the newest tools in systems biology can be applied to these microorganisms to enhance the production of chemical compounds from gaseous C1 compounds through metabolic and genetic manipulations (Köpke et al. 2010; Latif et al. 2014). Additionally, methods such as media and reactor design optimization are being pursued to enhance chemical production from existing and newly isolated CO-/CO_2-fermenting microorganisms. The application of all these technologies will contribute to make microbial fermentative production of chemical building block compounds cost-effective when compared with other technologies (Wilkins and Atiyeh 2011). To highlight the potential of syngas fermentation, in the last decade, we have witnessed its

application in the production of biofuels (mainly bioethanol) at pilot or industrial scales (e.g., LanzaTech Inc., USA).

The challenge of the bio-utilization of plastic waste is similar to that faced by lignocellulosic biotechnology, which relies mostly on enzymatic hydrolysis and monomer metabolism as key enabling factors. Therefore, much can be learned from developments in this field. We thus expect that major research efforts will be needed to find and engineer efficient plastic-degrading enzymes and associated processes. Also, research is needed toward characterizing the microbial degradation of a wide variety of plastic monomers, varying from long-chain aliphatics derived from PE to α,ω-alcohols and α,ω-acids, as well as complex aromatics derived from PET and PU. In principle, when these two steps, namely, the enzymatic hydrolysis and the subsequent degradation of the monomers, have been taken, the substrate in virtually any current sugar-based biotech process could be replaced with plastic waste. Initially, research will be focused on relatively pure plastics of a single type (i.e., PET), but the real strength of microbial plastic utilization will lie in the ability to utilize mixed waste streams which are inaccessible to traditional recycling techniques.

Acknowledgements The authors have received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no. 633962 for the project P4SB.

References

Abe M, Kobayashi K, Honma N, Nakasaki K (2010) Microbial degradation of poly(butylene succinate) by *Fusarium solani* in soil environments. Polym Degrad Stab 95:138–143

ACC, APR (2016) The 2015 US national postconsumer plastics bottle recycling rate report American Chemical Council & Assoc. Plastic Recyclers. https://plastics.americanchemistry.com/2015-United-States-National-Postconsumer-Plastic-Bottle-Recycling-Report.pdf. Accessed 09 Oct 2017

Agarwal M, Koelling KW, Chalmers JJ (1998) Characterization of the degradation of polylactic acid polymer in a solid substrate environment. Biotechnol Prog 14:517–526

Akutsu Y, Nakajima-Kambe T, Nomura N, Nakahara T (1998) Purification and properties of a polyester polyurethane-degrading enzyme from *Comamonas acidovorans* TB-35. Appl Environ Microbiol 64:62–67

Akutsu-Shigeno Y, Adachi Y, Yamada C, Toyoshima K, Nomura N, Uchiyama H, Nakajima-Kambe T (2006) Isolation of a bacterium that degrades urethane compounds and characterization of its urethane hydrolase. Appl Microbiol Biotechnol 70:422–429

Albertsson AC, Barenstedt C, Karlsson S, Lindberg T (1995) Degradation product pattern and morphology changes as means to differentiate abiotically and biotically aged degradable polyethylene. Polymer 36:3075–3083

Ali MI, Ahmed S, Robson G, Javed I, Ali N, Atiq N, Hameed A (2014) Isolation and molecular characterization of polyvinyl chloride (PVC) plastic degrading fungal isolates. J Basic Microbiol 54:18–27

Álvarez-Barragán J, Domínguez-Malfavón L, Vargas-Suárez M, González-Hernández R, Aguilar-Osorio G, Loza-Tavera H (2016) Biodegradative activity of selected environmental fungi on a polyester polyurethane varnish and polyether polyurethane foams. Appl Environ Microbiol 82:5225. https://doi.org/10.1128/aem.01344-16

Araujo R, Silva C, O'Neill A, Micaelo N, Guebitz G, Soares CM, Casal M, Cavaco-Paulo A (2007) Tailoring cutinase activity towards polyethylene terephthalate and polyamide 6,6 fibers. J Biotechnol 128:849–857

Arena U (2012) Process and technological aspects of municipal solid waste gasification. A review. Waste Manag 32:625–639

Arkatkar A, Juwarkar AA, Bhaduri S, Uppara PV, Doble M (2010) Growth of *Pseudomonas* and *Bacillus* biofilms on pretreated polypropylene surface. Int Biodeterior Biodegrad 64:530–536

Artham T, Sudhakar M, Venkatesan R, Nair CM, Murty KVGK, Doble M (2009) Biofouling and stability of synthetic polymers in sea water. Int Biodeterior Biodegrad 63:884–890

Babu RP, O'Connor K, Seeram R (2013) Current progress on bio-based polymers and their future trends. Prog Biomater 2:8. https://doi.org/10.1186/2194-0517-2-8

Baker PJ, Poultney C, Liu Z, Gross R, Montclare JK (2012) Identification and comparison of cutinases for synthetic polyester degradation. Appl Microbiol Biotechnol 93:229–240

Balasubramanian V, Natarajan K, Hemambika B, Ramesh N, Sumathi CS, Kottaimuthu R, Kannan VR (2010) High-density polyethylene (HDPE)-degrading potential bacteria from marine ecosystem of Gulf of Mannar, India. Lett Appl Microbiol 51:205–211

Barnes DKA, Galgani F, Thompson RC, Barlaz M (2009) Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc B 364:1985–1998

Barth M, Oeser T, Wei R, Then J, Schmidt J, Zimmermann W (2015a) Effect of hydrolysis products on the enzymatic degradation of polyethylene terephthalate nanoparticles by a polyester hydrolase from *Thermobifida fusca*. Biochem Eng J 93:222–228

Barth M, Wei R, Oeser T, Then J, Schmidt J, Wohlgemuth F, Zimmermann W (2015b) Enzymatic hydrolysis of polyethylene terephthalate films in an ultrafiltration membrane reactor. J Membr Sci 494:182–187

Barth M, Honak A, Oeser T, Wei R, Belisario-Ferrari MR, Then J, Schmidt J, Zimmermann W (2016) A dual enzyme system composed of a polyester hydrolase and a carboxylesterase enhances the biocatalytic degradation of polyethylene terephthalate films. Biotechnol J 11:1082–1087

BASF SE (2017) Biodegradable and compostable packaging solutions with ecoflex®. BASF. http://www.plasticsportal.eu/ecoflex. Accessed 11 Oct 2017

Bechthold I, Bretz K, Kabasci S, Kopitzky R, Springer A (2008) Succinic acid: a new platform chemical for biobased polymers from renewable resources. Chem Eng Technol 31:647–654

Beguin P, Aubert JP (1994) The biological degradation of cellulose. FEMS Microbiol Rev 13:25–58

Beneroso D, Bermúdez JM, Arenillas A, Menéndez JA (2015) Comparing the composition of the synthesis-gas obtained from the pyrolysis of different organic residues for a potential use in the synthesis of bioplastics. J Anal Appl Pyrolysis 111:55–63

Bengelsdorf FR, Straub M, Dürre P (2013) Bacterial synthesis gas (syngas) fermentation. Environ Technol 34:1639–1651

Billig S, Oeser T, Birkemeyer C, Zimmermann W (2010) Hydrolysis of cyclic poly(ethylene terephthalate) trimers by a carboxylesterase from *Thermobifida fusca* KW3. Appl Microbiol Biotechnol 87:1753–1764

Bombelli P, Howe CJ, Bertocchini F (2017) Polyethylene bio-degradation by caterpillars of the wax moth *Galleria mellonella*. Curr Biol 27:R292–R293

Brady L, Brzozowski AM, Derewenda ZS, Dodson E, Dodson G, Tolley S, Turkenburg JP, Christiansen L, Huge-Jensen B, Norskov L et al (1990) A serine protease triad forms the catalytic centre of a triacylglycerol lipase. Nature 343:767–770

Byun Y, Cho M, Hwang S-M, Chung J (2012) Thermal plasma gasification of municipal solid waste (MSW). In: Yun Y (ed) Gasification for practical applications. InTech, Rijeka, pp 183–210

Cacciari I, Quatrini P, Zirletta G, Mincione E, Vinciguerra V, Lupattelli P, Giovannozzi Sermanni G (1993) Isotactic polypropylene biodegradation by a microbial community: physicochemical characterization of metabolites produced. Appl Environ Microbiol 59:3695–3700

Calabia BP, Tokiwa Y (2006) A novel PHB depolymerase from a thermophilic *Streptomyces* sp. Biotechnol Lett 28:383–388

Carniel A, Valoni É, Nicomedes J, Gomes AC, Castro AM (2017) Lipase from *Candida antarctica* (CALB) and cutinase from *Humicola insolens* act synergistically for PET hydrolysis to terephthalic acid. Process Biochem 59:84–90

Cerrone F, Davis R, Kenny ST, Woods T, O'Donovan A, Gupta VK, Tuohy M, Babu RP, O'Kiely P, O'Connor K (2015) Use of a mannitol rich ensiled grass press juice (EGPJ) as a sole carbon source for polyhydroxyalkanoates (PHAs) production through high cell density cultivation. Bioresour Technol 191:45–52

Chen DR, Bei JZ, Wang SG (2000) Polycaprolactone microparticles and their biodegradation. Polym Degrad Stab 67:455–459

Chen S, Su L, Chen J, Wu J (2013) Cutinase: characteristics, preparation, and application. Biotechnol Adv 31:1754–1767

Choi D, Chipman DC, Bents SC, Brown RC (2010) A techno-economic analysis of polyhydroxyalkanoate and hydrogen production from syngas fermentation of gasified biomass. Appl Biochem Biotechnol 160:1032–1046

Deguchi T, Kitaoka Y, Kakezawa M, Nishida T (1998) Purification and characterization of a nylon-degrading enzyme. Appl Environ Microbiol 64:1366–1371

Derraik JGB (2002) The pollution of the marine environment by plastic debris: a review. Mar Pollut Bull 44:842–852

Diender M, Stams AJM, Sousa DZ (2015) Pathways and bioenergetics of anaerobic carbon monoxide fermentation. Front Microbiol 6:1275. https://doi.org/10.3389/fmicb.2015.01275

Do YS, Smeenk J, Broer KM, Kisting CJ, Brown R, Heindel TJ, Bobik TA, DiSpirito AA (2007) Growth of *Rhodospirillum rubrum* on synthesis gas: conversion of CO to H2 and poly-beta-hydroxyalkanoate. Biotechnol Bioeng 97:279–286

Dris R, Imhof H, Sanchez W, Gasperi J, Galgani F, Tassin B, Laforsch C (2015) Beyond the ocean: contamination of freshwater ecosystems with (micro-)plastic particles. Environ Chem 12:539–550

Drzyzga O, Revelles O, Durante-Rodríguez G, Díaz E, García JL, Prieto A (2015) New challenges for syngas fermentation: towards production of biopolymers. J Chem Technol Biotechnol 90:1735–1751

Dürre P (2016) Butanol formation from gaseous substrates. FEMS Microbiol Lett 363. https://doi.org/10.1093/femsle/fnw040

Dürre P, Eikmanns BJ (2015) C1-carbon sources for chemical and fuel production by microbial gas fermentation. Curr Opin Biotechnol 35:63–72

Eberl A, Heumann S, Bruckner T, Araujo R, Cavaco-Paulo A, Kaufmann F, Kroutil W, Guebitz GM (2009) Enzymatic surface hydrolysis of poly(ethylene terephthalate) and bis(benzoyloxyethyl) terephthalate by lipase and cutinase in the presence of surface active molecules. J Biotechnol 143:207–212

Ehara K, Iiyoshi Y, Tsutsumi Y, Nishida T (2000) Polyethylene degradation by manganese peroxidase in the absence of hydrogen peroxide. J Wood Sci 46:180–183

Ellen MacArthur Foundation, World Economic Forum, McKinsey & Company (2016) The new plastics economy: rethinking the future of plastics. Ellen MacARTHUR Foundation & World Economic Forum & McKinsey & Company. http://www.ellenmacarthurfoundation.org/publications. Accessed 09 Oct 2017

Emadian SM, Onay TT, Demirel B (2017) Biodegradation of bioplastics in natural environments. Waste Manag 59:526–536

European Bioplastics (2016) Driving the evolution of plastics. European Bioplastics, Berlin. http://docs.european-bioplastics.org/2016/association/EUBP_image_brochure.pdf. Accessed 15 Oct 2017

European Commission (2017) Strategy on plastics in a circular economy – roadmap. EC. http://ec.europa.eu/smart-regulation/roadmaps/docs/plan_2016_39_plastic_strategy_en.pdf. Accessed 15 Oct 2017

Farah S, Anderson DG, Langer R (2016) Physical and mechanical properties of PLA, and their functions in widespread applications – a comprehensive review. Adv Drug Deliv Rev 107:367–392

Fernandez Y, Arenillas A, Menendez JA (2011) Microwave heating applied to pyrolysis. In: Grundas S (ed) Advances in induction and microwave heating of mineral and organic materials. InTech, Rijcka, pp 723–752

Fontanella S, Bonhomme S, Koutny M, Husarova L, Brusson JM, Courdavault JP, Pitteri S, Samuel G, Pichon G, Lemaire J, Delort AM (2010) Comparison of the biodegradability of various polyethylene films containing pro-oxidant additives. Polym Degrad Stab 95: 1011–1021
Friedrich J, Zalar P, Mohorcic M, Klun U, Krzan A (2007) Ability of fungi to degrade synthetic polymer nylon-6. Chemosphere 67:2089–2095
Fujisawa M, Hirai H, Nishida T (2001) Degradation of polyethylene and Nylon-66 by the laccase-mediator system. J Polym Environ 9:103–108
Galloway TS, Cole M, Lewis C (2017) Interactions of microplastic debris throughout the marine ecosystem. Nat Ecol Evol 1:0116. https://doi.org/10.1038/s41559-017-0116
Gamerith C, Zartl B, Pellis A, Guillamot F, Marty A, Acero EH, Guebitz GM (2017) Enzymatic recovery of polyester building blocks from polymer blends. Process Biochem 59:58–64
Geyer R, Jambeck JR, Law KL (2017) Production, use, and fate of all plastics ever made. Sci Adv 3: e1700782. https://doi.org/10.1126/sciadv.1700782
Gilan I, Hadar Y, Sivan A (2004) Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. Appl Microbiol Biotechnol 65:97–104
Glass NL, Schmoll M, Cate JH, Coradetti S (2013) Plant cell wall deconstruction by ascomycete fungi. Annu Rev Microbiol 67:477–498
Goff M, Ward PG, O'Connor KE (2007) Improvement of the conversion of polystyrene to polyhydroxyalkanoate through the manipulation of the microbial aspect of the process: a nitrogen feeding strategy for bacterial cells in a stirred tank reactor. J Biotechnol 132:283–286
Greene JP (2014) Sustainable plastics: environmental assessments of biobased, biodegradable, and recycled plastics. Wiley. https://doi.org/10.1002/9781118899595
Gross C, Hamacher K, Schmitz K, Jager S (2017) Cleavage product accumulation decreases the activity of cutinase during PET hydrolysis. J Chem Inf Model 57:243–255
Guebitz GM, Cavaco-Paulo A (2008) Enzymes go big: surface hydrolysis and functionalization of synthetic polymers. Trends Biotechnol 26:32–38
Guzik MW, Kenny ST, Duane GF, Casey E, Woods T, Babu RP, Nikodinovic-Runic J, Murray M, O'Connor KE (2014) Conversion of post consumer polyethylene to the biodegradable polymer polyhydroxyalkanoate. Appl Microbiol Biotechnol 98:4223–4232
GVM (2016) Aufkommen und Verwertung von PET – Getränkeflaschen in Deutschland 2015. Gesellschaft für Verpackungsmarktforschung mbH, Mainz. http://www.kunststoffverpackungen.de/show.php?ID=5961&PHPSESSID=apceu6k6r1irm4q7qff60ofp50. Accessed 15 Oct 2017
Hadad D, Geresh S, Sivan A (2005) Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. J Appl Microbiol 98:1093–1100
Hajighasemi M, Nocek BP, Tchigvintsev A, Brown G, Flick R, Xu X, Cui H, Hai T, Joachimiak A, Golyshin PN, Savchenko A, Edwards EA, Yakunin AF (2016) Biochemical and structural insights into enzymatic depolymerization of polylactic acid and other polyesters by microbial carboxylesterases. Biomacromolecules 17:2027–2039
Hakkarainen M, Albertsson AC (2004) Environmental degradation of polyethylene. Adv Polym Sci 169:177–199
Herzog K, Müller RJ, Deckwer WD (2006) Mechanism and kinetics of the enzymatic hydrolysis of polyester nanoparticles by lipases. Polym Degrad Stab 91:2486–2498
Ho KLG, Pometto AL, Gadea-Rivas A, Briceno JA, Rojas A (1999) Degradation of polylactic acid (PLA) plastic in Costa Rican soil and Iowa State University compost rows. J Environ Polym Degrad 7:173–177
Horsman PV (1982) The amount of garbage pollution from merchant ships. Mar Pollut Bull 13:167–169
Hu X, Gao Z, Wang Z, Su T, Yang L, Li P (2016) Enzymatic degradation of poly(butylene succinate) by cutinase cloned from *Fusarium solani*. Polym Degrad Stab 134:211–219
Iiyoshi Y, Tsutsumi Y, Nishida T (1998) Polyethylene degradation by lignin-degrading fungi and manganese peroxidase. J Wood Sci 44:222. https://doi.org/10.1007/bf00521967

Jambeck JR, Geyer R, Wilcox C, Siegler TR, Perryman M, Andrady A, Narayan R, Law KL (2015) Plastic waste inputs from land into the ocean. Science 347:768–771

Jendrossek D, Handrick R (2002) Microbial degradation of polyhydroxyalkanoates. Annu Rev Microbiol 56:403–432

Jeyakumar D, Chirsteen J, Doble M (2013) Synergistic effects of pretreatment and blending on fungi mediated biodegradation of polypropylenes. Bioresour Technol 148:78–85

Kaplan DL, Hartenstein R, Sutter J (1979) Biodegradation of polystyrene, poly(metnyl methacrylate), and phenol formaldehyde. Appl Environ Microbiol 38:551–553

Kawai F (2002) Microbial degradation of polyethers. Appl Microbiol Biotechnol 58:30–38

Kawai F (2010) Polylactic acid (PLA)-degrading microorganisms and PLA depolymerases. In: Green polymer chemistry: biocatalysis and biomaterials. American Chemical Society, Washington, DC, pp 405–414

Kawai F, Oda M, Tamashiro T, Waku T, Tanaka N, Yamamoto M, Mizushima H, Miyakawa T, Tanokura M (2014) A novel Ca2+−activated, thermostabilized polyesterase capable of hydrolyzing polyethylene terephthalate from *Saccharomonospora viridis* AHK190. Appl Microbiol Biotechnol 98:10053–10064

Kenny ST, Runic JN, Kaminsky W, Woods T, Babu RP, Keely CM, Blau W, O'Connor KE (2008) Up-cycling of PET (polyethylene terephthalate) to the biodegradable plastic PHA (polyhydroxyalkanoate). Environ Sci Technol 42:7696–7701

Kenny ST, Runic JN, Kaminsky W, Woods T, Babu RP, O'Connor KE (2012) Development of a bioprocess to convert PET derived terephthalic acid and biodiesel derived glycerol to medium chain length polyhydroxyalkanoate. Appl Microbiol Biotechnol 95:623–633

Kim DY, Kim HW, Chung MG, Rhee YH (2007) Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. J Microbiol 45:87–97

Klemm D, Schmauder H-P, Heinze T (2002) Cellulose. In: Vandamme E, De Beats S, Steinbüchel A (eds) Biopolymers. Wiley-VCH, Weinheim, pp 290–292

Köpke M, Held C, Hujer S, Liesegang H, Wiezer A, Wollherr A, Ehrenreich A, Liebl W, Gottschalk G, Dürre P (2010) *Clostridium ljungdahlii* represents a microbial production platform based on syngas. Proc Natl Acad Sci U S A 107:13087–13092

Köpke M, Mihalcea C, Bromley JC, Simpson SD (2011a) Fermentative production of ethanol from carbon monoxide. Curr Opin Biotechnol 22:320–325

Köpke M, Mihalcea C, Liew F, Tizard JH, Ali MS, Conolly JJ, Al-Sinawi B, Simpson SD (2011b) 2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. Appl Environ Microbiol 77:5467–5475

Koutny M, Sancelme M, Dabin C, Pichon N, Delort AM, Lemaire J (2006) Acquired biodegradability of polyethylenes containing pro-oxidant additives. Polym Degrad Stab 91:1495–1503

Krasowska K, Janik H, Gradys A, Rutkowska M (2012) Degradation of polyurethanes in compost under natural conditions. J Appl Polym Sci 125:4252–4260

Krueger MC, Harms H, Schlosser D (2015a) Prospects for microbiological solutions to environmental pollution with plastics. Appl Microbiol Biotechnol 99:8857–8874

Krueger MC, Hofmann U, Moeder M, Schlosser D (2015b) Potential of wood-rotting Fungi to attack polystyrene sulfonate and its depolymerisation by *Gloeophyllum trabeum* via hydroquinone-driven Fenton chemistry. PLoS One 10:e0131773. https://doi.org/10.1371/journal.pone.0131773

Krueger MC, Seiwert B, Prager A, Zhang S, Abel B, Harms H, Schlosser D (2017) Degradation of polystyrene and selected analogues by biological Fenton chemistry approaches: opportunities and limitations. Chemosphere 173:520–528

Labow RS, Erfle DJ, Santerre JP (1996) Elastase-induced hydrolysis of synthetic solid substrates: poly(ester-urea-urethane) and poly(ether-urea-urethane). Biomaterials 17:2381–2388

Latif H, Zeidan AA, Nielsen AT, Zengler K (2014) Trash to treasure: production of biofuels and commodity chemicals via syngas fermenting microorganisms. Curr Opin Biotechnol 27:79–87

Lebreton LCM, van der Zwet J, Damsteeg J-W, Slat B, Andrady A, Reisser J (2017) River plastic emissions to the world's oceans. Nat Commun 8:15611. https://doi.org/10.1038/ncomms15611

Lim S-P, Gan S-N, Tan IKP (2005) Degradation of medium-chain-length polyhydroxyalkanoates in tropical forest and mangrove soils. Appl Biochem Biotechnol 126:23. https://doi.org/10.1007/s12010-005-0003-7

Lindman B, Karlström G, Stigsson L (2010) On the mechanism of dissolution of cellulose. J Mol Liq 156:76–81

Lucas N, Bienaime C, Belloy C, Queneudec M, Silvestre F, Nava-Saucedo JE (2008) Polymer biodegradation: mechanisms and estimation techniques. Chemosphere 73:429–442

Lülsdorf N, Vojcic L, Hellmuth H, Weber TT, Mussmann N, Martinez R, Schwaneberg U (2015) A first continuous 4-aminoantipyrine (4-AAP)-based screening system for directed esterase evolution. Appl Microbiol Biotechnol 99:5237–5246

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Marten E, Müller R-J, Deckwer W-D (2003) Studies on the enzymatic hydrolysis of polyesters I. Low molecular mass model esters and aliphatic polyesters. Polym Degrad Stab 80:485–501

Marten E, Müller R-J, Deckwer W-D (2005) Studies on the enzymatic hydrolysis of polyesters. II. Aliphatic–aromatic copolyesters. Polym Degrad Stab 88:371–381

Matsumiya Y, Murata N, Tanabe E, Kubota K, Kubo M (2010) Isolation and characterization of an ether-type polyurethane-degrading micro-organism and analysis of degradation mechanism by *Alternaria* sp. J Appl Microbiol 108:1946–1953

Matsumura S, Harai S, Toshima K (2001) Lipase-catalyzed transformation of poly(trimethylene carbonate) into cyclic monomer, trimethylene carbonate: a new strategy for sustainable polymer recycling using an enzyme. Macromol Rapid Commun 22:215–218

Messenger B (2012, Nov-Dec) Is waste gasification finally coming of age? Waste Manage World 18–23

Mor R, Sivan A (2008) Biofilm formation and partial biodegradation of polystyrene by the actinomycete *Rhodococcus ruber*: biodegradation of polystyrene. Biodegradation 19:851–858

Mukherjee S, Kundu PP (2014) Alkaline fungal degradation of oxidized polyethylene in black liquor: studies on the effect of lignin peroxidases and manganese peroxidases. J Appl Polym Sci 131. https://doi.org/10.1002/app.40738

Müller V (2003) Energy conservation in acetogenic bacteria. Appl Environ Microbiol 69:6345–6353

Müller CA, Perz V, Provasnek C, Quartinello F, Guebitz GM, Berg G (2017) Discovery of polyesterases from moss-associated microorganisms. Appl Environ Microbiol 83: e02641–e02616

Munasinghe PC, Khanal SK (2010) Biomass-derived syngas fermentation into biofuels: opportunities and challenges. Bioresour Technol 101:5013–5022

Murphy CA, Cameron JA, Huang SJ, Vinopal RT (1996) Fusarium polycaprolactone depolymerase is cutinase. Appl Environ Microbiol 62:456–460

Nakamiya K, Sakasita G, Ooi T, Kinoshita S (1997) Enzymatic degradation of polystyrene by hydroquinone peroxidase of *Azotobacter beijerinckii* HM121. J Ferment Bioeng 84:480–482

NatureWorks (2017) Composting Ingeo™. NatureWorks LLC. http://www.natureworksllc.com/What-is-Ingeo/Where-it-Goes/Composting. Accessed 09 Oct 2017

Negoro S, Shibata N, Tanaka Y, Yasuhira K, Shibata H, Hashimoto H, Lee YH, Oshima S, Santa R, Oshima S, Mochiji K, Goto Y, Ikegami T, Nagai K, Kato D, Takeo M, Higuchi Y (2012) Three-dimensional structure of nylon hydrolase and mechanism of nylon-6 hydrolysis. J Biol Chem 287:5079–5090

Nimchua T, Punnapayak H, Zimmermann W (2007) Comparison of the hydrolysis of polyethylene terephthalate fibers by a hydrolase from *Fusarium oxysporum* LCH I and *Fusarium solani* f. sp. pisi. Biotechnol J 2:361–364

Nishida H, Tokiwa Y (1993) Distribution of poly(β-hydroxybutyrate) and poly(ϵ-caprolactone) aerobic degrading microorganisms in different environments. J Environ Polym Degr 1:227–233

Oeser T, Wei R, Baumgarten T, Billig S, Follner C, Zimmermann W (2010) High level expression of a hydrophobic poly(ethylene terephthalate)-hydrolyzing carboxylesterase from *Thermobifida fusca* KW3 in *Escherichia coli* BL21(DE3). J Biotechnol 146:100–104

Otake Y, Kobayashi T, Asabe H, Murakami N, Ono K (1995) Biodegradation of low-density polyethylene, polystyrene, polyvinyl-chloride, and urea-formaldehyde resin buried under soil for over 32 years. J Appl Polym Sci 56:1789–1796

Phua SK, Castillo E, Anderson JM, Hiltner A (1987) Biodegradation of a polyurethane in vitro. J Biomed Mater Res 21:231–246

PlasticsEurope (2016) Plastics – the facts 2016. Düsseldorf. http://www.plasticseurope.org. Accessed 13 Oct 2017

Pometto AL III, Lee BT, Johnson KE (1992) Production of an extracellular polyethylene-degrading enzyme(s) by *Streptomyces* species. Appl Environ Microbiol 58:731–733

Pruter AT (1987) Sources, quantities and distribution of persistent plastics in the marine environment. Mar Pollut Bull 18:305–310

Rajandas H, Parimannan S, Sathasivam K, Ravichandran M, Yin LS (2012) A novel FTIR-ATR spectroscopy based technique for the estimation of low-density polyethylene biodegradation. Polym Test 31:1094–1099

Restrepo-Flórez J-M, Bassi A, Thompson MR (2014) Microbial degradation and deterioration of polyethylene - a review. Int Biodeterior Biodegrad 88:83–90

Revelles O, Beneroso D, Menéndez JA, Arenillas A, García JL, Prieto MA (2016a) Syngas obtained by microwave pyrolysis of household wastes as feedstock for polyhydroxyalkanoate production in *Rhodospirillum rubrum*. Microb Biotechnol 10:1412. https://doi.org/10.1111/1751-7915.12411

Revelles O, Tarazona N, García JL, Prieto MA (2016b) Carbon roadmap from syngas to polyhydroxyalkanoates in *Rhodospirillum rubrum*. Environ Microbiol 18:708–720

Rhee YH, Kim YH, Shin K-S (2006) Characterization of an extracellular poly(3-hydroxyoctanoate) depolymerase from the marine isolate, *Pseudomonas luteola* M13-4. Enzym Microb Technol 38:529–535

Ribitsch D, Heumann S, Trotscha E, Herrero Acero E, Greimel K, Leber R, Birner-Gruenberger R, Deller S, Eiteljoerg I, Remler P, Weber T, Siegert P, Maurer KH, Donelli I, Freddi G, Schwab H, Guebitz GM (2011) Hydrolysis of polyethyleneterephthalate by *p*-nitrobenzylesterase from *Bacillus subtilis*. Biotechnol Prog 27:951–960

Ronkvist ÅM, Xie W, Lu W, Gross RA (2009) Cutinase-catalyzed hydrolysis of poly(ethylene terephthalate). Macromolecules 42:5128–5138

Ruiz C, Main T, Hilliard NP, Howard GT (1999) Purification and characterization of two polyurethanase enzymes from *Pseudomonas chlororaphis*. Int Biodeterior Biodegrad 43:43–47

Ruiz-Dueñas FJ, Martinez AT (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2:164–177

Russell JR, Huang J, Anand P, Kucera K, Sandoval AG, Dantzler KW, Hickman D, Jee J, Kimovec FM, Koppstein D, Marks DH, Mittermiller PA, Núñez SJ, Santiago M, Townes MA, Vishnevetsky M, Williams NE, Vargas MPN, Boulanger L-A, Bascom-Slack C, Strobel SA (2011) Biodegradation of polyester polyurethane by endophytic fungi. Appl Environ Microbiol 77:6076–6084

Ryan PG, Moore CJ, van Franeker JA, Moloney CL (2009) Monitoring the abundance of plastic debris in the marine environment. Philos Trans R Soc B 364:1999–2012

Santana VT, Goncalves SPC, Agnelli JAM, Martins-Franchetti SM (2012) Biodegradation of a polylactic acid/polyvinyl chloride blend in soil. J Appl Polym Sci 125:536–540

Santo M, Weitsman R, Sivan A (2013) The role of the copper-binding enzyme – laccase – in the biodegradation of polyethylene by the actinomycete *Rhodococcus ruber*. Int Biodeterior Biodegrad 84:204–210

Schmidt J, Wei R, Oeser T, Belisário-Ferrari MR, Barth M, Then J, Zimmermann W (2016) Effect of Tris, MOPS, and phosphate buffers on the hydrolysis of polyethylene terephthalate films by polyester hydrolases. FEBS Open Bio 6:919–927

Schmidt J, Wei R, Oeser T, Dedavid e Silva L, Breite D, Schulze A, Zimmermann W (2017) Degradation of polyester polyurethane by bacterial polyester hydrolases. Polymer 9:65. https://doi.org/10.3390/polym9020065

Shah Z, Krumholz L, Aktas DF, Hasan F, Khattak M, Shah AA (2013) Degradation of polyester polyurethane by a newly isolated soil bacterium, *Bacillus subtilis* strain MZA-75. Biodegradation 24:865–877

Shinozaki Y, Morita T, Cao XH, Yoshida S, Koitabashi M, Watanabe T, Suzuki K, Sameshima-Yamashita Y, Nakajima-Kambe T, Fujii T, Kitamoto HK (2013) Biodegradable plastic-degrading enzyme from *Pseudozyma antarctica*: cloning, sequencing, and characterization. Appl Microbiol Biotechnol 97:2951–2959

Sielicki M, Focht DD, Martin JP (1978) Microbial degradation of [^{14}C]polystyrene and 1,3-diphenylbutane. Can J Microbiol 24:798–803

Sivan A (2011) New perspectives in plastic biodegradation. Curr Opin Biotechnol 22:422–426

Sridewi N, Bhubalan K, Sudesh K (2006) Degradation of commercially important polyhydroxyalkanoates in tropical mangrove ecosystem. Polym Degrad Stab 91:2931–2940

Succinity (2016) Biobased polybutylene succinate (PBS) – an attractive polymer for biopolymer compounds. Succinity GmbH & Nova-Institute. http://www.succinity.com/images/succinity_broschure.pdf. Accessed 09 Oct 2017

Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 25:1503–1555

Sulaiman S, Yamato S, Kanaya E, Kim JJ, Koga Y, Takano K, Kanaya S (2012) Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach. Appl Environ Microbiol 78:1556–1562

Suyama T, Tokiwa Y (1997) Enzymatic degradation of an aliphatic polycarbonate, poly(tetramethylene carbonate). Enzym Microb Technol 20:122–126

Suyama T, Tokiwa Y, Ouichanpagdee P, Kanagawa T, Kamagata Y (1998) Phylogenetic affiliation of soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics. Appl Environ Microbiol 64:5008–5011

Tanigaki N, Fujinaga Y, Kajiyama H, Ishida Y (2013) Operating and environmental performances of commercial-scale waste gasification and melting technology. Waste Manage Res 31:1118–1124

Then J, Wei R, Oeser T, Barth M, Belisário-Ferrari MR, Schmidt J, Zimmermann W (2015) Ca^{2+} and Mg^{2+} binding site engineering increases the degradation of polyethylene terephthalate films by polyester hydrolases from *Thermobifida fusca*. Biotechnol J 10:592–598

Then J, Wei R, Oeser T, Gerdts A, Schmidt J, Barth M, Zimmermann W (2016) A disulfide bridge in the calcium binding site of a polyester hydrolase increases its thermal stability and activity against polyethylene terephthalate. FEBS Open Bio 6:425–432

Thirunavukarasu K, Purushothaman S, Sridevi J, Aarthy M, Gowthaman MK, Nakajima-Kambe T, Kamini NR (2016) Degradation of poly(butylene succinate) and poly(butylene succinate-co-butylene adipate) by a lipase from yeast *Cryptococcus* sp. grown on agro-industrial residues. Int Biodeterior Biodegrad 110:99–107

Tokiwa Y, Pranamuda H (2002) Microbial degradation of polyesters. In: Steinbüchel A, Doi Y (eds) Polyesters II: properties and chemical synthesis. Wiley-VCH Verlag GmbH, Weinheim

Tokiwa Y, Suzuki T (1981) Hydrolysis of copolyesters containing aromatic and aliphatic ester blocks by lipase. J Appl Polym Sci 26:441–448

Tsuji H (2002) Polylactides. In: Steinbüchel A, Doi Y (eds) Biopolymers: polyesters III. Wiley-VCH, Weinheim, pp 129–177

UNESCO (1994) Marine debris: solid waste management action plan for the wider Caribbean. IOC Technical series, vol 41. UNESCO, Paris

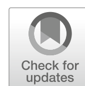
US EPA (2016) Advancing sustainable materials management: 2014 fact sheet. US EPA, Office of Land and Emergency Management, Washington, DC. Report: EPA530-R-17-01

Vertommen MAME, Nierstrasz VA, Veer M, Warmoeskerken MMCG (2005) Enzymatic surface modification of poly(ethylene terephthalate). J Biotechnol 120:376–386

Volke-Sepulveda T, Saucedo-Castaneda G, Gutierrez-Rojas M, Manzur A, Favela-Torres E (2002) Thermally treated low density polyethylene biodegradation by *Penicillium pinophilum* and *Aspergillus niger*. J Appl Polym Sci 83:305–314

Volova TG, Prudnikova SV, Vinogradova ON, Syrvacheva DA, Shishatskaya EI (2017) Microbial degradation of polyhydroxyalkanoates with different chemical compositions and their biodegradability. Microb Ecol 73:353–367
Walsh M, O'Connor K, Babu R, Woods T, Kenny S (2015) Plant oils and products of their hydrolysis as substrates for polyhydroxyalkanoate synthesis. Chem Biochem Eng Q 29:123–133
Wang GB, Labow RS, Santerre JP (1997) Biodegradation of a poly(ester)urea-urethane by cholesterol esterase: isolation and identification of principal biodegradation products. J Biomed Mater Res 36:407–417
Wang Y-W, Mo W, Yao H, Wu Q, Chen J, Chen G-Q (2004) Biodegradation studies of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Polym Degrad Stab 85:815–821
Wang S, Lu A, Zhang L (2016) Recent advances in regenerated cellulose materials. Prog Polym Sci 53:169–206
Ward PG, Goff M, Donner M, Kaminsky W, O'Connor KE (2006) A two step chemo-biotechnological conversion of polystyrene to a biodegradable thermoplastic. Environ Sci Technol 40:2433–2437
Wei R, Zimmermann W (2017a) Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we? Microb Biotechnol 10:1308–1322
Wei R, Zimmermann W (2017b) Biocatalysis as a green route for recycling the recalcitrant plastic polyethylene terephthalate. Microb Biotechnol 10:1302–1307
Wei R, Oeser T, Barth M, Weigl N, Lübs A, Schulz-Siegmund M, Hacker MC, Zimmermann W (2014a) Turbidimetric analysis of the enzymatic hydrolysis of polyethylene terephthalate nanoparticles. J Mol Catal B Enzym 103:72–78
Wei R, Oeser T, Then J, Kühn N, Barth M, Schmidt J, Zimmermann W (2014b) Functional characterization and structural modeling of synthetic polyester-degrading hydrolases from *Thermomonospora curvata*. AMB Express 4:44. https://doi.org/10.1186/s13568-014-0044-9
Wei R, Oeser T, Zimmermann W (2014c) Synthetic polyester-hydrolyzing enzymes from thermophilic actinomycetes. Adv Appl Microbiol 89:267–305
Wei R, Oeser T, Schmidt J, Meier R, Barth M, Then J, Zimmermann W (2016) Engineered bacterial polyester hydrolases efficiently degrade polyethylene terephthalate due to relieved product inhibition. Biotechnol Bioeng 113:1658–1665
White GF, Russell NJ, Tidswell EC (1996) Bacterial scission of ether bonds. Microbiol Rev 60:216–232
Wilkins MR, Atiyeh HK (2011) Microbial production of ethanol from carbon monoxide. Curr Opin Biotechnol 22:326–330
Witt U, Einig T, Yamamoto M, Kleeberg I, Deckwer WD, Müller RJ (2001) Biodegradation of aliphatic–aromatic copolyesters: evaluation of the final biodegradability and ecotoxicological impact of degradation intermediates. Chemosphere 44:289–299
Woodruff MA, Hutmacher DW (2010) The return of a forgotten polymer – polycaprolactone in the 21st century. Prog Polym Sci 35:1217–1256
World Economic Forum, Ellen MacArthur Foundation, McKinsey & Company (2015) Project MainStream – a global collaboration to accelerate the transition towards the circular economy. World Economic Forum, Geneva. REF 041214
Yamada-Onodera K, Mukumoto H, Katsuyaya Y, Saiganji A, Tani Y (2001) Degradation of polyethylene by a fungus, *Penicillium simplicissimum* YK. Polym Degrad Stab 72:323–327
Yang J, Yang Y, Wu WM, Zhao J, Jiang L (2014) Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. Environ Sci Technol 48:13776–13784
Yang Y, Yang J, Wu WM, Zhao J, Song Y, Gao L, Yang R, Jiang L (2015a) Biodegradation and mineralization of polystyrene by plastic-eating mealworms: part 1. Chemical and physical characterization and isotopic tests. Environ Sci Technol 49:12080–12086
Yang Y, Yang J, Wu WM, Zhao J, Song Y, Gao L, Yang R, Jiang L (2015b) Biodegradation and mineralization of polystyrene by plastic-eating mealworms: part 2. Role of gut microorganisms. Environ Sci Technol 49:12087–12093

Yoon MG, Jeon HJ, Kim NM (2012) Biodegradation of polyethylene by a soil bacterium and *alkB* cloned recombinant cell. J Bioremed Biodegrad 3:145. https://doi.org/10.4172/2155-6199.1000145
Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, Toyohara K, Miyamoto K, Kimura Y, Oda K (2016) A bacterium that degrades and assimilates poly(ethylene terephthalate). Science 351:1196–1199
Zhang L, Liu H, Zheng L, Zhang J, Du Y, Feng H (1996) Biodegradability of regenerated cellulose films in soil. Ind Eng Chem Res 35:4682–4685
Zhang Q, Dor L, Fenigshtein D, Yang W, Blasiak W (2012) Gasification of municipal solid waste in the plasma gasification melting process. Appl Energy 90:106–112
Zimmermann W, Billig S (2011) Enzymes for the biofunctionalization of poly(ethylene terephthalate). Adv Biochem Eng Biotechnol 125:97–120



Determination of Kinetic Parameters and Metabolic Modes Using the Chemostat

19

Rich Boden and Lee P. Hutt

Contents

1 Introduction 364
2 Chemostat Kinetic Theory 366
3 Technical Considerations 368
3.1 Organisms and Media 368
3.2 Preventing Misbehavior 377
3.3 Analytical Chemistry 379
3.4 Automatic Titration 380
3.5 Starting, Running, and Finishing 383
4 Determination of Parameters 386
4.1 Maximum Specific Molar Growth Yield (Y_{MAX}) and Maintenance Coefficient (m_S) 386
4.2 Metabolic Quotients (q) and the Respiration Rate (q_{O2}) 390
4.3 ATP Yield of Biomass (Y_{ATP}) 391
4.4 The Monod Constant (K_S) 391
4.5 Oxygen Transfer Coefficient (k_La) 392
4.6 Comparing Metabolic Modes 393
4.7 Analogous to Inverted Mixed Inhibition 394

R. Boden (✉)
School of Biological and Marine Sciences, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK

Sustainable Earth Institute, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK
e-mail: rich.boden@plymouth.ac.uk

L. P. Hutt
School of Biological and Marine Sciences, Faculty of Science and Engineering, University of Plymouth, Plymouth, UK
e-mail: lee.hutt@plymouth.ac.uk

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9_24

4.8 Analogous to Inverted Uncompetitive Inhibition 397
4.9 Analogous to Inverted Competitive Inhibition 399
4.10 Analogous to Inverted Noncompetitive Inhibition 399
5 Conclusions 400
6 Research Needs 400
References 401

Abstract

Continuous cultures in the form of chemostats limited by carbon source, energy source, nitrogen, phosphate, or oxygen are commonly used where reproducible growth or a continuous supply of biomass is needed. Originally, they were developed as means to determine a range of kinetic parameters such as specific molar growth yield (Y) and derived parameters such as maximum specific molar growth yield (Y_{MAX}), maximum specific growth rate (μ_{MAX}), maintenance coefficient (m_S), specific maintenance rate (a), the Monod constant (K_S), etc. These parameters afford very useful means of comparing organisms or determining metabolic modes within an organism but are sadly no longer widely used, largely for reasons of skill sets disappearing. In this chapter we cover the basics of running a chemostat to give high-quality growth data and manipulation thereof to obtain growth kinetic parameters.

1 Introduction

The most commonly used form of growth of *Bacteria* and *Archaea* in liquid culture is the batch culture, a closed system in which a finite amount of electron donor (which is also the carbon source, for growth of heterotrophs) is supplied in some manner of liquid medium. The organism grows until some nutrient runs out – either the carbon source or a trace metal, commonly zinc – or a toxic or inhibitory by-product of growth builds up in the medium (e.g., hydroxonium ions or "protons" as we conventionally simplify to in biology). Batch cultures can also be undertaken in bioreactors ("fermenters" in common lab parlance, regardless of their metabolic mode) that afford larger volumes along with pH control, oxygenation control, stirring, etc.

Batch culture has a number of limitations, viz.:

- **Cell "happiness"** – for wont of a better word! When cells are grown in a closed system such as batch culture, they are swimming in their own waste – be that protons, fatty acids, side products, secondary metabolites, etc. – and this can cause metabolic stress and raise the maintenance needs of the cells, leading to low enzyme activities and low protein yields.
- **Inability to change one variable** – for example, if one wanted to examine expression of a gene that one thinks may be involved in temperature tolerance, one could grow shake-flask batch cultures or batch bioreactors at the optimum growth temperature and 5 °C higher. In changing the temperature, one also changes the specific growth rate (μ) as the hotter flask will be growing slower.

Does the gene expression change owing to temperature or growth rate? One cannot elucidate this in batch culture.

- **Unrealistic carbon source/electron donor levels** – in the batch culture, the organism is supplied with, e.g., 20 mM acetate at the beginning of culture, and this goes down over time. Even in high-acetate environments such as peat bogs, concentrations do not usually exceed 1 mM (Avery et al. 1999); thus one has to consider how environmentally or "real-world" relevant the data collected are, since the cells are swimming in their electron donor or carbon source.
- **Limited range of parameters determinable** – useful parameters concerning maintenance or normalized forms of yields that are directly comparable with organisms from other genera or even other domains cannot be determined from batch cultures.

Various types of continuous culture exist, and most are based around vessels with some manner of input and output (the latter can be a weir in the wall or a drop tube set at a given depth with a pump attached) to maintain a constant volume – for every mL that enters the system, an mL leaves the system. Of the variations that exist, some serve to keep the cell number constant in the vessel at all times (turbidostat, cytostat) or to keep the level of cellular substrate consumption constant (auxostat) or the concentration of carbon source or electron donor constant (nutristat). In a system that keeps a given *limiting substrate* (be that the carbon source, electron donor, nitrogen source, phosphorus source, or even a metal) **absent** from the vessel at all times since it is wholly consumed, the amount of biomass is also constant when the system is in steady state – this is the chemostat.

Chemostat setups vary enormously and can be as basic as a sidearm test tube with medium dripped in from a Pasteur pipette pushed through a foam bung – while such systems of *c.* 50 mL volume are excellent teaching tools and can be handy for keeping "fussy" organisms alive, they are not suited to the much more rigorous conditions needed in the research laboratory. Chemostat setups of such rigor have been reported with volumes on the μL scale using microfluidics (Groisman et al. 2005; Long et al. 2013), with devices printable using stereolithography methods. Semimicro setups with volumes of as little as 10 mL can be produced by a glassblower with relative ease and can, with the right pumps, etc., allow a laboratory to run dozens of experiments at the same time (e.g., the Pirtian Mini-Loop system is very useful for aerobic phototrophs in particular, Pirt et al. 1979). The more typical systems are purpose-built, well-controlled devices that can be procured from specialist suppliers and which have a range of vessels that can hold cultures of 0.1–10 L. Large chemostat volumes can be useful when the goal is to produce a steady-state culture and sacrifice it for protein purification, but for kinetic work, a small culture volume is much more convenient as it reduces the amount of time and medium consumed during equilibration. In our laboratory, we use *c.* 2500 mL vessels with *c.* 500 mL medium for kinetic studies, and we find this very convenient, with the large headspace being particularly useful when growing organisms on more challenging (to handle) substrates that require a long batch culture phase without air sparging,

such as carbon disulfide – e.g., the *c.* 2000 mL volume of air contains about 17 mmol oxygen, which is enough to oxidize about 8 mmol carbon disulfide without the need to sparge the culture – headspace volume is an important consideration in the setting up of a reactor, which we discuss in due course.

In this chapter we will cover the theory of Monodian and Pirtian kinetics that underpins cultivation in the chemostat and the methodologies for determination of these parameters – we have split this into two separate sections with a technical section on how to actually do it all in between so that the reader can make proper consideration of ensuring their cultivation method will provide them with the data that they need that those data will not be subject to artifacts.

2 Chemostat Kinetic Theory

The basic parameters of the chemostat that require consideration are that a culture of V (mL) volume is contained in a vessel with an inflow and outflow. Fresh medium containing the limiting substrate of concentration S (mM) is pumped into the vessel at a flow rate of F (mL/h). The hydrodynamic dilution rate of the culture D (h^{-1}) can be determined since:

$$\boldsymbol{D = F/V}$$

If the growth rate of the organism in the culture μ (h^{-1}) – the rate of cell division – is slower than D, the number of cells leaving in the outflowing medium will exceed the number of new cells from cell division; thus the culture turbidity will fade and the culture will "wash out." If μ exceeds D, more cells will be produced in the reactor than will leave in the outflowing medium; thus the culture turbidity will increase. The latter scenario is very common in the early stages of a continuous culture, but eventually μ will equal D, and a steady state will form in which the cell number remains constant – every cell division owing to substrate entering the vessel is balanced by a cell leaving in the outflowing medium. In this state, [*substrate*] is zero, not "below the detection limit," not "< 0.01 mM," but absolutely 0.000 mM – since that is the very definition of the chemostat: a steady-state culture at which the limiting substrate is absent in the vessel. In such a condition, every molecule of the substrate that falls into the vessel is immediately consumed and immediately permits just enough growth to occur to balance to loss of cells. This gives a more environmentally relevant scenario since in many environments, a substrate may not be detectable, but the flux can be very high, as is the case in the chemostat at steady state (cf. oxygen in activated sludge). Many useful parameters can be determined from a chemostat culture, and they will be discussed in Sect. 4.

For chemostat theory to apply absolute, there are conditions that must be met:

(1) **All growth must be planktonic and without foam.** Wall growth or biofilms forming on probes, etc., will prevent a true, stable steady-state culture from forming. Foam rafts form owing to lysed cells, biosurfactant buildup, etc., and are more common in cultures growing on lipids, fatty acids, or hydrocarbons

than on low-concentration sugars or inorganics – they cause issues with maintaining a constant volume and can also provide a surface for biofilms to colonize when they dry on the vessel wall. Silicone antifoam agents can control or prevent foam, and both this and mechanisms to prevent wall growth and biofilms are covered in Sect. 3.

(2) **The culture must be very well mixed**. Effectively "infinite mixing" of the culture is an absolute necessity, since the drops of substrate-bearing medium falling into the vessel must be disbursed instantaneously such that their consumption is instantaneous.

(3) **Aside from the limiting substrate, everything else must be in excess.** That is to say that metals, nitrogen, phosphorus, sulfur, etc., must all be in excess but so too must be the terminal electron acceptor – if this is molecular oxygen, then gassing must be very vigorous. In cultures of chemolithoautotrophs, it is important to insure that the limiting electron donor at, e.g., 20 mM is truly limiting by ensuring a vast supply of carbon dioxide/(bi)carbonate.

(4) **The medium must be entirely defined and should contain a single carbon source or a single electron donor**. A basal salt medium, usually with a phosphate buffering system, is used and is supplemented with the carbon source or electron donor. While vitamins may be provided to ensure they are not limiting growth, there should not be any carbon sources present. It is not suitable to have any complex components in the medium, e.g., brain-heart infusion is commonly used in batch cultures at concentrations of 0.002% (*w/v*) when growing autotrophs as a "vitamin" – in the chemostat, using proper vitamin solutions is very important as they are fully defined and cannot be taken up as co-substrates to produce biomass. It is not necessary to use a water-soluble limiting substrate – alkanes, elementary sulfur, etc., can be used.

(5) **All parameters must be tightly controlled with feedback systems**. For example, a thermistor probe in the culture feeds back to a microprocessor control unit that has been programmed by the user to hold the culture at 43 °C. If the temperature momentarily drops owing to a draft or cool medium being fed in, the control unit turns on a heated probe or jacket and briefly warms the culture. If the culture becomes too warm owing to biogenic heat, the control unit opens a solenoid valve, running cold water through a cold finger to cool the culture. Similar systems are required for proton concentration (a pH probe and reservoirs of acid and base) and dissolved oxygen (dO_2) concentration as a minimum (for anaerobes, a redox potential (E_h) probe may be more useful). To maintain culture volume, a Liebig or Allihn condenser is attached to the outflowing airline so that water vapor is returned to the vessel – at higher temperatures (>50 °C), a Dimroth or Friedrichs condenser may be necessary as the efficiency will need to be higher. Often tap water is used to feed these condensers, but for high-temperature work, a chilling unit that can supply water at 2 °C is much more effective.

(6) **The limiting substrate, other media components, and the production of biomass must be accurately quantifiable.** It is quite impossible to run a chemostat if one cannot quantify the depletion of the limiting substrate – and

for that matter, the phosphorus and nitrogen sources (to ensure they are not limiting) – and the production of biomass. For some electron donors that are difficult to measure without specialist equipment, such as dimethyl sulfone ($DMSO_2$), but for which the end products (sulfate) are stoichiometric and measurable, one can effectively measure substrate by proxy, as one can measure the sulfate being produced (Boden et al. 2011a; Borodina et al. 2000). It is important that all analytical work be done with great care and precision, appropriate glassware, etc. There is an emphasis on, e.g., kits provided by manufacturers for use by biologists to be high-throughput and fast, often working in very small volumes in plate readers where tiny pipetting errors change the path length; it is prudent to work like an analytical chemist with proper volumetric glassware and proper calibrants and – for colorimetric assays – using the largest path length cuvettes possible so as to extend accuracy.

3 Technical Considerations

In this section we discuss the setup and running of the chemostat as well as its rescue if things go awry.

3.1 Organisms and Media

Organisms wont to forming biofilms, rosettes, etc., can be difficult to manage in the chemostat in terms of keeping all growth planktonic, but we give herein methods for preparing glassware to avoid biofilm formation, and rapid stirring is often sufficient to reduce clumping of cells.

3.1.1 Biomass Determination

It is critical that the amount of biomass is determined accurately and that optical density is not used as raw data, but is converted to a meaningful unit. In our hands, both colorimeters and spectrophotometers are equally useful for biomass determination as long as they are well calibrated. Many users use an arbitrary wavelength and determine optical density (*OD*) at, e.g., 600 nm (OD_{600}) without giving it much thought, but it is absolutely critical that proper calibrations are made for every organism used, (1) to determine the proper wavelength to use and (2) to determine the *OD* conversion to biomass factor. This is achieved by growing flasks of the organism on various substrates (since accumulation of volutin, etc., can alter the results) to late-exponential phase. Cells are harvested, washed, and resuspended in the same basal medium they were grown in or in a suitable buffer and are then diluted to a range of at least five arbitrary turbidities – 3×1 L of each turbidity is required, and they are diluted in volumetric flasks – the optical density of each being checked in replicate cuvettes at a range of wavelengths from 440 nm to 740 nm (below 440 nm the Soret peak of cytochrome *c* tends to cause interference – we usually use 440, 540, 640, and 740 nm, as a minimum) then returned to the flask.

A set of 0.2 μm filters (45 mm diameter) are placed in all-glass filter holders on top of 0.45 μm filters as supports and are dried overnight at 95 °C – this is necessary to remove any moisture and must be done within the holders; otherwise, they curl and then crack when used. The filters are removed and weighed on an analytical balance and replaced in the holders. Each is used to filter one of the replicate turbidities, and the cells on the filter are then washed with 500 mL of the same basal medium, then with 100 mL ice-cold water. Filters are dried in crystallizing dishes at 70–95 °C to constant mass (up to a week – a vacuum oven can speed this up) and are weighed. From the mean amount of biomass at each optical density, one can plot the *OD* amount of biomass (ordinate, no units) against the concentration of biomass (abscissa, mg dry biomass/L) with different lines for each *OD* wavelength used. From these data, the linearity of the data can be examined – we usually look for one linear to $OD = 0.9$ as we routinely dilute all samples >0.9 for analysis anyway since spectrophotometer inaccuracy begins to interfere. The conversion ratio (e.g., for *Thermithiobacillus tepidarius* strains, $OD_{440} = 0.1$ is 23 mg dry biomass per L) can then be determined. In Fig. 1, we show representative data for *Pseudomonas*, *Achromobacter*, and *Thermithiobacillus* – it can be seen that in all cases, 440 nm permits the largest change in *OD* per unit dry biomass, thus affords the most useful data. Koch (1981) also found that low wavelengths were also the most useful for small cell sizes. The linearity of the data does vary, particularly for *Pseudomonas* and *Achromobacter* at low densities, but the standard error of mean is usually consistently small within each organism's data. The exception to this is the *Thermithiobacillus* sp., which has greater standard error of mean, potentially owing to carboxysomes ("polyhedral bodies") present in the cells being at variable number and the very high cytochrome *c* content of sulfur-oxidizing *Bacteria*, for which the tail end of the Soret peak will possibly give some minor interference at 440 nm and the beginning of the α peak will possibly interfere at 540 nm, but these absorbances are usually very low versus optical densities, and the latter has the greater impact on the value obtained.

Some workers use the amount of protein or amount of DNA in lieu of the amount of dry biomass, but these are very prone to change during growth and should be avoided. Cell carbon is a useful unit if one has access to a total organic carbon (TOC) analyzer or can have CHNS analyses performed on a sample, but we have found for various members of the *Proteobacteria* that they contain 48% C by mole in every case. Cell number is quite a useless unit in chemostat kinetics since 20 small cells could have the same amount of biomass as five large cells or the culture could contain a range of cell sizes in a pleomorphic organism; thus we strongly recommend dry biomass or cell carbon aredetermined.

3.1.2 Protein Determination

While we strongly recommend cell protein not be used as the unit of growth, it can be useful to determine as a secondary factor, and the bicinchoninic acid assay (Smith et al. 1985) is generally the most useful; however, calibration should be carefully considered since the de rigueur bovine serum albumen does not always give a particularly good calibration. Microtiter plate assays should be avoided as the path

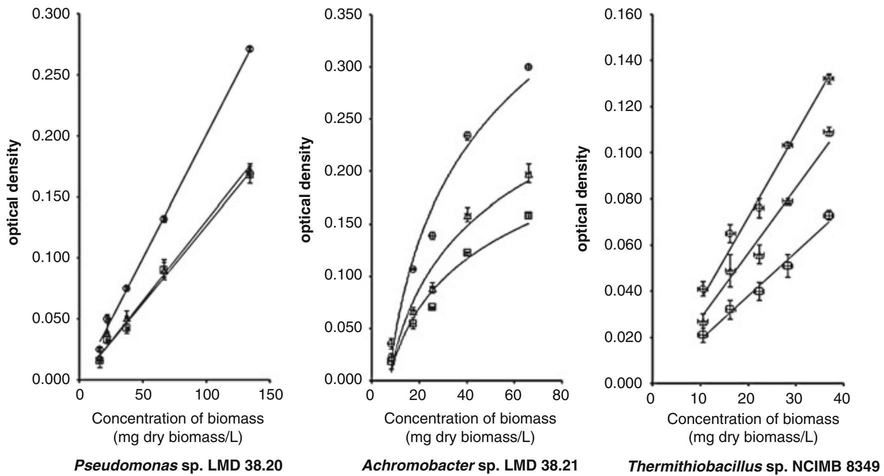


Fig. 1 Relationships between concentration of dry biomass and optical density at a range of wavelengths for members of the genera *Pseudomonas* (*Gammaproteobacteria*), *Achromobacter* (*Betaproteobacteria*), and *Thermithiobacillus* (*Acidithiobacillia*). Optical density determination wavelengths are indicated by circles (440 nm), triangles (540 nm), and squares (600 nm). Error bars are standard error of mean ($n = 3$). Briefly, cells obtained from glucose, succinate, or thiosulfate-limited chemostats (respectively) were washed in phosphate-buffered saline to give a range of turbidities. Optical density was determined at three wavelengths for each suspension before drying to constant mass at 70 °C in pre-weighed glass scintillation vials versus a cell-free control. None of the organisms produced endospores, exospores, or cysts. They do not contain stacked internal membranes nor do they produce intracellular volutin (polyphosphate), lipid (*β*-poly-hydroxybutyrate), or polysaccharide granules. *Thermithiobacillus* sp. NCIMB 8349 contains carboxysomes (polyhedral bodies) at variable numbers per cell in common with other members of the genus (cf. Boden et al. 2016) but does not produce elementary sulfur granules in continuous culture (Hutt 2016). Cell were short slender rods – *Pseudomonas* sp. LMD 38.20 (0.5 × 2.6 μm), *Achromobacter* sp. LMD 38.21 (0.8 × 1.2 μm), and *Thermithiobacillus* sp. NCIMB 8349 (0.5 × 1.5 μm)

length is not consistent – 1 mm path length optical glass cuvettes may be a slight inconvenience, but they are more precise. For calibration, proteins are best extracted at the French press and debris *not* removed, but the lysate is then treated with trichloroacetic acid (TCA) for which many protocols exist and the protein precipitate washed in acetone and redissolved before determination – this removes almost all interfering factors.

3.1.3 Cell Counting

Many workers count cells even though this is not a useful parameter for kinetic work really. This can be done by flow cytometry or using a Neubauer hemocytometer – whatever is used, vital or live-dead staining must be used to separate biomass from necromass – many commercial kits based on fluorescent dyes such as SYTO® 9 and propidium iodide are available. Alternatively dyes such as *N*-(3-tri-ethylammoniumpropyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide

(FM 4-64) can be used in "DIY" approaches (Pogliano et al. 1999). A complex of Ponceau S (Acid Red 112) and depleted uranyl sulfate or uranyl acetate can be used for vital staining of some unicellular *Eukarya* and *Bacteria* – we have used it with *Methylobacterium* spp. – and it can be visualized with a normal microscope (van Steveninck and Booij 1964). Sunset Yellow FCF (Orange Yellow S) has recently been reported as a "vital stain" for the *Bacteria* although the original publication does not show any live-dead staining of cells – this may simply be a stain that does not *kill* the cell, rather than being a true vital stain (Gao et al. 2017).

3.1.4 Basal Media

In this section we cover preparation of media and some example media that we use in our laboratory. For the very best data to be obtained, analytical grade chemicals should be used for all media components and for the limiting substrate and for the acid and base used for pH control – while this is costly, the data quality is worth it, and after the initial outlay, the chemicals will keep for many batches of medium.

Media for the chemostat should be made in glass-distilled water (or equivalent – we have found amino acid contamination in "Milli-Q" water, however) and use Class A volumetric glassware to minimize variation. All carboys and glassware should be cleaned by soaking overnight in 2% (*v/v*) nitric acid and then washing in glass-distilled water until the washings are neutral to pH paper. To make, e.g., a 20 L Nalgene carboy of medium that requires phosphates to be autoclaved separately in 25% of the final volume, the phosphate stock is made up of a 5 L Class A volumetric flask and is autoclaved in a 10 L carboy that has a one-port lid with a large 0.2 μm pore size filter attached, which is wrapped in foil for autoclaving. The ingredients for the remainder of the medium are added to the 20 L carboy with a very large glass-coated "flea" and 15 L glass-distilled water is added in the form of 3×5 L volumetric flasks as before. The carboy is stirred until everything has dissolved and placed on a level bench (checked with a spirit level), and once settled, a very fine permanent marker is used to mark the bottom of the meniscus at intervals around the vessel. The lid of the vessel should have three ports – one is connected to a drop tube leading to the bottom of the vessel – this is silicone tubing that is weighted with a short length of glass tubing at the lower end to keep it in the medium and prevent it from floating. The outside of this port is connected to silicone tubing and terminated with tubing connector, which is wrapped in foil. This tube is clamped off with Spencer Wells artery forceps or with gate clamps – we use the former as they are easier to operate with one hand. The next port has no interior connection, and the exterior is connected to a large diameter 0.2 μm pore size filter, which is wrapped in foil – this connection must *not* be clamped when autoclaved! The final port is connected to the bottom sidearm of a large aspirator bottle or 1 L or 2 L volume, using a long length of tubing that is terminated half way with a clamp and foil wrapped as before. The top of the bottle is plugged with a foam bung and is wrapped in foil. When autoclaving media, it is critical that the autoclave is used properly – viz., that a second 20 L carboy is filled to the same volume with water and is autoclaved with a wad of cotton wool instead of the stopper and with the "load" thermistor of the autoclave sitting in the bottom of it – this ensures that the medium is

properly heated for the right amount of time (usually 10 psi for 15 mins, but it may take 12 h for a large volume to come to temperature): do not rely on autoclave tape as an indicator! If the medium is $<$ pH 6.5, it is important that no proteinaceous broths or wastes are autoclaved at the same time as their (poly)amine fumes may pull the pH of the medium up. Autoclaving a large funnel wrapped in foil is very useful for the next stage. The autoclaved medium and phosphate solution are allowed to cool fully – usually overnight – we place them in the fume hood as the draft speeds up this process somewhat.

The 20 L carboy is positioned on a bench or on the floor, with the aspirator bottle on the bench close to a Bunsen burner. The level of the liquid in the carboy is checked against the line and carefully topped up with sterile distilled water if need be. Foil coverings on filters are removed, and aseptically, using the sterile funnel, the phosphate solution is poured into the aspirator bottle until it is full. The foam bung is put back in place and the clamp is then removed, and the bottle is raised to dispense phosphate into the medium carboy. This is repeated until all of the phosphate has been added. If any substrate or vitamin stock is to be added to the carboy after autoclaving, we add it to the aspirator within the first aliquot of the phosphate solution so that the later aliquots ensure it is fully washed in. The clamp between the aspirator and the carboy is tightly closed and the tubing severed on the aspirator side – razor blades are the best thing for cutting tubing as they give a straight cut. The carboy is then placed adjacent to the reactor on top of a magnetic stirrer, and it is stirred continually during use to prevent any sediments forming. The connection to the vessel and the determination of flow rate are covered in Sect. 3. For some extreme thermophiles, a stirring hotplate is useful to preheat the medium.

Basal media for use in the chemostat do not require vast buffering capacities, since the system is under automatic proton concentration control – being able to reduce this allows phosphate limitation to be undertaken with ease. For some work, using organic Good buffers (Good et al. 1966) is useful but with caution. Tris is always to be avoided – most autoclave-safe pH electrodes cannot determine pH in Tris-buffered solutions and can be damaged by it, plus there is a lot of variance of pH with temperature in this system. Tightly controlled batch culture work is needed to determine if they have any inhibitory effect or can be consumed as a carbon or nitrogen source. It is worth noting that piperazine-*N*-*N′*-bis(2-ethanesulfonic acid) (PIPES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (HEPPS), and piperazine-*N*-*N′*-bis(2-hydroxypropanesulfonic acid) (POPSO) can form radicals and thus disrupt redox chemistry or oxidize some substrates and should be avoided (Grady et al. 1988). Bicine and tricine should also be avoided as they inhibit flavin and flavone-dependent enzymes in the presence of light, during which they are oxidized themselves, thus lowering buffering capacity (Soni and Kapoor 1981). Consideration must also be made of any unwanted effects of buffers on downstream methods for quantification of the limiting substrate. A wider discussion of the full gamut of buffering systems including bicarbonate, Good buffers, succinate, etc., is given in Good and Izawa (1972).

Table 1 Composition of E basal salts (EBS) for use in the chemostat as a generalist basal medium for aerobic organisms. The phosphates are autoclaved separately in 25% of the final volume and are combined once totally cold – the vitamin stock is added to the combined medium once cold. Trace metal solution T and vitamin solution VJK are given in Tables 2 and 3, respectively

Component	Concentration (g/L)
Potassium dihydrogen phosphate	4.00
Dipotassium hydrogen phosphate	4.00
Ammonium chloride	0.40
Magnesium sulfate heptahydrate	0.80
Trace metals solution "T"	10.00 mL
Vitamin solution "VJK"	0.10 mL

We tend to use phosphate buffering, and our usual medium for continuous culture for non-halophiles is E-basal salts (EBS, modified from Kelly and Syrett (1964)) which is given in Table 1. EBS can be used as is with up to 60 mM substrate carbon (e.g., 10 mM glucose) under carbon limitation. For phosphate limitation, the two phosphate salts are reduced to 0.4 g each. For nitrogen limitation, we increase the substrate carbon to 90 mM total. If used with chemolithoautotrophs, 20 mM thiosulfate or 10 mM tetrathionate, etc., can be used in place of the carbon source for electron donor limitation. The pH of EBS is 7.0 but can be varied from pH 5.0 to pH 8.5 by varying the phosphate ratios on the basis of the standard buffer tables provided in most biochemical and chemical texts. The trace metals solution "T" is modified from Tuovinen and Kelly (1973) and is given in Table 2. It is useful to model the solution chemistry of defined media during autoclaving, cooling, and mixing to ensure that the final solution concentrations of all components are sufficient for growth and to understand the composition of any precipitates that may form – PHREEQC (Parkhurst and Appelo 1999) is a very useful platform for this and can give important insight, particularly when considering critical trace metal ions such as Cu(II) versus Cu(I) versus Cu mineral speciation in media for the growth of methanotrophs or when studying cytochrome *c* oxidases.

We discuss issues with foam in Sect. 3.2.1, but in the absence of a foam controller, it is prudent to add a non-emulsion non-silicone antifoam such as Antifoam 289 or Antifoam 204 (Sigma-Aldrich) to each carboy before autoclaving at a final concentration of about 0.002% (*v/v*) – these should be checked in batch culture to determine if they have any inhibitory effects on the organism.

For the acid and base solutions required for the autotitrator to maintain proton concentration, these are usually "matched" to the medium – e.g., for EBS we would usually use 0.500 M sulfuric acid and 1.000 M potassium hydroxide as EBS is high in sulfate and potassium already. For autotrophs growing under electron donor limitation on, e.g., thiosulfate, where sulfuric acid is produced by the culture, we use 1.000 M potassium carbonate so that the dissolved inorganic carbon (DIC) in the culture is kept high and non-limiting. All acid and base solutions should be made in volumetric flasks and standardized by titration against a standard acid or base purchased – as we do – at a precise concentration rather than made up in the laboratory – this can be surprisingly economical as many suppliers hold annual

Table 2 Composition of trace metal solution T, modified from Tuovinen and Kelly (1973). The sodium hydroxide is dissolved in about 400 mL glass-distilled water, and the disodium EDTAate is added. Once dissolved, each of the remaining ingredients is added, in order, after dissolving in about 40 mL glass-distilled water, with washings. The solution is then adjusted to pH 6.0 using 1 M sodium hydroxide solution (20–25 mL) added slowly with stirring and is then diluted to 1 L in a volumetric flask. It is stored at room temperature in non-actinic glass, and the color may vary from green to red without any effect on performance

Component	Concentration (g/L)
Sodium EDTAate dihydrate	50.00
Sodium hydroxide	9.00
Zinc sulfate heptahydrate	5.00
Calcium chloride	5.00
Manganous chloride tetrahydrate	2.50
Cobaltous chloride hexahydrate	0.50
Ammonium heptamolybdate tetrahydrate	0.50
Ferrous sulfate heptahydrate	5.00
Cupric sulfate pentahydrate	0.20

sales of titration supplies, including standardized acid and base solutions. For determination of the precise concentration of potassium hydroxide solutions, one would titrate against standardized 1.000 M hydrochloric acid using phenolphthalein; for potassium carbonate, the same acid is used with methyl orange. For sulfuric acid, use standardized 1.000 M sodium hydroxide and phenolphthalein. The great advantage of standardizing is the absolute ability to determine how much acid/base was consumed by the chemostat, thus how much was produced per mole of substrate, ultimately. This is particularly useful in fermentations and in growing sulfur autotrophs.

3.1.5 Vitamin Solutions

There are many published vitamin stock solutions in common use, such as the one we give in Table 3. This solution is light and heat labile when concentrated and should only be added to media after autoclaving. For truly auxotrophic organisms (e.g., *Thiomicrospira pelophila* DSM 1534^{T} for vitamin B_{12}), individual vitamins should be added which is both less expensive and less wasteful. Our practice is that for all organisms, we use a vitamin solution at 0.1 mL/L as this ensures nothing can run out and become limiting during growth.

3.1.6 Carbon Sources

If it exists, it is probably a carbon source for something. Carbon sources can be solids, liquids, or gases at room temperature and have varying levels of solubility.

For gaseous carbon sources, two options are available – one is for, e.g., monomethylamine (MMA), where a solid hydrochloride salt exists and can be dissolved in media instead. For gases that do not have soluble salts, e.g., methane, the only option re: a chemostat is to limit by oxygen, phosphate, or nitrogen as methane limitation is very hard to achieve, technically. For an, e.g., phosphate-limited system, the

Table 3 Composition of vitamin solution VJK, given to us by Dr. Jan Kuever, MPA Bremen (Germany), and which we have used for some time. The vitamins are added in order either directly or from stock solutions to about 400 mL glass-distilled water. Once all have dissolved, the pH is *very carefully* adjusted to pH 4.0 using 0.1 M NaOH and is then diluted to 1 L in a volumetric flask. The solution is sterilized by passage through a 0.2 μm filter and is stored in non-actinic glass at 4 °C. It is our practice to store it as many 50 mL aliquots, which are used by individual workers, and prevent contamination of a large batch of very costly solution

Component	Concentration (mg/L)
Cyanocobalamin (B_{12})	100
p-Aminobenzoic acid (B_{10})	80
Biotin (B_7)	20
Nicotinic acid (B_3)	200
Calcium pantothenate (B_5)	100
Pyridoxine hydrochloride (B_6)	300
Thiamine hydrochloride (B_1)	200
Lipoic acid	50
Riboflavin (B_2)	50
Folic acid (B_9)	20

chemostat is sparged with air at about 2 L/min for a 500–1000 mL culture, and the air feed is supplemented with methane at 20–30 mL/min. Note that for methane specifically, during the first 24–48 h of growth, carbon dioxide should also be added, at 20 mL/min, but this is not needed once the culture is established.

For water-soluble solids and liquids, if they are stable and will remain in solution during autoclaving (e.g., glucose or glycerol), they can be added to the medium carboy. If they are likely to volatilize (e.g., methanol) or degrade (e.g., cysteine), they should be filter-sterilized as a stock solution and added to the cooled medium with the phosphates. For some very pungent and/or toxic volatile organics (e.g., dimethylsulfide, carbon disulfide), special procedures must be adhered to, which we describe elsewhere. For less soluble solids and liquids, they can be added to very low concentrations or added until they saturate the medium completely.

For very unstable compounds, it is prudent not to add them to the medium but to meter them into the reactor from a separate stock as described in Sect. 3.1.7.

Carbon Sources for Generalist Heterotrophs

When selecting appropriate substrates for "generalist" organisms, we have found it most useful to use intermediates of Krebs' cycle, if the organism will grow on them, such as succinate. This enables relatively easy determination of the theoretical yields. Batch culture experiments are needed to determine how fast it will grow, approximately, and what kinds of yield can be obtained. If the carbon source cannot be accurately quantified, it cannot be used. It is obviously important to have a firm view of what the pathway of dissimilation to carbon dioxide is so that theoretical yields can be determined.

Carbon Sources for Specialist Groups

For methanotrophs, methane should be of very high purity as contaminating alkanes will kill the culture. For methylotrophs, very high purity substrates are needed – if formaldehyde is used, it should be made from the thermal depolymerization of paraformaldehyde (Chongcharoen et al. 2005) as procurement of methanol-free formaldehyde is very expensive and only small-volume ampoules are sold.

Dimethylsulfide (DMS) and dimethylsulfoxide (DMSO) should be from ultra-pure stocks. The latter must be kept over molecular sieve to ensure that it does not absorb water. For volatiles like DMS, Viton tubing should be used throughout, and medium should be in a glass carboy as the substrate will escape through Nalgene.

Thiol stock solutions are made by adding to well-deoxygenated water (boiled while bubbling with argon, under reflux for 15 min with a drying tube on the top of the reflux condenser, then cooling fully under an atmosphere of dry argon). Most are liquid, but for the gaseous methanethiol, this is done by bubbling through the water in a Drechsel bottle and then destroying the "off-gas" by passage through household bleach (as thiols are extremely pungent and even if the work is done in the fume hood, the local streets will smell of it! There is a serious danger when working with thiols of causing panic of a gas leak as in many countries, and ethanethiol is added to the natural gas supply to give it a detectable smell – the first author speaks from experience of having done this!). A saturated solution made in this way is about 0.3 M methanethiol, and this can be quantified and then diluted in medium (in a glass carboy, never Nalgene) appropriately before use. It will dimerize into dimethyl disulfide (DMDS) over time, and this must be monitored and the medium changed as necessary – a key point in kinetic chemostats is the need to monitor medium continually.

3.1.7 Electron Donors for Chemolithoautotrophs

Electron donors such as thiosulfate, sulfide, sulfite, and tetrathionate of high purity can be obtained from most chemical suppliers. Sodium polythionates must be synthesized by the user (Boden et al. 2010 gives details and references) as must dithionate (more details in: Boden and Hutt 2019). Polythionates, sulfide, and sulfite are filter sterilized and added to media just before use – sulfide is usually neutralized with sulfuric acid to the same pH as the medium. We flush the headspace of the medium carboys with argon and attach a balloon of argon to the air filter on the carboy so that the headspace stays full of argon to minimize auto-oxidation, and this slight overpressure prevents leaks.

Elementary sulfur can be added as a Weimarn sol or a Reimi sol since a solid substrate is not practical (von Weimarn 1926; Raffo 1908; Steudel 2003). If the medium needs to be pumped into the reservoir at F mL/h to achieve the required D, when working with sulfur sols (which should be made aseptically in sterile carboys as they cannot be autoclaved), one should reduce the medium flow to $0.9F$ and meter the sol into the vessel at $0.1F$ using tubing with a very narrow caliber to minimize settling. This gives the same dilution rate but avoids issues of instability by keeping

the sols away from the medium. It is wise to wash all glassware and plasticware overnight in concentrated hydrochloric acid if using the Weimarn sols as alkaline debris on the glass will make them less stable (Steudel 2003). The same "fractional flow rate" method can be used to add labile substrates such as sulfide or polythionates from separate stock solutions.

Carbon disulfide should be purified according to Skidmore (1979), and in the final step, the distillation should be done under argon and the collected product stored under argon at −80 °C and then added to the medium just before use (in glass carboys). There will still be *some* oxidation to carbonyl sulfide, but purification in this way removes most of it, which has metabolic importance but also reduces the smell in use – purified carbon disulfide smells similar to diethyl ether, rather than of decomposing vegetables.

3.2 Preventing Misbehavior

A kinetic run of the chemostat can last some months if well-managed, but issues can and do arise – we strongly advise that foam, wall growth, and contamination contingencies are in place before a run starts – some substrates are very costly, and it is not worth wasting money, quite simply.

3.2.1 Foam

Foam can be both prevented and cured and we usually use both systems. For prevention, a low concentration (0.002% *v/v*) of Antifoam 289 or Antifoam 204 (Sigma-Aldrich) added to the medium reservoir before autoclaving tends to prevent foam formation. Foam probes are comprised of an electrode in the medium and another in the lid of the vessel such that a foam raft would link them and complete an electrical circuit, which triggers a pump connected to a reservoir of antifoam. We use 0.5% (*v/v*) of the same antifoam agent in this reservoir – though some workers prefer the emulsion-based Antifoam A (Sigma-Aldrich) for the same purpose – our intention is that the foam probe and dosing system only operates in extremis and that the low level of antifoam in the medium is the main preventative measure. Pirt and Callow (1958) found that antifoam added as a bolus when foam rafts appeared actually led to more severe foaming and that antifoam added to the medium or meted into the vessel at regular intervals was superior. Whatever antifoam is used, it must be tested with the organism to ensure it does not inhibit growth.

3.2.2 Wall Growth

Growth on the walls of the vessel destroys the steady state and makes it very difficult to obtain any useful data during kinetic runs. For vessels with overflow weirs, biofilm growth in the weir can block the overflow and cause floods and damage to the equipment. This can be prevented by treating the glass to render it hydrophobic, but for some organisms like *Hyphomicrobium* spp., if they cannot colonize the glass, they will tend to form flocs in the culture, which can be just as difficult to obtain useful data from; however, very violent stirring (>400 rpm) combined with rapid

aeration is usually enough to create so much turbulence that they are unable to aggregate into rosettes and larger structures.

Before use, after thorough washing with 2% (*v/v*) nitric acid, the vessel should be soaked overnight in glass-distilled water and then drained and wiped dry with lens tissue. Is it then coated with 0.15 M dimethyldichlorosilane in octamethylcyclotetrasiloxane (“D4”) or chloroform (1.8 mL of the former is diluted to 100 mL with either solvent) on tissue (a pipe cleaner should be used to attend to overflow weirs) and left in the fume hood for 1 h (hydrogen chloride gas is evolved) before thoroughly washing with 1% (*v/v*) Neutracon® (Decon Laboratories Ltd, Hove, UK) and then deionized water and then autoclaving. This renders the glass hydrophobic even in 1-year runs (Boden et al. 2010, 2011a, b) and minimizes wall growth significantly. Domestic silicone glass treatments, such as Rain-X® Rain Repellent (Illinois Tool Works (USA) or Kraco Enterprises (UK)), which is mostly polydimethylsiloxane, are potentially a significantly more economical alternative, and while we have used Rain-X® Rain Repellent to coat glass plates for acrylamide gel casting (it works very well), we have not tried using it on culture vessels ourselves.

Biofilm growth in the tube from the vessel to the waste pot is also a cause of problems such as blockages and flooding, and this is easy to remove by massaging the tubing between the finger and thumb from top to bottom every day or every few days. This tubing must be very straight as any sag will harbor strong biofilm growth. If there is a continual flow of off-gas through this tubing (as there will be in most systems), this will usually prevent a lot of biofilm growth, but not all of it. If Viton or other opaque tubing is used, some short glass sections as “windows” will enable the worker to identify if biofilm is building up by massaging the region above the tubing and then watching for chunks of biofilm to wash down.

3.2.3 Grow Back

It is not uncommon for the medium feed line to get contaminated by cells splashing up from the vessel, and if minor, this can be ignored, but if it starts to grow back toward the medium reservoir, it becomes a major issue. Two main methods of prevention are in common use. A glasstundish, more commonly called an “anti-grow-back trap,” can be added to the medium feed just above the vessel. This comprises a glass bulb (spherical or cylindrical – usually about 2.5 cm diameter) with olives top and bottom, and the medium flow is simply broken by it dripping through an air space. This is our most commonly used option, but since they must be made by a scientific glassblower and the latter are becoming hard to find outside of major glassblowing companies (cf. Xia 2016) that charge a high premium on small jobs, they are not economical to produce individually – some basic glassblowing skills would allow the worker to make their own from a test tube and two pipettes without much effort – they don’t need to look perfect; they just need to function! The second option also requires a glassblower unless a suitable pre-made product can be found. Leibig condensers of slightly wider caliber than the outside diameter of the medium feed are obtained and the socket and cone cut off so that they can be slide over the feed line before autoclaving. Afterward, hot silicone oil (60 °C for

mesophiles) is recirculated through the glass jacket – this is enough to kill organisms trying to colonize the feed line, and the heat of the medium is dissipated during the drop into the vessel and landing in temperature-controlled medium. This same setup can be used to preheat medium for extreme thermophiles.

3.2.4 First Aid Kit

If the chemostat becomes contaminated, it is honestly best to abandon ship and just start over, but if the contamination is very low level, it can sometimes be rescued. We keep a "first aid kit" in the fridge comprising 10 mL syringes with 19 gauge syringe needles, loaded with sterile solutions of (a) 100× stocks of antibiotics that the organism under test can resist, (b) soluble antifoam at 10× the usual strength, and (c) 100× stock of the carbon source or electron donor based on double that at which is used in the basal medium. Between these, we can usually remedy low-level contamination by switching the reactor into batch mode and injecting antibiotics and monitoring the contamination level; if there is excessive foaming as a result, we can handle that as well. The carbon source or electron donor stock is useful during this process to add as a bolus to bulk up the culture again before switching back to continuous culture. Obviously after treatment in this way, the waste pot should be changed and the contents destroyed appropriately so as to ensure antibiotic waste does not enter drains, in keeping with good antibiotic stewardship principles.

We also keep several spare pH electrodes (autoclaved in glass measuring cylinders with damp cotton at the bottom) to hand, since they frequently fail during long runs, and a spare Clark oxygen cell probe treated in the same way, though they usually fail on the first day, if at all.

3.3 Analytical Chemistry

Very good determinations of the carbon source or electron donor are needed for the chemostat to be of any valid use for kinetic purposes. A lot of kits from, e.g., Sigma-Aldrich are based on enzymatic reactions and are quite specific but offer microtiter plate methods – these should be avoided at all costs since tiny pipetting errors change the path length and 1 mm path length cuvettes are far superior for reproducible assays. In this section we summarize some of the methodologies we have found to be reliable.

It is absolutely critical that determinations are done using "Class A" volumetric glassware and glass pipettes (or very well-calibrated "Gilson" micropipettors) and with the precision one would anticipate in the analytical chemistry laboratory. External standards in the form of certified reference materials or standard solutions should always be used and should not be made by the user. These are separate from calibrants and should not be used to make them.

3.3.1 Carbohydrates and Intermediates of Krebs' Cycle

While high-performance liquid chromatography (HPLC) is useful as it can be automated, it is not well-suited to obtain regular data ad hoc owing to setup,

calibration, time, etc., particularly on communal instrumentation. Colorimetric tests that are done stringently and with good calibration can be as precise – they can be calibrated against HPLC to understand their error margin. Carbohydrate and Krebs' cycle intermediate kits are available from Sigma-Aldrich, Megazyme, and many others, which are very reliable and economical (and can be made oneself for even less money), and they can be made even more so by using smaller reaction volumes, etc.

3.3.2 Common Electron Donors

Kelly and Wood (1994) outlined methodologies for determination of thiosulfate, trithionate, and tetrathionate in mixtures and a second for tetrathionate, pentathionate, and hexathionate – between them, they allow all of these oxyanions to be quantified. For elementary sulfur sols, sulfide, sulfite, and thiocyanate, Kelly and Wood (1998) curate methods, along with those for various volatiles – carbon disulfide, dimethylsulfide, etc.

3.3.3 Phosphate

For phosphate-limited chemostats (or to demonstrate phosphate is not limiting), we determine phosphate using malachite green phosphate methods as we have found them to be very reliable in the 0–100 mM range – they are interfered with by arsenite so for arsenite-limited chemostats, chromatographic separation may be needed. Many commercial kits are sold – we have found the one from R&D Systems (Abingdon, UK) to be very satisfactory.

3.3.4 Nitrogen Sources

The phenol hypochlorite method of Solórzano (1969) is probably the most precise colorimetric method for ammonia determination. Nitrate and nitrite methods as given by Kelly and Wood (1998) are the ones that we use.

3.4 Automatic Titration

Most parameters of the chemostat are controlled by a microprocessor control unit, with various elaborate systems that log all parameters every 5 s and report them in a spreadsheet, etc. now available.

Very useful testing devices can be obtained from, e.g., Electrolab Biotech (Tewksbury, UK) which comprise resistors that are connected to probe ports on the controller and should give a reading of, e.g., pH 7.00 – these make it possible to determine cable, controller, and probe faults with rapidity. Variable devices with multiple resistors in parallel can "fake" every pH unit or dO_2 concentration.

3.4.1 Temperature

A good thermocouple probe is necessary, and while they do not need calibration, they should be checked periodically in baths of water of known temperature so that the user is aware of "drift." For workers with thermophiles, specialist probes may be

needed versus the <50 °C probes usually sold with most systems. Temperature can be controlled with a heated probe or a heated jacket – we have come to prefer the latter – oil, water, or electrically heated options are possible; the latter is much more costly, but it makes it easier to see the culture as the jacket can be removed very quickly.

3.4.2 Oxygenation

Clark oxygen cell probes require a lot more care than they are often given – the terminal membranes need cleaning in enzymatic solutions periodically, and the electrolyte needs replacement after every run. Such probes are calibrated after autoclaving by purging the system (basal medium and substrate already in the vessel) with oxygen-free nitrogen for at least an hour at 1 L/min to give a base line "$dO_2 = 0\%$" value to the control unit. It is then purged with laboratory air for a further 1–2 h to give a "$dO_2 = 100\%$" value to the control unit. A properly calibrated probe is essential for determination of, e.g., K_La during a run but can also be a valuable tool for determining reaction stoichiometry and for checking culture health – if the air supply and stirring are turned off and a bolus of carbon source added, the dO_2 should fall – if it doesn't, the culture may be dead. A similar test is done at each steady state – at, e.g., a C-limited steady state, a bolus of medium containing vitamins, metals, nitrogen, and phosphate is injected – if the dO_2 falls and then the amount of biomass increases, the culture was not limited by carbon. Specialist enzyme-based cleaners are available for the membranes on Clark oxygen cell probes, but we have used "protein remover tablets" for hard contact lenses with equal effect!

Pirt (1975) gives a lot of detail re: modes of oxygenation, and the reader should consult Pirt for any complex questions re: oxygenation – Pirt gives a lot of detail on stirring rates, headspace volumes, and gas sparging as means of oxygenation. As a basic rule, the air sparge should be at the bottom of the vessel, directly under the impellor. We use two Rushton pattern impellers, one directly above the other with the blades out of step, positioned directly over the sparge arm. In our laboratory for a *c*. 500 mL culture volume, we use 2–4 L/min of laboratory air sparged into the bottom of the vessel through a punctured blind-ended tube to maintain dO_2 at 50.0% for autotrophs on nonvolatile substrates – heterotrophs need less as they are not dependent on the 0.04% (*v/v*) carbon dioxide but the 21% (*v/v*) molecular oxygen. If working with, e.g., carbon disulfide, we would use 1 L/min to avoid pushing the electron donor out of the medium before it can be consumed. In the Scott Laboratory at the University of South Florida (Tampa, FL), where *Thiomicrospira*, *Thiomicrorhabdus*, and *Hydrogenovibrio* spp. are cultivated at very low dO_2, this is achieved by sparging briefly with a pulse of pure molecular oxygen, followed by rapid stirring. The stirring then stops, and at intervals, pure molecular oxygen pulses, each followed by brief stirring, are provided – this keeps the dO_2 very low in a highly reproducible manner (Kathleen M. Scott, personal communication).

A common issue when growing organisms on gaseous carbon sources (e.g., methane), or when growing obligate autotrophs that will be attracted to the carbon dioxide in the air feed, is biofilm formation in the holes of the sparge arm, which can eventually block it. While this can sometimes be rescued by turning the air up to a

high velocity, more often than not, this fails – those biofilms are surprisingly strong! We have found that a Bunsen valve over the sparge arm will prevent this – a piece of silicone tubing is slid over the arm and tightly attached with cable ties and each end. A razor blade is then used to slit a wiggly line that weaves in and out between the sparge holes. During use, this creates two flaps that will flap open and closed continually as gas enters the reactor, completely preventing biofilms from taking hold and blocking gas ingress even in runs of >1 year in length.

3.4.3 Proton Concentration

Proton concentration is usually monitored using a gel-filled (i.e., autoclavable) combination pH electrode. This is calibrated *before* the system is autoclaved, using the typical pH 4, 7, and 10 colored buffers found in most laboratories. After calibration, it should be checked with a pH 5 or pH 8 buffer *not* used for calibration to ensure it is working correctly. It is autoclaved in the vessel, usually with medium present to prevent damage to the probe. After autoclaving, the system is cooled completely before connecting the probe to the control unit. We have found it critical to have two to three spare probes to hand which can be calibrated and then autoclaved in measuring cylinders in about 1 h to rescue the system if the probe starts to malfunction – biofilm growth on the probe junction is the most common reason for this, which cannot be prevented, but is usually so minimal that it does not disrupt the steady state. During routine daily monitoring of the system, samples can be removed for external pH determination using a pH electrode or by titration, and in most modern systems, these data can be used to correct a drifting pH electrode very easily but are also useful data.

The microprocessor control unit controls two pumps – one that adds acid and one for base – that respond to fluctuations in pH. Typically the acid and base are matched to the medium, e.g., if using thiosulfate as an electron donor for which the end product of growth is sulfate, sulfuric acid is usually used. We typically use 1 N acid and base for pH control – larger reactors, however, need stronger solutions to avoid excess dilution of the culture. For growth of autotrophs under electron donor limitation, the base used is commonly potassium carbonate since this ensures dissolved inorganic carbon is not the limiting substrate. The tubing from the acid and base reservoirs should be very long and moved along every week as the pump tends to "chew" the tubing resulting in non-addition or leaks. We have found the surgical steel used to manufacture the "triple addition" ports on many systems is prone to acid attack and greasing it with high-vacuum grease tends to minimize this, but having a spare, sterilized port on hand is important in these types of reactor as it is a common point of failure! Using a weaker acid is an option but does dilute the culture excessively.

Where precise determination of acid/base volume addition is needed (which is the case when any kinetic parameters are to be calculated), two options are used – one is an autotitrator that records the distance the pump has traveled; thus from tubing of fixed caliber, it calculates how much acid/base has been added since last reset. Alternatively, 500 mL or 1000 mL Class B burettes are readily available for about US$260–300 and can simply be used as acid/base reservoirs with the tap left open

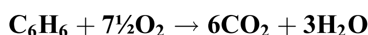
and cotton wool stuffed into the neck. We have used both options, and providing there is an autoclave big enough for these burettes, they are a good option that is more economical in the long run and perhaps less prone to error. They are simply topped up during use and their volume recorded during daily monitoring.

3.5 Starting, Running, and Finishing

3.5.1 Starting a Run

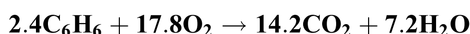
The beginning of a run is commenced immediately after polarization of the oxygen probe. The medium in the vessel is fully aerated at this stage, and a late-exponential phase batch culture of about 10% (*v/v*) of the culture volume is added to the vessel. This can be done via addition ports from an inverted universal bottle or by injection through a septum port – the latter can kill a lot of cells if done too rapidly, however. For a nonvolatile, nongaseous substrate, the culture is aerated and sparged without any control (i.e., without attempting to keep dO_2 at 50%), but the pH is monitored and controlled. Usually we then run as a standard batch culture for 24–48 h until we are confident the amount of biomass is rising and that acid or base has been consumed by the culture. At this stage, we begin to move to a steady state as described later in this section. For volatile substrates, after inoculating the fully aerated, substrate-free medium, the air sparging is turned off, and the stirring is reduced to 50 rpm to just keep the culture moving. Substrate is injected into the vessel, and the batch culture phase is continued without adding any addition of air. How much substrate is added at this stage is determined as follows:

If the culture is of volume V and the vessel is of volume L, the headspace volume H is determined by subtracting V from L. Assuming laboratory air to be about 20% molecular oxygen, the volume of oxygen is $0.2H$ at the point of inoculation. The stoichiometry of total oxidation of substrate to carbon dioxide, water, etc. (i.e., the full oxidation as if all of the substrate was being used for energy and not for biomass, thus the hypothetical maximum amount of oxygen required) is determined – for example, for benzene:



$$\mathbf{C_6H_6 + 7\tfrac{1}{2}O_2 \rightarrow 6CO_2 + 3H_2O}$$

The amount of molecular oxygen in the headspace is determined from $0.2H/22.4$, for example, in a vessel where $L = 6$ L, $V = 4$ L, and $H = 2$ L; thus there is 17.8 mmol O_2 present; given this, the stoichiometry can be adjusted to:



$$\mathbf{2.4C_6H_6 + 17.8O_2 \rightarrow 14.2CO_2 + 7.2H_2O}$$

Thus it is determined that 2.4 mmol benzene is the maximum that can be utilized for the given amount of headspace molecular oxygen, and this amount (165 μL) is injected into the vessel, giving a final concentration of 0.6 mM. Obviously, sensu stricto, one should also take into account the dissolved oxygen in the medium; however, the purpose of this step is merely to avoid "overfeeding" the culture and risking killing it but give it enough substrate to grow well given the amount of

oxygen present – so we ignore the *relatively* small fraction of oxygen dissolved in the medium. The concentration of benzene is then monitored over 24–48 h (or longer), and once totally or almost totally consumed, the culture is sparged with air for 1 h to fully aerate it, and a further 2.4 mmol is injected. Once this process has been repeated three times, the culture is usually sufficiently "bulked up" and "hungry" to begin conversion to continuous flow as described later in this section.

For gaseous substrates it is usual to mix them into the air supply – for example, methane is typically added at 30 mL/min to 2 L/min air – it is *critical* with flammable gases to ensure that the mixture produced is outside of the explosive limits (for methane, in the above example, it is 1.5% (*v/v*) in air, where the explosive range is 5–15% in air). This is simply sparged through the culture, and it is to all intents and purposes treated like a nonvolatile substrate, as described above. For most methanotrophs, carbon dioxide would also be added at about 5 mL/min for the first 24–48 h – it is not entirely known why this is required, but it is the case that most of them require it to kick-start growth in some way.

The conversion to continuous flow is best done slowly. We make use of a "timer plug" of the ilk used to switch on lights when on holiday at set intervals, with a peristaltic pump connected. This allows flow to be continued for blocks of time without any hands-on work. We usually begin by determining a low dilution rate – around $D = 0.01\ h^{-1}$ or so – sometimes lower. In the tubing running from the medium reservoir to the vessel, we have a "T" connector to which the "stem" of the "T" is connected to a 10 mL analytical quality graduated pipette or burette via a short length of tubing that can be clamped. The top of the pipette has another length of tubing connected to a filter. This enables the flow rate (F) of the medium to be determined. The pump is positioned between the "T"-piece and the vessel. Briefly, one clamps the tube close to the pump then attaches a syringe to the filter and draws up medium from the reservoir into the pipette. The tubing between the "T" and the reservoir is then clamped and the pump turned on at various speeds, and triplicate determinations of F are made by timing how long it takes to pump 5 mL out of the pipette and dividing down to give F in mL/min. If we then want $D = 0.01\ h^{-1}$ initially and we have a 2000 mL culture volume (unlikely – ours is actually 492.5 mL and precise volume must be known with all probes in place!), we can then ascertain (since $D = F/V$) that we need F at 20 mL/h, or 0.33 mL/min, and the pump set appropriately. We then have the pump running as "1 hour on, 1 hour off" for about 12 h or so and then "3 hours on, 1 hour off" for 12 h, and after that, there is usually enough biomass to support running the pump continually – if the culture washes out completely, reinoculate and repeat but run "3 hours on, 1 hour off" for 1–2 days before going continuous. It is worth noting that some models of reactor (e.g., Fermac models from Electrolab Biotech Ltd) have a "medium" pump built in that is set to deliver x mL/min by running at one speed for short lengths of time and then being off – thus the medium is being added in pulses – this must be avoided as it will not give any useful data. We find it useful to ensure the tubing from reservoir to vessel has 3–4 lengths of tubing of different caliber in series so that we can work at very low and very high F by moving the position relative to the pump.

3.5.2 Steady State

Once the culture has been running at a fixed D for several days, the amount of biomass (x) will start to stabilize, and the concentration of the limiting substrate will deplete to 0 M (thus the change in the amount of limiting substrate (ΔS) is the same as the initial concentration thereof) – at this stage, the yield (Y) should be calculated every time the amount of biomass is monitored – on the basis of all units being converted to "per liter" rather than "per chemostat volume":

$$\boldsymbol{Y} = \Delta\boldsymbol{x}/\Delta\boldsymbol{S}$$

The unit of Y can be g dry biomass/mol electron donor, g dry biomass/mol glucose, g dry biomass/mol glucose carbon, etc. – we tend to use all of them, depending on the precise context. A steady state is achieved when Y stabilizes, not X – the substrate consumed must be considered. We have a "T" connector in the tube just after the medium reservoir with a vaccine stopper on the "stem" of the "T" so we can withdraw sterile medium and monitor it for the precise substrate concentration over time – this is important as many substrates will oxidize to some degree in air. Once Y is stable, 5 V of medium is passed through the vessel before a steady state is stable and can be made use of – some workers use 3 V, but we have found in our hands that 5 V is more reliable, even if it does consume much more medium. At this point, replicate determinations of Y are made. D is similarly determined based on both F and the volume per hour of acid/base being consumed since they add to the dilution of the culture – they are added to F to give the true flow rate, and thus D is determined. For kinetic studies, about ten steady states at a range of D are determined by increasing/decreasing flow – we usually start low and speed up – and from these data, various useful parameters can be determined as discussed later in this chapter.

If removing samples from the chemostat at steady state for other work, a maximum of 0.1 V should be removed per day (Pirt 1975). We find it more convenient to connect a small (2 L) sterile waste container buried in wet ice and to collect overflow when we need larger amounts of biomass for work. For thermophiles, putting this bottle in cold water is enough to cool the overflow. From a $D = 0.15\ h^{-1}$ run of our *c*. 500 mL culture volume system, we can obtain 1.2 L of overflow in a 16-h overnight collection ready for work the next day.

At steady state, contamination checks must be carried out daily. In our laboratory we mostly work on obligate autotrophs, and our checks would be to streak out onto, e.g., EBS agar containing thiosulfate on which the autotroph will grow and Reasoner's 2A agar (Reasoner and Geldreich 1985) on which heterotrophic contaminants will grow. We also produce and examine Gram-stained slides daily and keep these for the duration of the run so that we can go back and check for any low-level contamination retrospectively. In a log book, we note temperature, pH, dO_2, E_h, volume of acid and base added thus far, medium flow rate (it should be checked periodically as pumps can "slip" during long runs), optical density (thus the amount of biomass), and substrate concentration in the reservoir and the vessel. While we use a computer to automatically log data from the pH probe directly, it is still essential to log manually lest many months of data are lost if the software crashes.

3.5.3 Washout Kinetics: μ_{MAX}

The parameter of critical dilution rate (D_{crit}) is that at which the culture begins to washout rather than increase in Y. It can be hard to determine directly since when a culture is growing at $D \geq 0.9D_{crit}$, erratic behavior and a fall in Y are not uncommon (Pirt 1975), so while D_{crit} is hypothetically equal to the maximum specific growth rate of the organism (μ_{MAX}), it is hard to determine D_{crit} simply by trial and improvement until a washout is observed: it is best determined from washout kinetics. We do this at the end of a run, usually, and it can be done within a working day. We use the method of Karagouni and Slater (1978), in which firstly a steady state is established at any D at which the culture is stable but is reasonably high, so that Y is also high enough that falls are easy to measure. Based on the specific growth rate (μ) from shake-flask batch culture on the same substrate, the μ_{MAX} is estimated to be *roughly* in the same region – thus if working at $D = 0.08\ h^{-1}$ and shake-flask culture gave $\mu = 0.12\ h^{-1}$, for the purposes of the next step, we make the assumption that this is *somewhere around* μ_{MAX}. D is then increased to, e.g., $0.20\ h^{-1}$ so that it is well above the estimated μ_{MAX}, and Y is determined every hour for 5–10 h. By plotting the decrease in Y against D, it is possible to determine the actual, transient μ of the organism at each point of the washout by regression. Further increasing D allows this to be determined several times – this could continue until the culture washes out to the point at which one cannot determine Y any more. From these data, it is possible to determine the highest μ evident during washout, which is the true μ_{MAX}.

An alternative method for the determination of μ_{MAX} is that of Pirt and Callow (1960), in which we establish a steady state at a moderate D and determine x there (which we call x_i). D is then increased to a much higher value, and x is determined at intervals over 5–10 h. A plot of ln x versus time (t) is then produced, for which:

$$\ln \boldsymbol{x} = (\boldsymbol{\mu}_{\mathbf{MAX}} - \boldsymbol{D})\boldsymbol{t} + \ln \boldsymbol{x}_{\mathbf{i}}$$

Thus, the gradient of the line is μ_{MAX}-D, and since the latter is known, the former can be calculated easily.

4 Determination of Parameters

We have already given the determination of μ_{MAX} in Sect. 3.5.3, since it is determined experimentally rather than from the examination of data from many experiments. In this section, we consider the determination of other parameters from chemostat yield data.

4.1 Maximum Specific Molar Growth Yield (Y_{MAX}) and Maintenance Coefficient (m_S)

The maximum specific molar growth yield (Y_{MAX}) is known by many names – maximum yield coefficient, theoretical yield, true yield, and so on. To understand

what this coefficient means and how it is determined, it is best considered alongside the maintenance coefficient (m_S).

The concept of what we refer to as "Pirtian maintenance," sensu the maintenance coefficient (m_S) has been argued against by many authors over the years, and the validity of the concept and the methods of determination have both come under scrutiny – von Bodegom (2007) gives an excellent critical evaluation of the concept. It is important the note that m_S in the Pirtian sense is not the only interpretation of or means to evaluate maintenance as an overall concept, and thus while some shun this particular parameter in terms of others, it still has purpose – should the reader wish to explore the full gamut of maintenance parameters, von Bodegom's (2007) review is an excellent springboard. Pirt (1965) gives a good overview of the derivations and significance of Pirtian maintenance parameters and, along with Pirt (1975), provides a very useful canon of Pirtian kinetic theory.

Pirt stated that a fraction of the total electron donor consumed (ΔS_E) was used in part for growth (ΔS_G) and in part for maintenance (ΔS_M), the latter being for repair and replacement of damaged cell components. Since ΔS_M is probably always >0, it is impossible to determine a true specific molar growth yield in the sense of just growth; since the ΔS component of the parameter (cf. Sect. 3.5.2) is total substrate (ΔS_E, if an electron donor/energy source) consumed, Y is effectively "amount of biomass formed per unit substrate consumed, regardless of what for." If it were possible to view $\Delta S_M = 0$, we would see the "true" growth yield Y_{MAX}:

$$\boldsymbol{Y_{MAX} = \Delta x / \Delta S_G}$$

Y_{MAX} is the highest possible yield parameter for growth per unit electron donor, since no substrate is consumed for maintenance – obviously this cannot be determined directly experimentally. Y_{MAX} is instead determined from Y at a range of μ:

$$\boldsymbol{1/Y = m_S(1/\mu) + (1/Y_{MAX})}$$

After determining Y at a range of steady states each at a different μ (i.e., D), Y_{MAX} can be determined from the above relationship. Historically, this was done using graphical methods akin to the Lineweaver-Burk plot (Lineweaver and Burk 1934) in enzymology (compare $1/V$ vs. $1/S$ and $1/Y$ vs. $1/\mu$), but they are highly error laden, and hyperbolic fitting is now so much easier to undertake. Rather than explain at length how to go about doing it, there are many YouTube resources explaining how to determine V_{max} from enzymology data using hyperbolic fitting, for example, using the "SOLVER" add-on for Microsoft Excel, which reduces the task to a few minutes work – these should be consulted if the reader is not familiar with reducing the sum of squared residuals, etc. – alternative methods using R, Python, etc., are well-suited to batch processing of datasets or for particularly large datasets. Hyperbolic fitting, if done following the Michaelis-Menten methodologies, will not give m_S directly, but via a second parameter, the specific maintenance rate (a), which can be considered as

a turnover rate for biomass – on its own it is not particularly useful, but it can be used to determine m_S since:

$$\boldsymbol{m_S = a/Y_{MAX}}$$

Again, historically m_S was determined graphically, usually from plots of the metabolic quotient (q) versus μ. Since:

$$\boldsymbol{q = \mu/Y}$$

this plot is analogous to the Hanes-Woolf plot from enzymology (Hanes 1932) and thus is prone to the same errors (Haldane 1957). The metabolic quotient q is sometimes termed the specific rate of substrate uptake or the specific metabolic quotient; it is often written in terms of the limiting substrate – $q_{thiosulfate}$, $q_{glucose}$, etc. – if the limiting substrate is respiratory molecular oxygen, q_{O2} is properly termed the "respiration rate." The same could be said of any anaerobic terminal electron acceptor, though while q_{NO3-} is a respiration rate, q_{O2} is the respiration rate! q is usually given in mmol substrate per gram dry biomass – we consider these parameters in Sect. 4.2.

The maintenance coefficient m_S is usually given in g electron donor per g dry biomass.h and is a measure of how much of the energy source is required to maintain a gram of dry biomass for an hour. It can be converted to m_{ATP}, which is given in mmol ATP per g dry biomass.h – the amount of ATP required to maintain a gram of dry biomass for an hour – though this conversion requires understanding of the production rates of ATP per unit electron donor. This can be easily determined in resting cell suspensions held in a thermostatted vessel at the usual growth temperature (we use the chamber of a Rank Brothers Ltd (Bottisham, Cambridge, UK) Clark-type oxygen cell along with a recirculating water bath as an easy option, but small flasks in a water bath may suffice, though they will take longer for the contents to reach temperature). To these cells, a known amount of pre-warmed electron donor is added, and aliquots are then removed at 10-s intervals into 10% of the aliquot volume of 1.45 M perchloric acid containing 10 μM xylenol blue (Cohen 1922) and are held on ice for at least 10 min for digestion to complete. This all must be carried out in hypochlorite-washed, baked glass containers to ensure background ATP is fully removed. After incubation on ice, 1.00 mL 0.3 M potassium hydroxide is added per 0.2 mL perchloric acid solution used in the digestion. This brings about neutralization, as indicated by the indicator changing from red/yellow to just violet – if it is not neutralized, further alkali should be added. The heavy white precipitate formed is allowed to settle and the concentration of ATP is determined in the decanted supernate using commercial firefly luciferase kits and a luminometer (though x-ray film or a scintillation counter can also be used for light detection – cf. Boden et al. 2010) – it is important that the ATP calibrants are subjected to perchloric acid and potassium hydroxide since potassium perchlorate does afford some quenching of the luminescence (Kelly 1965; Boden et al. 2010). The final cell suspension left in the incubation chamber is rapidly filtered to remove biomass, and the filtrate is assayed

to determine the concentration of the electron donor – from these data, one can determine both the ATP production rate versus a control incubated without electron donor and the amount of ATP formed per mol electron donor – the latter allowing determination of m_{ATP} (the methods we summarize here are from Boden et al. 2010, 2011b; Kelly 1965; Kelly and Syrett 1966; Hutt 2016 – the reader should consult these texts for full technical detail and example datasets).

m_S and m_{ATP} are measures of the "cost of living" of the organism – if a non-halophile is subjected to salinity, for example, the amount of biomass formed (x) decreases, but the amount of electron donor consumed stays the same or can increase – and compared to growth without salt stress, there is more electron donor consumed per unit biomass (since the latter has decreased); this additional consumption is owing to a rise in m_{ATP}, thus m_S; the organism demands more electron donor turnover to simply stay alive. As to what maintenance is comprised of, we know from the work of Stouthamer and Bettenhausen (1973) and Watson (1970) on solute stress in *Bacteria* and unicellular *Eukarya* that maintaining osmotic balance and gradients requires a significant fraction of the maintenance energy. Studies by Harrison and Loveless (1971) working at or below the optimum temperature of *Escherichia coli* showed no increase in m_S with temperature change; thus they (and later Pirt (1975)) assumed that m_S was not effected by temperature – though since they were not working at temperatures likely to cause *significant damage* (i.e., toward the very upper limits of growth), there is no reason why m_S would increase at the temperatures examined. Maintaining proton gradients and repairing damage induced by pH changes resulted in a reasonably high increase in m_{ATP} (Harrison and Loveless 1971), indicating that this is a major use of maintenance energy.

One of us studied the effects of Hg(II) ions on *Methylococcus capsulatus* bath in cell suspensions using [^{14}C]-methane as a tracer (Boden and Murrell 2011). In the absence of Hg(II), the partitioning of methane-carbon between biomass and carbon dioxide was 61 ± 4% assimilated to biomass and 23 ± 3% dissimilated to carbon dioxide over 1 h, with the remainder presumed to be as soluble intermediates. When 10 mM Hg(II) was added, all of the methane was very rapidly oxidized to carbon dioxide within 30 min, and absolutely none was assimilated into biomass. These data demonstrate a very sharp rise in the maintenance cost of the organism – when Hg(II) is present, the constitutively expressed mercuric reductase (EC 1.16.1.1) reduces the ion to elementary mercury to detoxify it, requiring 1 mol NADH per mol Hg(II) detoxified – in this case, 0.5 mmol NADH would be needed by the 50 mL culture. As the flasks contained 9 mL methane (0.4 mmol), and we know that methane dissimilation in this organism using the particulate methane monooxygenase (EC 1.14.18.3) generates a net 1 mol NADH per mol methane fully dissimilated (Anthony 1982), it is easy to see that during that 30-min period, about 80% of the mercury would have been detoxified owing to this rapid oxidation of the electron donor purely for energy. In cases like these where a bolus of electron donor is rapidly "burnt" to yield energy for a specific detoxification – be that of metal ions or of protons or of a toxic organic – the maintenance coefficient of the organism must rise such that $\mathbf{\Delta S_M} = \mathbf{\Delta S}$; the amount of electron donor required to maintain the organism has become equal to the total amount of electron donor consumed, as

absolutely none is being used for growth. Such situations are impossible to monitor in the chemostat since if no growth occurs, no change in Y can be measured, of course – thus they must be derived as we have here from batch culture work and use of sensitive tracers – carbon-14 detection allows use at fM concentrations whereas carbon-13 tracing is often limiting and requires use at, e.g., 1–5% of the total carbon source; so for a 10 mM glucose culture, there would be 0.1–0.5 mM carbon-13 present. A thorough (if dated) review of radiotracers in cell physiology can be found in Aronoff (1957), which gives useful technical detail missing from later works.

A very elegant example of the use of Y_{MAX} in physiology is that of Wood and Kelly (1986), who made use of comparison of Y_{MAX} and a "corrected" version that accounted only for electron donor oxidized for ATP synthesis. This was done during aerobic growth of the obligate autotroph *Thermithiobacillus tepidarius* on a range of homologous electron donors – the polythionates, viz., trithionate, tetrathionate, hexathionate, and heptathionate – which increase by one interior sulfane sulfur at each step in the series (pentathionate was not used owing to complications of synthesis at the time). In theory, the ratio of "corrected" yields between adjacent polythionates in the chain will be consistently 7:4 if only oxidative phosphorylation contributes to ATP synthesis but would skew as chain length increased if substrate-level phosphorylation also contributed – this allowed a very elegant determination that only oxidative phosphorylation contributed to ATP synthesis in this organism. Similarly, Y_O (cf. Sect. 4.2) was fairly consistent between polythionates examined, suggesting that molecular oxygen as a terminal electron acceptor is the only route of ATP synthesis in this organism. The "corrected" Y referred to here is specifically Y_G and is determined from:

$$Y_{\mathbf{G}} = \Delta \boldsymbol{x} / \Delta S_{\mathbf{G}}$$

which is the same means of determination of Y but with consideration only of the amount of electron donor consumed for growth and not for maintenance.

4.2 Metabolic Quotients (q) and the Respiration Rate (q_{O2})

We have covered in Sect. 4.1 the means of determination of q and of what q and q_{O2} are in basic terms, but herein we cover their use.

The respiration rate (q_{O2}) can be determined from the maximum respiration rate (q_{O2MAX}) in oxygen-limited chemostats – the latter is determined from $q_{O2MAX} = \mu_{MAX}/Y_O{}^{MAX}$ – the maximum specific molar growth rate with respect to oxygen and the maximum yield with oxygen as the limiting substrate:

$$\boldsymbol{q}_{\mathbf{O_2}} = \boldsymbol{q}_{\mathbf{O_2}\mathrm{MAX}} \times \boldsymbol{p}_{\mathbf{O_2}} / \left(\boldsymbol{p}_{\mathbf{O_2}} + \boldsymbol{K}_{\mathbf{p}}\right)$$

where p_{O2} is the dissolved oxygen partial pressure (usually in mmHg) and K_p is the Monod constant (K_S, cf. Sect. 4.4) for oxygen, but given in pressure units (mmHg is the most commonly used unit in the literature, since most studies are in older texts – as long as the units used for p and K_p are consistent, anything can be

used – the Pascal (Pa) is probably the most convenient, as the standard atmosphere (atm) is not very convenient for dissolved gas partial pressures). If we then make the assumption that the specific molar growth yield per mole of oxygen (Y_O) is constant, we can change this to:

$$\mu = \mu_{MAX} \times p_{O_2}/(p_{O_2} + K_p)$$

This gives the relationship between specific molar growth rate (per mole of oxygen in this case, μ) and the concentration of dissolved oxygen as the limiting substrate. At a critical dissolved oxygen partial pressure (p_{crit}), q_{O2} ceases to be coupled to p_{O2} – p_{crit} is dependent entirely on μ under air at atmospheric pressure, but under hyperbaric conditions, the relationship between q_{O2} and p_{O2} becomes unpredictable and unstable – for further information on growth at elevated pressures, the reader should consult MacLennan et al. (1971), which uses *Methylobacterium extorquens* AM1 as a model organism during growth on methanol, or for relations between p_{crit} and μ in general, Harrison et al. (1969).

4.3 ATP Yield of Biomass (Y_{ATP})

The ATP yield of biomass (Y_{ATP}) was originally defined (Bauchop and Elsden 1960) as:

$$Y_{ATP} = M_W Y_E/n$$

where M_W is the molecular weight of the electron donor (in Daltons), n is the number of moles ATP produced by the metabolism of 1 mol electron donor, and Y_E is the specific molar growth yield expressed in g dry biomass per g electron donor. We would now of course simplify this to:

$$Y_{ATP} = Y/n$$

where Y is expressed in g dry biomass per mole electron donor. We have already outlined how n can be determined in Sect. 4.1.

The magnitude and meaning of Y_{ATP} have changed over the years since 1960, with it originally being thought to have a fixed value of about 10.5, but this was later found by Stouthamer and Bettenhausen (1973) to be vastly underestimated. In various studies, Stouthamer estimated the maximum Y_{ATP} to be 28.8–32.1 (Stouthamer 1973, 1976), but since chemostat kinetics has largely gone out of fashion in recent decades, the true gamut of Y_{ATP} and influencing factors has been largely neglected.

4.4 The Monod Constant (K_S)

The Monod constant (K_S) is also known as the saturation constant and is the equivalent of the Michaelis-Menten constant (K_M, Michaelis and Menten 1913) in

as much as that it is the concentration of substrate at which μ is half of μ_{MAX}. It is termed the Monod constant after the "Monod relation" in which it appears (Monod 1942, 1949):

$$\mu = \mu_{MAX} \times S/(S + K_S)$$

where S is the concentration of the limiting substrate in the medium reservoir. An extension of the Monod relation by consideration of Y at the same time gives the "Pirt equation":

$$\mu = \mu_{MAX} \times (S/(S + K_S)) - m_S \times Y_{MAX}$$

The Monod constant has an inverse relationship with the affinity of an organism for its substrate – a high K_S refers to a low affinity:

$$1/\mu = (K_S/S\mu_{MAX}) + (1/\mu_{MAX})$$

As such, hyperbolic fitting as outlined elsewhere in this chapter can be used to determine K_S from the specific growth rates in (pH and oxygenation-controlled) batch culture at a range of substrate concentrations. Historically, this was done from a plot of $1/\mu$ versus $1/S$, in which the x-intercept is $-1/K_S$ and the y-intercept is $1/\mu_{MAX}$ – such plots are still useful for visualizing data particularly when considering mixed metabolic modes (cf. Sect. 4.6). In spite of the relative ease of determination, K_S values are not widely reported in the literature – historically this was owing to understanding that the double-reciprocal plots gave high-error estimations for a largely very low parameter (Pirt 1975), though this has changed significantly in the twenty-first century with computational means of solving hyperbolic data. Most determinations for carbon sources are in the range of tens of μM – that for respiratory oxygen is near to 1 μM and those for macronutrients such as magnesium and phosphate are similarly in the tens of μM. Those for vitamins are as low as hundreds of pM (i.e., very high affinity), but trace metal values are seldom reported, and there is some evidence that the Monod relation breaks down at such low concentrations of a substrate (Pirt 1975).

4.5 Oxygen Transfer Coefficient (k_La)

Particularly important in biotechnology, the oxygen transfer coefficient (k_La) is the proportionality constant for the rate of oxygen mass transfer into a liquid relative to the concentration gradient. k_La can be determined through abiotic methods such as by the reaction of sulfite with dissolved oxygen to yield sulfate in the presence of Cu(II) or Co(II) ions as a catalyst (Cooper et al. 1944). The reactor is filled with 1 N sodium sulfite solution (freshly prepared!) containing 1 mM cupric sulfate or cobaltous sulfate (it is prudent to add the sulfite solution to the reactor slowly and gently to avoid too much air mixing in, and to then add the Cu(II) or Co(II) stock

solution and gently stir in using a glass rod), and then the air sparging is commenced, with a timer started the moment the first bubble leaves the sparge arm. At intervals, sparging is stopped, the solution stirred briefly, and an aliquot removed. Sulfite is determined in samples by adding excess standard iodine solution and titrating against thiosulfate, or by colorimetric means – both are given in Kelly and Wood (1998). From the determined sulfite concentrations, the rate of consumption (v) can be determined (importantly not forgetting to convert from concentration to amount first – a common error!):

$$2k_{L}a \times c_{S} = -v$$

where c_S is the solubility of oxygen in the liquid under test (506 μM at 25 °C in pure water – but this will vary in culture media, obviously). Pirt (1975) gives various methods for the determination of $k_{L}a$ in cultures, and a detailed overview with an industrial process viewpoint can be found in Garcia-Ochoa and Gomez (2009).

4.6 Comparing Metabolic Modes

While we have noted above the errors in the q versus μ and $1/Y$ versus $1/\mu$ plots, we do find them very useful for comparing growth conditions and in the determination of metabolic modes. In this regard, they can be used, e.g., akin to the inverse of the enzyme inhibitor methods of Cornish-Bowden (1974), when considering chemolithoheterotrophy or mixotrophy – we will come back to this later in this section. To do this, data are obtained by hyperbolic fitting and then replotted on the above axes, such that, e.g., the y-intercept on the $1/Y$ versus $1/\mu$ plot is the reciprocal of the Y_{MAX} value determined by hyperbolic fitting and not the natural y-intercept of the data points. This is often most useful when comparing data with older studies, and this can be done directly by reanalysis of the original work by using the WebPlotDigitizer tool (http://www.automeris.io), which has been demonstrated as reliable for extracting raw data from original plots and enables reevaluation (Drevon et al. 2016). Using this tool or equivalent, it is possible to extract raw data from every data point of $1/Y$ versus $1/\mu$ plots and to reanalyze them using hyperbolic fitting, which gives better determination of Y_{MAX} and m_S both directly and because in many $1/Y$ versus $1/\mu$ plots, best-fit lines were drawn in by hand and may not be the mathematical best fit of the dataset. To illustrate the value in these reanalyses, Fig. 2 shows data from chemostat cultures of *Halothiobacillus neapolitanus* NCIMB 11333 (Kelly and Syrett 1963) grown by Mason (1986) under electron donor limitation. Mason's original data are shown (points) with trend lines (black) based on her original Y_{MAX} determinations from graphical methods and our reanalysis (red) from extracted raw data reanalyzed using hyperbolic fitting. It can be seen that there is very little difference between Mason's mid-1980s graphical determinations and our reanalyses with very little variance (new vs. old, g/mol) in Y_{MAX} for tetrathionate (17.3 vs. 17.2), but more for trithionate (9.3 vs. 9.1) and thiosulfate (8.3 vs. 8.0). m_S was more variable (new vs. old, mmol/g.h) but again less variable for tetrathionate (3.34 vs. 3.58) than trithionate (3.23 vs. 3.04) and thiosulfate

(5.53 vs. 6.31), even though the original values are from q versus μ plots. While these differences are small, they are important; thus it is good practice to reanalyze data where possible, and plots were made available by the original authors than to merely compare the coefficients produced with those from older studies.

There are a number of mixed metabolic modes that can be examined usefully using these graphical methods, such as with chemolithoheterotrophy (cf. Boden and Hutt 2019), where heterotrophic growth is supplemented by the oxidation of an auxiliary substance as an electron donor but there is no concomitant autotrophy. Endo-chemolithoheterotrophy is the variant in which an organism takes in, e.g., dimethylsulfide and grows on it heterotrophically (per *Methylophaga* spp.) and then endogenously generates a sulfide or thiosulfate moiety from the compound and in turn oxidizes that chemolithotrophically as an auxiliary substance (i.e., source of electrons), producing sulfate, thiosulfate, or tetrathionate as the end product – which has been observed in *Methylophaga thiooxydans* and other *Methylophaga* spp., as well as in some *Hyphomicrobium* spp. (Boden et al. 2010). Exo-chemolithoheterotrophy is the more commonly observed variety in which, for example, a *Methylophaga* sp. is grown heterotrophically on methanol and thiosulfate is supplied as an exogenous auxiliary substance, which is oxidized chemolithotrophically. Mixotrophy (often – but wrongly – used as a blanket term for all or any mixed metabolic mode) occurs when an organism grows both heterotrophically, e.g., on glucose while growing simultaneously autotrophically at the expense of light or an auxiliary substance such as thiosulfate or formate, as is the case in *Paracoccus versutus* A2^{T} (Wood and Kelly 1981). A further, more complex scenario occurs and has been observed in *Xanthobacter tagetidis* TagT2C^{T} (Padden 1997; Padden et al. 1998) growing on substituted thiophenes in which carbon from the thiophene backbone is assimilated heterotrophically, while the sulfide moiety is oxidized chemolithotrophically as an electron donor. Some of the energy conserved from the latter oxidation supplements the heterotrophic growth as endo-chemolithoheterotrophy and some fuels the assimilation of carbon dioxide (some exogenous, some produced from the thiophene backbone) – thus we have mixotrophy in which the heterotrophic component is being supplemented with electrons from an auxiliary substance – endochemolithoheterotrophy in this case. This combination of mixotrophy in which the heterotrophy is chemolithoheterotrophy does not thus far have a name but we propose **voracotrophy** (Latin adj. *vorax*, voracious, inclined to devour; Gr. n. fem. *trophê*, food, nourishment), literally "feeding voraciously" or "feeding greedily" for this scenario in which an organism is literally using every metabolic mode that it has all at once. In this section, we will consider several common patterns observed in graphical representations of data and their equations, based on the Michaelis-Menten kinetic analogies for inhibitor studies.

4.7 Analogous to Inverted Mixed Inhibition

Returning to the *H. neapolitanus* data given in Fig. 2, these afford an example of using graphical methods to consider mixed metabolic modal kinetics using the

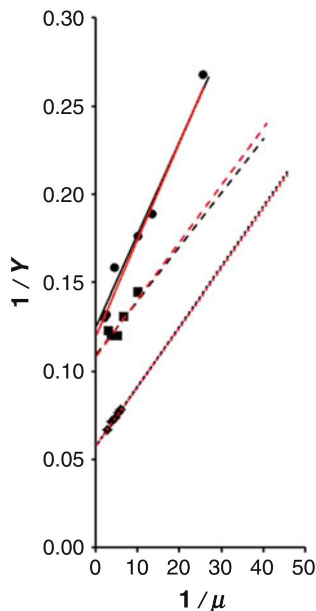


Fig. 2 Reconstructed $1/D$ versus $1/Y$ plot for *Halothiobacillus neapolitanus* NCIMB 11333 (Kelly's "*Thiobacillus* sp. C" (Kelly and Syrett (1963)) grown in electron donor-limited chemostats on thiosulfate (circles, solid trend lines), trithionate (squares, broken trend lines), and tetrathionate (lozenges, dotted trend lines) at a range of dilution rates by Mason (1986). Raw data were extracted from the same plot by Mason (1986) using the WebPlotDigitizer package (http://www.automeris.io) based on scanned images from the original and trend lines applied (black) based on Mason's Y_{MAX} determinations (from $1/D$ versus $1/Y$ graphical methods) to set the y-intercept. Mason's raw data were reanalyzed by hyperbolic fitting using the SOLVER add-on for Microsoft Excel and the GRG nonlinear engine to reduce the sum of squared residuals. Newly determined Y_{MAX} and m_S data for each electron donor were used to construct new $1/D$ versus $1/Y$ series (red) by calculating $1/Y$ values at a range of D given the new Y_{MAX} and m_S

inverse of common enzymology graphical methods. In Lineweaver-Burk plots, an uncompetitive inhibitor gives a parallel line to that of the uninhibited system, with a higher $1/V_{MAX}$, whereas a competitive inhibitor gives a nonparallel line that shares the same $1/V_{MAX}$ as uninhibited enzyme. A noncompetitive inhibitor gives a nonparallel line that has a different $1/V_{MAX}$ but converges at the same x-intercept as the uninhibited system, and a mixed inhibitor gives a nonparallel line that crosses the uninhibited system left of the ordinate. For growth stimulated by an auxiliary substance, obviously, the stimulated condition takes the place of the "uninhibited" line in enzymology, and the control condition takes the place of the "inhibited" line. So, if we consider the thiosulfate and trithionate data of Fig. 2, we must first consider the species – where thiosulfate is $S_2O_3^{2-}$ and trithionate is $S_3O_6^{2-}$, the latter behaves as $(S_2O_3^-)SO_3^-$ or $S(SO_3^-)_2$ in terms of energetics and reactivity, particularly in cleavage reactions (Lyons and Nickless 1968) – since the substance is cleaved to thiosulfate and sulfite during metabolism by trithionate hydrolase (EC 3.12.1.1), thus we can consider it as the former: "thiosulfate

plus sulfite," in which the latter is the auxiliary substance versus growth on thiosulfate alone. It should be noted that some reports on trithionate hydrolase suggest the sulfite moiety is immediately oxidized by the oxygen of water into sulfate – were this the case, Y on trithionate and thiosulfate would be identical, since the only energetically active part of the oxyanion would be thiosulfate moiety, unless the protons released from water could be coupled to energy metabolism – the observation of the production of sulfate could simply be due to experimental issues owing the low stability of sulfite and tendency to oxidize immediately, and that is what we consider to be the case; it clearly warrants further study. One would expect a higher Y_{MAX} for trithionate versus thiosulfate if all three sulfurs were released from it in an oxidizible state (i.e., thiosulfate and sulfite), which is the case in Fig. 2. The trithionate line (broken) crosses that of thiosulfate (solid) somewhere left of the ordinate and that of trithionate will cross the abscissa much further left than that of thiosulfate: per a mixed inhibitor but with the lines inverted since it is stimulatory not inhibitory. Similarly, if plotted per an Eadie-Hofstee plot (Hofstee 1959 – Y vs. Y/μ for chemostat data), nonparallel lines per the inverse of competitive inhibition (i.e., trithionate on top) are seen but with them crossing left of the ordinate (*data not shown*) – congruent with mixed inhibition. If we analogize with the inverse of the enzymological parameters of a mixed inhibitor, one would expect to see m_S decrease for growth on trithionate and see a decrease in the organism for the substrate – an increase in k_S for trithionate versus thiosulfate, both accompanied by an increase in Y_{MAX}. In the same way as mixed inhibition works, sulfite (the "extra" part – acting as the inverse of an inhibitor) is metabolized via a different pathway to thiosulfate and thus is not bound by the same site – there is no competition. In terms of kinetic parameters, the extended Michaelis-Menten equation used for mixed inhibitor studies can be used to derive the inverse equivalent for Monod kinetics:

$$\boldsymbol{v} = ((\mathbf{1}/\boldsymbol{\alpha}') \times \boldsymbol{v}_{\mathbf{MAX}} \times \boldsymbol{S})/((\boldsymbol{\alpha}/\boldsymbol{\alpha}') \times \boldsymbol{K}_{\mathbf{M}} + \boldsymbol{S})$$

where α and α' are modifying factors defined as:

$$\boldsymbol{\alpha} = \mathbf{1} + (\boldsymbol{I}/\boldsymbol{K}_{\mathbf{i}})$$
$$\boldsymbol{\alpha}' = \mathbf{1} + \left(\boldsymbol{I}/\boldsymbol{K}'_{\mathbf{i}}\right)$$

where I is the concentration of the inhibitor and K_i and K'_i are the dissociation constants for the enzyme-inhibitor and enzyme-substrate-inhibitor complexes, respectively. Analogizing into the Monod kinetic, since we are dealing with stimulation of growth and not inhibition, the modifying factors are reciprocated:

$$\boldsymbol{Y} = ((\boldsymbol{\beta}') \times \boldsymbol{Y}_{\mathbf{MAX}} \times \boldsymbol{D})/((\boldsymbol{\beta}/\boldsymbol{\beta}) \times \boldsymbol{m}_{\mathbf{S}} + \boldsymbol{D})$$

where β and β' are modifying factors for the stimulatory factor – be that an additional electron donor or an additional carbon source, though what they related to physiologically is unknown, however, one would anticipate they are defined:

$$\boldsymbol{\beta} = \mathbf{1} + (\boldsymbol{B}/\boldsymbol{K}_{\mathbf{B}})$$
$$\boldsymbol{\beta}' = \mathbf{1} + \left(\boldsymbol{B}/\boldsymbol{K}'_{\mathbf{B}}\right)$$

where B is the concentration of the auxiliary substance and k_B and k'_B are probably Monod constant-like parameters for the auxiliary substance. For many auxiliary substances, particularly exogenous sugars and electron donors, this may all simplify down to a single modifying factor β defined by a single Monod parameter k_B, but there will be more complex scenarios such as that of the above example of "forked" chemolithoautotrophic growth of *H. neapolitanus* on trithionate, if we consider the latter to be "thiosulfate plus sulfite," where the compound is split into these components and they are oxidized either (the latter) to sulfate via sulfite dehydrogenase (EC 1.8.2.1) or (the former) to sulfate via tetrathionate and/or through protein bound intermediates in the Kelly-Friedrich complex – in both of the latter include sulfite dehydrogenase also; thus the pathways do converge eventually, and all are coupled to the respiratory chain at the level of cytochrome *c*, which is directly oxidized by the terminal oxidase; thus the energy metabolisms of the sulfite and thiosulfate components of trithionate converge at this point.

4.8 Analogous to Inverted Uncompetitive Inhibition

As a second example, we can consider the exochemolithoheterotrophic growth of *Sagittula stellata* E37^{T} on fructose with dimethylsulfide as the auxiliary substance, which is oxidized to dimethylsulfoxide (Boden et al. 2011b); m_S and μ_{MAX} do not vary between heterotrophic and exochemolithoheterotrophic growth, but Y_{MAX} is 14% higher in the latter situation. Using graphical methods, on a $1/Y$ versus $1/\mu$ plot, two parallel lines are seen since m_S is constant, as would be the case in enzymology with an uncompetitive inhibitor – a is increased, m_S stays the same, and Y_{MAX} is increased. Analogizing the modified Lineweaver-Burk kinetic for uncompetitive (anti-competitive) inhibitors:

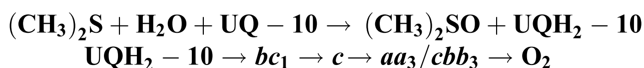
$$\mathbf{1/\mathit{v} = \mathit{K}_M/(v_{MAX} \times \mathit{S}) + (1 + \alpha')/\mathit{v}_{MAX}}$$

which we can analogize into the Monod kinetic, reciprocating the modifying factor since we are concerned with stimulation:

$$\mathbf{1/\mathit{Y} = \mathit{m}_S \times 1/\mu + \mathit{Y}_{MAX}/\beta'}$$

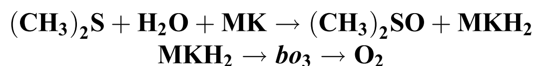
This allows us to determine Y on fructose plus dimethylsulfide at any given μ, using Y_{MAX} and m_S from growth on fructose alone. Using data from Boden et al. (2010), we can determine that β' is 4805; thus K'_B is 416 nM – presumably this is a Monod constant for dimethylsulfide and is indeed in the right order of magnitude for the K_S of high-affinity substrates such as oxygen or vitamins (Pirt 1975) – one would anticipate that high affinity was necessary for highly volatile substances such as this. If the organism were grown at a range of concentrations of the auxiliary substance (B), each at a range of μ, the Dixon plot (Dixon 1953) and its associated modifications (Cornish-Bowden 1974) can also be used to determine the magnitude

of K'_B for mixed metabolic modes that fit the inverted uncompetitive inhibitor kinetic (such as *Methylophaga thiooxydans* DMS010^T on methanol/thiosulfate or dimethylsulfide/thiosulfate, Boden et al. 2010). The precise nature of the interaction of the substrate versus the auxiliary substance at energetic level is not clear; however, it is worth noting that in the examples of *S. stellata* and *M. thiooxydans*, there is no overlap between the pathways for the main substrate oxidation and for the auxiliary substance oxidation. In the former, fructose is oxidized via glycolysis and Krebs' cycle, with NAD(P)H formation at the former being used to create proton motive force (Δp) at the ubiquinone-10-coupled NADH dehydrogenase (EC 1.6.5.3), with addition being generated at the bc_1 complex from electrons coupled at the level of succinate dehydrogenase and the quinone pool. Dimethylsulfide in some *Alphaproteobacteria* is oxidized to dimethylsulfoxide via the NADPH coupled trimethylamine monooxygenase (EC 1.14.13.148, Lidbury et al. 2016); however, since NADPH is *consumed* during this oxidation and there is no further downstream metabolism in *S. stellata*, this enzyme cannot be responsible for DMS oxidation when acting as an electron donor for energy metabolism. A more likely scenario is a ubiquinone-10 (UQ-10) coupled dimethylsulfoxide reductase (EC 1.8.5.3) acting in the reverse direction, generating ubiquinol-10 (UQH_2-10), which donates to the bc_1 complex (EC 1.10.2.2), which in turn donates to cytochrome c, which donates to the cbb_3- or aa_3-type cytochrome c oxidases (both proton-translocating, EC 1.9.3.1) found in this genus, followed by reduction of molecular oxygen:



$$\mathbf{(CH_3)_2S + H_2O + UQ - 10 \rightarrow (CH_3)_2SO + UQH_2 - 10}$$
$$\mathbf{UQH_2 - 10 \rightarrow \mathit{bc_1} \rightarrow \mathit{c} \rightarrow \mathit{aa_3/cbb_3} \rightarrow O_2}$$

Alternatively, as is the case with the same enzyme in *Rhodobacter* spp., also from the *Alphaproteobacteria*, the enzyme is coupled to a menaquinone (MK), forming menaquinol (MKH_2) which donates directly to the proton-translocating bo_3-type menaquinol oxidase (EC 1.10.3.x), followed by reduction of molecular oxygen:



$$\mathbf{(CH_3)_2S + H_2O + MK \rightarrow (CH_3)_2SO + MKH_2}$$
$$\mathbf{MKH_2 \rightarrow \mathit{bo_3} \rightarrow O_2}$$

If menaquinone is the site of coupling of electrons from dimethylsulfide to the respiratory chain and the bo_3 oxidase (versus the aa_3 and cbb_3 oxidases used in fructose metabolism) is thus used, there is no overlap between the entire pathway of fructose oxidation and that of dimethylsulfide oxidation – thus, the two energy metabolisms – whereas in the previous example of trithionate versus thiosulfate oxidation in *H. neapolitanus*, the energy metabolisms overlap. A further example of this type of kinetic is observed in *Beggiatoa alba* ATCC 33555^T grown on acetate with or without sulfide as an auxiliary electron donor (Güde et al. 1981).

4.9 Analogous to Inverted Competitive Inhibition

The final example we will consider is the analogy of competitive inhibition, which is observed in *Acidithiobacillus ferrooxidans* grown on tetrathionate as the electron donor and carbon dioxide as the carbon source (Eccleston and Kelly 1978) at 0.03% (*v/v*) in air or at 9.00% (*v/v*) in air. In this case, the $1/Y$ versus $1/\mu$ plot shows two lines that cross at the *y*-intercept, i.e., share the same Y_{MAX} (12.5 g dry biomass/mol tetrathionate) but have different gradients – m_S (mmol tetrathionate) at the elevated carbon dioxide partial pressure being 0.55 mmol/g.h versus 1.02 mmol/g.h at atmospheric levels. These data follow the expected shape of the Lineweaver-Burk plot of a competitive inhibitor but inverted. The modified Michaelis-Menten kinetic for this inhibition is:

$$\mathbf{1/v = ((K_M \times \alpha)/v_{MAX}) \times 1/S + 1/v_{MAX}}$$

thus for a stimulated growth kinetic:

$$\mathbf{1/Y = ((m_S \times (1/\beta))/\mu + 1/Y_{MAX}}$$

Therefore, for the Eccleston and Kelly (1978) data, β is 1.77; thus at 9% (*v/v*) carbon dioxide, with the concentration in solution determined by Henry's law, K_B is 43.67 µM. This presumably represents a Monod constant-like coefficient for the carbon source – carbon dioxide – which is in the same order of magnitude as reported values (Pirt 1975).

4.10 Analogous to Inverted Noncompetitive Inhibition

In this scenario, Y_{MAX} and m_S would be different, but the specific maintenance rate a would be identical (on a $1/Y$ vs. $1/D$ plot, the *x*-intercept is $-1/a$) – but this is in part owing to the paucity of chemostat data available for which there are raw data to examine. It is seen in *Acidithiobacillus ferrooxidans* on thiosulfate versus tetrathionate limitation (Kelly et al. 1987) – in the former condition, Y_{MAX} was 8.2 g/mol and in the latter, 11.9 g/mol; m_S was also different – 1.45 and 1.00 mmol/g.h, respectively. The specific maintenance rate a was 11.9 in both conditions. There is no straightforward set of equations for the derivation of v in enzymology for an inhibited state of this kind working from v_{MAX} for the uninhibited version. In this example, thiosulfate is oxidized to tetrathionate, and the downstream metabolism is largely the same; *pace* the electrons from that initial oxidation. If we consider tetrathionate as two thiosulfates and redetermine Y_{MAX}, we have 5.95 g/mol thiosulfate equivalents. Thus, we could subtract this from Y_{MAX} for thiosulfate, finding the difference between the two is 2.25 g/mol thiosulfate – if the catabolism of exogenous and endogenous tetrathionate were identical and had an identical cost (e.g., transport across membranes, etc.), 2.25 g dry biomass would be the amount

obtained purely from the initial oxidation of 1 mol of thiosulfate to tetrathionate. Since m_S varies between the two electron donors – albeit not by very much – with tetrathionate having the lower maintenance cost, this could reflect not needing to make and maintain the enzymes of the initial thiosulfate oxidation or the need to undertake two transport steps; thiosulfate is bought into the periplasm and oxidized, and tetrathionate is transported to the cytoplasm for further metabolism; in tetrathionate-grown cells, tetrathionate is taken to the cytoplasm directly, cutting down on transport costs. It is worth noting that thiosulfate was supplied at 20 mM versus10 mM tetrathionate, meaning the ionic strength when the latter electron donor was used is lower, which could also account for the lower m_S since the osmotic balance costs will be lower.

5 Conclusions

In this section we have demonstrated that graphical methods can be useful for determining how different metabolic modes relate – all truly chemolithoheterotrophic growth we observed followed inverted uncompetitive inhibition; changing the carbon source concentration at a fixed electron donor level in autotrophic growth followed inverted competitive inhibition; electron donors that are metabolized largely separately followed inverted mixed inhibition, whereas those metabolized sequentially followed inverted noncompetitive inhibition. With further data and studies that follow the Dixon analysis method (Dixon 1953), it should be possible to determine the factors β and β' and the constants K_B and K'_B for a range of organisms and conditions, which will better enable us to understand what these constants mean in physiological terms and how they can be used to better model the effects of mixed metabolism.

6 Research Needs

- Determination of chemostat kinetic parameters Y_{MAX}, m_S, Y_{ATP}, m_{ATP}, a, E_G, Y_O, K_S, etc., for a wider range of organisms and growth conditions, using the latest technologies for more precise data
- Where raw data are available or can be extracted from plots, a reanalysis of older studies using hyperbolic fitting to yield more precise data
- A move to the determination of more meaningful growth parameters in batch and continuous culture – specifically dry weight or total organic carbon – and an end to the publication of raw optical densities without proper analysis

Acknowledgments RB thanks those who originally taught him the mathematical basis of chemostat kinetics that inspired him in the first place – Professor Jeremy Mason and Dr. Ann P Wood (both formerly of King's College London, London, UK) – and those from whom he learnt the practicalities, Professor Donovan P Kelly (*Professor Emeritus*, University of Warwick, Coventry, UK), Dr. Elena Borodina (Lecturer in Science, Weston College of Further and Higher Education,

Weston-super-Mare, UK), Mr. Gez Chapman, Mrs. Jane Green, and the late Dr. Steve Stanley (all formerly of University of Warwick, Coventry, UK). LPH and RB both thank our collaborators Dr. Kathleen M Scott (Associate Professor, University of South Florida, Tampa, FL, USA) and Dr. Jan Kuever (Head of Department of Microbiology, Official Material Testing Institute of the Free Hanseatic City of Bremen, Germany) for continued stimulating discussions on continuous culture methodology and kinetics and the wider field of growth physiology.

References

Anthony C (1982) The biochemistry of methylotrophs. Academic, London

Aronoff S (1957) Techniques of radiobiochemistry. The Iowa State College Press, Ames

Avery GB Jr, Shannon RD, White JD, Martens CS, Alperin MJ (1999) Effect of seasonal changes in the pathways of methanogenesis on the $\partial^{13}C$ values of pore water methane in a Michigan peatland. Global Biogeochem Cycles 13:475–484

Bauchop T, Elsden SR (1960) The growth of micro-organisms in relation to their energy supply. J Gen Microbiol 23:457–469

Boden R, Murrell JC (2011) Response to mercury (II) ions in *Methylococcus capsulatus* (bath). FEMS Microbiol Lett 324:106–110

Boden R, Hutt LP (2019) Chemolithoheterotrophy: means to higher growth yields from this widespread metabolic trait. In: Rojo F (eds) Aerobic utilization of hydrocarbons, oils, and lipids. Handbook of Hydrocarbon and Lipid Microbiology. Springer, Cham

Boden R, Kelly DP, Murrell JC, Schäfer H (2010) Oxidation of dimethylsulfide to tetrathionate by *Methylophaga thiooxidans* sp. nov.: a new link in the sulfur cycle. Environ Microbiol 12:2688–2699

Boden R, Borodina E, Wood AP, Kelly DP, Murrell JC, Schäfer H (2011a) Purification and characterization of dimethylsulfide monooxygenase from *Hyphomicrobium sulfonivorans*. J Bacteriol 193:1250–1258

Boden R, Murrell JC, Schäfer H (2011b) Dimethylsulfide is an energy source for the heterotrophic marine bacterium *Sagittula stellata*. FEMS Microbiol Lett 322:188–193

Boden R, Hutt LP, Huntemann M, Clum A, Pillay M, Palaniappan K, Varghese N, Mikhailova N, Stamatis D, Reddy T, Ngan CY, Daum C, Shapiro N, Markowitz V, Ivanova N, Woyke T, Kyrpides N (2016) Permanent draft genome of *Thermithiobacillus tepidarius* DSM 3134T, a moderately thermophilic. Stand Genomic Sci 11:74

Borodina E, Kelly DP, Rainey FA, Ward-Rainey NL, Wood AP (2000) Dimethylsulfone as a growth substrate for novel methyl-tropic species of *Hyphomicrobium* and *Arthrobacter*. Arch Microbiol 173:425–437

Chongcharoen R, Smith TJ, Flint KP, Dalton H (2005) Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification. Microbiology 151:2615–2622

Cohen A (1922) Xylenol blue and its proposed use as a new and improved indicator in chemical and biochemical work. Biochem J 16:31–34

Cooper CM, Fernstorm GA, Miller SA (1944) Performance of agitated gas-liquid contactors. Ind Eng Chem 36:504–509

Cornish-Bowden A (1974) A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem J 137:143–144

Dixon M (1953) The determination of enzyme inhibitor constants. Biochem J 55:170–171

Drevon D, Fursa SR, Malcolm AL (2016) Intercoder reliability and validity of WebPlotDigitizer in extracting graphed data. Behav Modif 41:323–339

Eccleston M, Kelly DP (1978) Oxidation kinetics and chemostat growth kinetics of *Thiobacillus ferrooxidans* on tetrathionate and thiosulfate. J Bacteriol 134:718–727

Gao P, Sun C, Li Y, Zou X, Wu X, Ling Y, Luan C, Chen H (2017) Vital staining of bacteria by sunset yellow pigment. Pol J Microbiol 66:113–117

Garcia-Ochoa F, Gomez E (2009) Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. Biotechnol Adv 27:153–176

Good NE, Izawa S (1972) Hydrogen ion buffers. Methods Enzymol 24:53–68

Good NE, Winget DG, Winter W, Connolly TN, Izawa S, Singh RMM (1966) Hydrogen ion buffers for biological research. Biochemistry 5:467–477

Grady JK, Chasteen ND, Harris DC (1988) Radicals from "Good's" buffers. Anal Biochem 173:111–115

Groisman A, Lobo C, Cho H, Campbell JK, Dufour YS, Stevens AM, Levchenko A (2005) A microfluidic chemostat for experiments with bacterial and yeast cells. Nat Methods 2:685–589

Güde H, Strohl WR, Larkin JM (1981) Mixotrophic and heterotrophic growth of *Beggiatoa alba* in continuous culture. Arch Microbiol 129:357–360

Haldane JBS (1957) Graphical methods in enzyme chemistry. Nature 179:832–832

Hanes CS (1932) Studies on plant amylases: the effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. Biochem J 26:1406–1421

Harrison DEF, Loveless JE (1971) The effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes in chemostat culture. J Gen Microbiol 68:35–43

Harrison DEF, MacLennan DG, Pirt SJ (1969) Responses of bacteria to dissolved oxygen tension. In: Perlman D (ed) Fermentation advances. Academic, New York, pp 117–143

Hofstee BHJ (1959) Non-inverted versus inverted plots in enzyme kinetics. Nature 184:1296–1298

Hutt LP (2016) Taxonomy, physiology and biochemistry of the sulfur bacteria. PhD thesis, University of Plymouth

Karagouni AD, Slater JH (1978) Growth of the blue-green alga *Anacystis nidulans* during washout from light- and carbon dioxide-limited chemostats. FEMS Microbiol Lett 4:295–299

Kelly DP, Syrett PJ (1964) The Effect of Uncoupling Agents on Carbon Dioxide Fixation by a Thiobacillus. J Gen Microbiol 34(2):307–317

Kelly DP (1965) Energy metabolism of the chemoautotrophic bacterium, *Thiobacillus*. PhD thesis, University College London

Kelly DP, Syrett PJ (1963) Effect of 2:4-dinitrophenol on carbon dioxide fixation by a *Thiobacillus*. Nature 197:1087–1089

Kelly DP, Syrett PJ (1966) Energy coupling during sulphur compound oxidation by *Thiobacillus* sp. strain c. J Gen Microbiol 43:109–118

Kelly DP, Wood AP (1994) Synthesis and determination of polythionates and thiosulfate. Methods Enzymol 243:475–501

Kelly DP, Wood AP (1998) Microbes of the sulfur cycle. In: Burlage R, Atlas R, Stahl D, Geesey G, Gayler G (eds) Techniques in microbial ecology. Oxford University Press, New York

Kelly DP, Mason J, Wood AP (1987) Energy metabolism in chemolithotrophs. In: van Verseveld HW, Duine JA (eds) Microbial growth on C_1 compounds. Proceedings of the 5th international symposium. Martinus Nijhoff Publishers, Dordrecht

Koch AL (1981) Growth measurement. In: Gerhardt P, Murray RGE, Costilow RN, Nester EN, Wood WA, Krieg NR, Phillips GB (eds) Manual of methods for general bacteriology. American Society for Microbiology, Washington, pp 182–207

Lidbury I, Kröber E, Zhang Z, Zhu Y, Murrell JC, Chen Y, Schäfer H (2016) A mechanism for bacterial transformation of dimethylsulfide to dimethylsulfoxide: a missing link in the marine organic sulfur cycle. Environ Microbiol 18:2753–2766

Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658–666

Long Z, Nugent E, Jager A, Cicuta P, Sclavi B, Consentino Lagomarsino M, Dorfman KD (2013) Microfluidic chemostat for measuring single cell dynamics. Lab Chip 13:947–945

Lyons D, Nickless G (1968) The lower oxy-acids of sulphur. In: Nickless G (ed) Inorganic sulphur chemistry. Elsevier, Amsterdam, pp 509–534

MacLennan DG, Ousby JC, Vasey RB, Cotton NT (1971) The influence of dissolved oxygen on *Pseudomonas* AM1 grown on methanol in continuous culture. J Gen Microbiol 69:395–404

Mason J (1986) Microbial growth and the oxidation of inorganic sulphur compounds. PhD thesis, University of Warwick

Michaelis L, Menten ML (1913) Die Kinetik der Invertinwirkung. Biochem Z 49:333–339

Monod J (1942) Recherches sur la croissance des Cultures Bactériennes. Hermann, Paris

Monod J (1949) The growth of bacterial cultures. Annu Rev Microbiol 3:371–394

Pirt SJ (1965) The maintenance energy of bacteria in growing cultures. Proc R Soc B 163:224–231

Padden AN (1997) Microbial degradation of organic sulfur compounds. PhD thesis, King's College London

Padden AN, Kelly DP, Wood AP (1998) Chemolithoheterotrophy and mixotrophy in the thiophene-2-carboxylic acid-utilizing *Xanthobacter tagetidis*. Arch Microbiol 169:249–256

Parkhurst DL, Appelo CAJ (1999) User's guide to PHREEQC (Version 2) – a computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations: U. S. Geological Survey Water-Resources investigations report 99-4259

Pirt SJ (1975) Principles of microbe and cell cultivation. Halsted Press, New York

Pirt SJ, Callow DS (1958) Observations on foaming and its inhibition in a bacterial culture. J Appl Microbiol 21:211–216

Pirt SJ, Callow DS (1960) Studies on the growth of *Penicillium chrysogenum* in continuous flow culture with reference to penicillin production. J Appl Bacteriol 23:87–98

Pirt SJ, Panikov N, Lee Y-K (1979) The miniloop: a small-scale air-lift microbial culture vessel and photo biological reactor. J Chem Technol Biotechnol 29:437–441

Pogliano J, Osborne N, Sharp MD, Abanes-De Mello A, Perez A, Sun Y-L, Pogliano K (1999) A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation. Mol Microbiol 31:1149–1159

Raffo M (1908) Ueber kolloiden Schwefel. Kolloid Z 2:358–360

Reasoner DJ, Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49:1–7

Skidmore DW (1979) Purification of carbon disulphide for use as a solvent in gas chromatography. Ann Occup Hyg 22:181–182

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Solórzano L (1969) Determination of ammonia in natural waters by the phenol hypochlorite method. Limnol Oceanogr 14:799–801

Soni ML, Kapoor RC (1981) Some thermodynamic parameters for hydroxyl amino acids: bicine and tricine. Int J Quantum Chem 20:385–391

Steudel R (2003) Aqueous sulfur sols. In: Steudel R (ed) Elemental sulfur and sulfur-rich compounds I. Springer, Berlin, pp 156–166

Stouthamer AH (1973) A theoretical study on the amount of ATP required for synthesis of microbial cell material. Anton van Leeuwenhoek 39:545–565

Stouthamer AH (1976) Yield studies in microorganisms, Patterns of Progress PP/M/3. Meadowfield, Shildon

Stouthamer AH, Bettenhausen C (1973) Utilization of energy for growth and maintenance in continuous and batch cultures of microorganisms. Biochim Biophys Acta 301:53–70

Tuovinen OH, Kelly DP (1973) Studies on the growth of *Thiobacillus ferrooxidans* I. Use of membrane filters and ferrous iron agar to determine viable numbers, and comparison with $^{14}CO_2$-fixation and iron oxidation as measures of growth. Arch Mikrobiol 88:285–298

van Steveninck J, Booij HL (1964) The role of polyphosphates in the transport mechanism of glucose in yeast cells. J Gen Physiol 48:43–60

von Bodegom P (2007) Microbial maintenance: a critical review on its quantitation. Microb Ecol 53:513–523

von Weimarn PP (1926) Über schwefellsungen von allen furben de spektrums. Kolloidchemische Beiheffe band XXII. Booklet 1–2

Watson TG (1970) Effects of sodium chloride on steady-state growth and metabolism of *Saccharomyces cerevisiae*. J Gen Microbiol 64:91–99

Wood AP, Kelly DP (1981) Mixotrophic growth of *Thiobacillus* A2 in chemostat culture on formate and glucose. J Gen Microbiol 125:55–62

Wood AP, Kelly DP (1986) Chemolithotrophic metabolism of the newly-isolated moderately thermophilic, obligately autotrophic *Thiobacillus tepidarius*. Arch Microbiol 144:71–77

Xia R (2016) The dying breed of craftsmen behind the tools that make scientific research possible. LA Times, 25 June 2016. http://www.latimes.com/local/education/la-me-caltech-glassblower-20160613-snap-story.html

Index

A
ACC-deaminase, 136
Acetogens, 350
Acetoin, 136
Acidithiobacillus ferrooxidans, 399
Adaptation, to rhizosphere, 161
Adsorption, 165
Aerobic biodegradation, ether pollutants
    alkyl ethers, 305–308
    branched alkyl ethers, 312–319
    chloroalkyl ethers, 308–311
    cyclic ethers, 319–323
Aerobic microbial approaches, 348
Alcanivorax, 55, 58, 59
Aliphatic-aromatic co-polyesters, 346
Aliphatic building blocks, 342
Aliphatic polyesters, 346
Alkane(s), 251
    degradation, 139
Alkane hydroxylase gene (alkB), 139
Alkane hydroxylases, 139
Alkyl ethers, 305, 307–308
    DME cometabolism, 307
    DME metabolism, 305–307
Alkylsuccinates, 274
Alkylsuccinate synthase gene, 260, 264, 270
Alphaproteobacteria, 80
Amazonian Deep-Sea Basin, 80
Ambient geochemistry, 206
1-Aminocyclopropane-1-carboxylate (ACC)
        deaminase activity, 131, 135
Ammonia, 207
Ammonia monooxygenase (AMO), 243
Ammonium, 56
Amplicon sequencing, 259
    See also Targeted metagenomics (amplicon
        sequencing)
Anaerobic dechlorination, 26
Anaerobic hydrocarbon
    biodegradation, 251, 274
    degradation, 260–262, 273
    transformations, 273
Anaerobic methane oxidation (AOM), 271
Analytical methods, 164
Angola Basin, 78
Antibiotic(s), 144
    resistances, 12, 13
Antifoam, 373, 377
Anti-grow-back-trap, 378
API gravity, 47, 49, 51
Aromatic fraction, 48
Aromatic hydrocarbons, 46
    effects of metabolism, 293
    heat shock response, 293–295
    stressor and nutrient, 290–292
Asphaltenes, 46, 48
*ass*A/*mas*D, 257, 260, 264
Associative diazotrophic bacteria, 133
ATP yield of biomass, 391
Atrazine, 235
Autotrophs, 374
Auxin, 131, 135
Auxostat, 365
Auxotrophic organisms, 374

B
Bacterial cutinases, 345
Bacteriophage, 211
Barbados trench, 79
Barite, 229
Basal media, 371–374
Battelle, 34
Beggiatoa alba, 398
Benchmarking information, 261
Benz[a]anthracene, 143

© Springer Nature Switzerland AG 2019

R. J. Steffan (ed.), *Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids: Biodegradation and Bioremediation*, Handbook of Hydrocarbon and Lipid Microbiology, https://doi.org/10.1007/978-3-319-50433-9

Benzene, 261
Benzoyl-CoA reductase, 270
Benzylsuccinate(s), 274
  synthase, 264, 270
Betaproteobacteria, 222
Bicinchoninic acid assay, 369
Bicine, 372
Bio-adsorption, 3
Bio-attenuation, 3
Bioaugmentation, 3, 37–39, 57–58, 110, 163, 205, 207, 208
Bioavailability, 95, 100, 165, 340
  weathered hydrocarbons bioremediation, 108–111
Bioavailable fraction, 165
BIOCHLOR, 210
Biocontrol mechanism, 137
Biocurtain, 207
Biodegradable plastics, 337
  cellulose, 339
  polybutylene adipate terephthalate, 340
  polybutylene succinate, 339
  polycaprolactone, 339
  polyhydroxyalkanoates, 338–339
  polylactic acid, 338
Biodegradation, 4, 34, 48, 144, 176–179
  challenges, 8
  hydrocarbons, 218
  properties, 160
  rates, 95
  science of, 5
  superbugs, 6
  systemic biology, 9–14
  See also Aerobic biodegradation, ether pollutants
Bioemulsans, 118
Biofilms, 160
Biological parts, 10
Biological up-cycling processes, 348
Biomarkers, 90
Biomass, 368–369, 389
BIOPLUME model, 210
Biopolymers, 349
Bioremediation, 34, 60, 109, 120, 159, 160, 165, 166
  challenges, 8–9
  chlorinated solvents, 23
  Dehalococcoides sp., 27
  developing markets, 30
  developments, 6–7
  Envirogen, Inc, 22
  EPA regulations, 27
  genetically engineered microorganisms, 30, 31
  limitations of, 8
  methyl-tert-butyl ether, 26
  modeling of, 209
  money rules, 23–24
  PCE and TCE, 26
  polychlorinated biphenyls, 25–26
  polymerase chain reaction, 29
  research reqiurement, 31
  strategy, 5
  sustainability, 28
  systemic biology, 9
  systems-focused approach, 27
  technology developer, 28–29
  technology of, 2, 5
  waste treatment, 28
Bioremediation Consortium, 35
BIOSCREEN, 210
Biosensors, 164
Biostimulants, 90
Biostimulation, 3, 7, 49, 91, 110, 205–208
Biosurfactants, 94, 119, 165
  bioremediation, 120
  low molecular weight, 117
Biotransformation, 109–110, 198
Bis(2-chloroethyl) ether (BCEE)
  cometabolism, 310
  metabolism, 309
Bis(2-chloroisopropyl) ether (BCIP), 308, 310, 311, 324
Bisphenol, 8
Bitumens, 47
Bizoaugmentation, 110
Black Swan, 14
Branched alkyl ethers, 312–319
  MTBE cometabolism, 315–317
  MTBE metabolism, 312–314
Brazil, 80
*bss*A, 257, 264
BTEX, 39
Buffering, 372
Bunsen valve, 382
2,3-butanediol, 136
γ-Butyrolactone, 311
bvcA, 37

C

C4 compounds, 349
C:N:P ratio, 202
Carbamate linkage, 342

Carbohydrates, 380
Carbon-carbon bonds, 342
Carbon disulfide, 377
Carbon limitation, 373
Carbon tetrachloride (CT), 38
Carboxylation, 257
Carboxylesterases, 346
Carboxysomes, 369
Caspian Sea, 76
Catabolic genes
  expression of, 165
  induction of, 163
Catabolic operons of TOL system, 297–299
Catabolic pathways, 160
Catabolic properties, 164
Catabolic repression, 163
Cell membrane, 292
Cell mobility, 129
Cellulase, 129, 339
Cellulose-based bioplastics, 339
Central Mediterranean and the Sirte Basin, 77
Certainty of containment (CoC), 14
Certified reference materials, 379
Challenging environment, 161
Chemolithoheterotrophy, 393, 394
Chemostat, 365
Chemostat kinetic theory, 366–368
Chemotaxis, 165
Chemotopes, 5
Chitinases, 138
Chlorinated benzenes, 39
Chlorinated solvents, 23
Chloroalkyl ethers, 308, 310–311
  BCEE cometabolism, 310
  BCEE metabolism, 309
Chlorodioxins, 4
Chlorofluorocarbons, 8
Chloroform (CF), 38
Chronically polluted, 95
Circular economy, 351
Clark oxygen cell probes, 379, 381
Clostridium, 222
Coal, 250, 265
Coal Oil Point, 267, 271
Coal reservoir, 270
Coal seams, 274
Cold seeps, 250, 271
Colwellia, 52, 53, 58
  C. psychrerythraea, 80
Colwelliaceae, 52
Combustion, 48
Cometabolism, 308, 311, 318–319, 323
  BCEE, 310
  biodegradation of oil, 81
  bioremediation, 234, 243
  14D, 321–323
  DME, 307
  MTBE, 314–317
Comparative metagenomic analysis, 261
Compound specific isotope analysis (CSIA), 38
Consumer packaging polymers, 337
Contamination
  environments, 163
  groundwater aquifers, 273
  checks, 385
Continuous culture, 365
Control planes, 174
Conventional methods, 90
Conventional pyrolysis, 348
Co-occurrence analysis, 263
Core microbiome, 263
COREXIT 9500, 51
Costs, 23–24
Crenarchaeota, 78
CRISPR/Cas9, 10, 15
Critical dilution rate, 386
Crude oil, 250
  production, 73
  reservoir, 269
Cultivation-dependent techniques, 127
Cultivation-independent methods, 127, 251
Culture-independent tools, 127–128
Customblen, 53, 54
Customizable microbial platform, 351
Cyanobacteria, 77
Cyclic ethers, 319, 323
  14D cometabolism, 321–323
  14D metabolism, 319–321
Cycloclasticus, 52, 53, 58, 60
Cytochrome P450s, 139
Cytokinins, 131, 135
Cytostat, 365

D

Deep sea plume of oil, 52
Deep subsurface, 265
Deep subsurface crude oil-containing reservoirs, 265
Deepwater Horizon, 50, 52, 58, 72, 81, 272
Deepwater plume, 52

Degradation
alkane(s), 251
anaerobic hydrocarbon, 260–262, 273
environmental, 337
and heat shock response, 294
Degradative enzymes, 145
Degrader endophytes (engineered), 144
Dehalobacter, 38
Dehalobacterium formicoaceticum, 39
Dehalococcoides, 27, 37, 38, 40
Dehalococcoides ethenogenes, 208
Delaware Bay, 56, 59
Delaware estuary, 56
Deltaproteobacteria, 58
Depolymerases, 338
Desulfococcus oleovorans, 58
2,4-Diacetyl phloroglucinol (DAPG), 137
Diagenesis, 72
Diagnostic strategy tool box, 111
Diazotrophy, 132–133
1,2-Dichloroethane, 38
Dichloroethene, 35
Dichloroethylene or vinyl chloride stall, 204
Dichloromethane, 39
Dichloro vinyl ether (DVE), 310
Dicyclopropylketone (DCPK), 315
Diesel range organic compounds, 102
Diethyl ether (DEE), 305, 307, 308, 310, 311
Dilution rate, 366
Dimethyldichlorosilane, 378
Dimethyl ether (DME)
cometabolism, 307
metabolism, 305–307
role of, 304
1,4-Dioxane, 40, 235
cometabolism, 321–323
metabolism, 319–321
Dioxygenase, 160, 162
Dispersants, 47, 49–53, 59, 60
Dispersions, 52
Dissolved inorganic carbon, 373
Dissolved oxygen, 94, 367
Diversity, 162
Dixon analysis method, 400
Dixon plot, 397
DNA amendments, 17
Drastic shifts, 222
Drilling, 220
Drilling mud, 220–224

E

E. coli, 223
Eadie-Hofstee plot, 396
Eastern Mediterranean's Nile Deep Sea Fan, 76
E-basal salts, 373
Ecological hazard assessment, 111
Economic cost, 166
Ectopic metabolism, 11
Electrolab Biotech, 380, 384
Electron donors, 234, 376
limitation, 373
Electrostimulation, 12
Elementary sulfur, 376
Emulsification, 49
Emulsified oil, 82
Endochemolithoheterotrophy, 394
Endocrine disruptors, 8, 9, 13
Endophytes, 145, 164
bacterial communities, 126
site remediation, 143
Engineered bioremediation, 205, 209
Engineering, rhizosphere, 144
Enhanced oil recovery, 205
Entner-Doudoroff (ED) pathway, 288
Envirogen, Inc, 22
Environment, 334
Environmental degradation, 337
Environmental DNA shot-gun metagenomics, 140
Environmental fate, 107
Environmental Galenic Science, 6
Environmental genomics, 145
Environmental monitoring, 54
Environmental remediation, 23
Enzymatic degradability, 342
Enzymatic hydrolysis, 345, 352
Episodic seeps of oil, 83
Erika, 59
Ethylene, 136
Evaporation, 47, 49
Evapotranspiration, 142, 144
Exochemolithoheterotrophy, 394
External standards, 379
Exxon Valdez oil spill, 51, 53, 55, 57

F

Fatal flaw, 204
Fenton reaction, 340
Fertilizers, 53–56, 60
Field deployment, 199
Firmicutes, 222, 226, 227
Flagellar biosynthesis, 129
Flexible Liner Underground Technologies Ltd. (FLUTe), 38
Flocs, 377
Fluoranthene, 143
mineralization, 119

Fluorescence in situ hybridisation-confocal laser scanning microscopy, 125
Foam, 377
Foam-probes, 377
Formaldehyde, 376
Fossil energy reservoirs, 265
Fractional flow-rate method, 377
Fracturing, 220
Fugacity, 108
Fumarate addition, 257, 274
  metabolites, 274
Functional genes, 259, 275
Fungal cutinases, 345

G
Gammaproteobacteria, 52, 58, 78, 222
Gas chromatography, 46, 101
Gaseous carbon sources, 374, 381
Gasification, 348
Gasoline, 198
Gene drives, 12
Generalist organisms, 375
Genetically engineered microorganisms (GEMs), 23, 208
Genetically engineering, 346
Genomics, 125, 131, 291–292
Geosyntec, Inc, 27
Gibberellines, 131
Gibberellins, 136
Gibbs free energy, 178
Glass-blower, 378
Glass transition temperature, 338
Global oil discoveries, 73
Gravimetric loss, 341
Great Australian Bight, 77
Greenhouse gases, 8, 13
Green liver, 159
Groundwater, 124
Guaymas Basin, 271
Gulf of Mexico, 74, 244, 272

H
Halanaerobium, 227
Halophilic bacteria, 227
Halothiobacillus neapolitanus, 393, 395
Hanes-Woolf plot, 388
Heat shock (HS) response, 293–295
Henry's law, 399
HEPES, 372
HEPPS, 372
Heterogeneity, 182–186
Heterotrophs, 364, 375
Hexachlorobenzene, 40
n-Hexadecane-degrading culture, 261
High-performance liquid chromatography (HPLC), 379
Horizontal gene transfer, 145, 164
Human health risk assessments, 101
Hydraulic conductivity, 202
Hydraulic fracturing, 224–228
Hydrocarbon compounds in groundwater, natural attenuation, *see* Natural attenuation, hydrocarbon compounds
Hydrocarbon, 250
  contamination, 145
  impacted environments-shotgun metagenomics, 265–270
  releasing thermal vent, 272
  seeps, 270–272
  utilizing isolates, 251
Hydrolases, 138, 344
Hydrolysable bonds, 342
Hydrolytic enzymes, 138
Hydrothermal vents, 250, 271, 272
2-Hydroxyethoxyacetic acid (2HEAA), 321
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 372
3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid, 372
Hydroxylation, 257
Hyperbaric conditions, 391
Hyperbolic fitting, 387
Hyphomicrobium, 377

I
Impellors, 381
Incineration, 337
Indicator compounds, 106
Indole-3-acetic acid (IAA), 135
Induced systemic resistance (ISR), 138
Industrial composting, 338
Industrial waste, 158
Inipol EAP22, 53, 55, 93
Inoculation, 144
Inorganic nutrients, 91–92
In situ anaerobic metabolism, 275
In situ hydrocarbon metabolism, 274
In situ techniques, 159
Insoluble crystalline microfibrils, 339
Interaction between plants and bacteria, 160
Inter-kingdom communication, 162
Intraspecific communication, 162
Intrinsic bioremediation, 90, 209–210
Ionic liquids, 8, 9
Iron, 134
Isotope fractionation, 217
IXTOC-I, 51

K
KB-1, 37
Kerosene range organic compounds, 102
Krebs' cycle, 375, 380
Kuwaiti oil wells, 51

L
Landfill, 335
Lecithin, 93
Light nonaqueous phase liquid, 202
Limiting substrate, 365
Lineweaver-Burk kinetics, 397
Lineweaver-Burk plot, 387, 399
Lipases, 346
Lipopeptides surfactants, 137
Louisiana, 56
Low energy environments, 95
luxR-solos, 162

M
Maintenance coefficient, 387, 388
Marine pollution, 335
Marine snow, 273
Marinobacter, 52, 80
Mass discharge, 184
Mass flux, 184
Mass transport heterogeneity, 182–185
Matrix Diffusion Toolkit, 210
Maximum specific molar growth yield, 386–390
Maximum yield coefficient, 386
Mealworm gut bacteria, 340
Mega Borg, 57
Metabolic burden, 6
Metabolic quotient, 388
Metabolic system, 293
Metabolism, 307–308, 310–311, 317–318, 323
  BCEE, 309
  14D, 319–321
  DME, 305–307
  MTBE, 312–314
Metabolites, 273
Metabolomics, 251, 274
Metagenome, 259
Metagenomics, 5, 143, 226, 227, 251, 272, 273
  analysis, 270
  experiments, 163
Metaorganism, 140
Metatranscriptomics, 140, 144, 261, 272
Methane-limitation, 374
Methane monooxygenase (MMO), 82, 139, 239, 305–307
Methane-oxidizing bacteria, 82
Methanogenesis, 270, 274
Methanogenic consortia, 251
Methanogenic hydrocarbon-degrading cultures, 261
Methanotrophic assemblages at seeps, 77
Methanotrophs, 239–242, 373, 376
  type I and type II, 79
Methylobacterium extorquens, 391
Methylococcus
  M. capsulatus, 242, 389
  M. trichosporium, 240
Methylophaga, 394
  M. thiooxydans, 394, 398
Methylotrophs, 376
Methyl *tertiary* butyl ether (MTBE), 26, 235
  cometabolism, 314–317
  metabolism, 312–314
Michaelis-Menten constant, 387, 391, 396, 399
Microaerophilic environment, 244
Microbial communities, 145
Microbial lipases, 346
Microbial plastic utilization, 352
Microbiome, 9, 12
Microcosm, 3
Micronization plastic material, 346
Micropollutants, 4, 14, 17
Microwave-induced pyrolysis, 349
Mineral nutrients, 133
Mineral oil range organic compounds, 102
Mississippi Canyon, 50
Mixed waste streams, 352
Mixotrophy, 393, 394
Modifying factors, 396
Molecular barcoding, 145
Molecular breeding, 15
Monitored natural attenuation (MNA), 172, 209
Monitorization, 164
Monoaromatic hydrocarbons, 251
Monod constant, 390–392
Monod kinetics, 396
Monod relation, 392
Monomer metabolism, 352
Monooxygenase, 160, 162
Mousse, 47, 49
MTBE, *see* Methyl *tertiary* butyl ether (MTBE)
Multi-objective optimization, 16
Multi-scale complexity, 13
Mutual benefit, 162

N
N_2-fixing bacteria, 133
Nakhodka, 57, 59
Naphthalene, 143

Naphthylmethylsuccinates, 274
Natural attenuation, 244
Natural attenuation, hydrocarbon compounds
biodegradation, 176
conceptual model, 173–175
implementation of, 173
microbial interactions, quantitative description of, 190
performance assessment, 179–189
sorption, 175–176
Natural gene transfer, 146
Natural oil seeps, 46, 72
Natural selection and neutral evolution, 80
Necromass, 370
Net environmental benefit, 59
Network analysis, 263
New sequencing technologies, 146
Nitrate, 56, 207
Nitrification, 243
Nitrite-oxidizing bacteria, 243
Nitrogen limitation, 373
Nitrosomonas europaea, 243
Nitrous oxide, 207
*nms*A, 257
Nonaqueous phase liquid (NAPL), 108
Non-biodegradable plastics
polyethylene, 341
polypropylene, 341
polystyrene, 340
polyurethane, 342
polyvinyl chloride, 342
Non-enzymatic hydrolysis, 338
Non-hydrolysable plastics, 340
Nonoxidizing biocides, 229
Non-sulfide producing, thermophilic microorganisms, 223
North Sea, 78
Nutrients, 50, 56
Nutristat, 365

O
Obligate autotrophs, 385
Ocean Drilling Program, 80
Oceanospirillaceae, 78
Oceanospirillales, 52, 53
n-Octacosane-degrading culture, 261
Octanol-water partition coefficient (Kow, 140
Oil-contaminated marine sediments, 274
Oil-Mineral Fines Interactions, 47
Oil sands tailings ponds, 273
Oil tankers, 46
Oleophilic biostimulants, 93
Oligotrophic environment, 81
On-site techniques, 159
Open sea, 92, 94
Optical density, 368
Organic contaminants, 124
Organic xenobiotics, 142
Orinoco and the Amazon Rivers, 79
Osmotic balance, 389
Oxidation capacity, 179
Oxidative biodegradation, 340
Oxidative phosphorylation, 390
Oxidative stress, 161
Oxidoreductases, 347
Oxygenation, 381
Oxygen dip, 52
Oxygen transfer coefficient ($k_{L}a$), 392–393

P
Paracoccus versutus, 394
Particulate methane monooxygenase (pMMO), 240
Patchy distributions, 208
Pavements, 48, 60
PCBs, *see* Polychlorinated biphenyls (PCBs)
Pectinase, 129
Pelagibacter, 52
2,3,4,5,6-Pentachlorobiphenyl, 40
Perchloroethylene (PCE), 23
Perfluorooctanoic acid (PFOA), 40
Permeable reactive barriers, 207
Pesticides, 3, 8
Petroleum hydrocarbons, 90, 124, 140–145
remediation, 124
Petroleum reservoirs, 274
pH electrode, 379, 382
Phenanthrene, 143
Phosphate-limited chemostats, 380
Phosphate mobilization, 134
Phospholipid ester-linked fatty acids, 79
Phosphorus, 82
Photoautotrophic, 142
Photo-oxidation, 341
Photooxidation, 47
PHREEQC, 373
Phyllosphere, 125
Phytoextraction, 159
Phytohormones, 135
Phytopathogenic bacteria, 135
Phytoremediation, 140, 145, 159
Phytostabilization, 159
Phytovolatilization, 159
Piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (PIPES), 372
Piperazine-*N*-*N*′-bis(2-hydroxypropanesulfonic acid) (POPSO), 372

Pirt equation, 392
Pirtian maintenance, 387
Pirtian Mini-Loop system, 365
Plant-associated (endophytic) bacteria, 145
Plant-bacteria combination, 162
Plant-endophyte associations, 143
Plant ethylene, 131
Plant growth promoting endophytes (PGPE), 131
Plant growth promoting rhizobacteria, 131
Plant growth promotion (PGP), 125
Plant growth regulators, 131
Plant-microbe communication, 162
Plant-microbe sitmulated phytoremediaton, 145
Plant microbiomes, 162
Plant rhizodeposition, 165
Plastics, 4, 5, 8, 13, 14, 17
  eating organisms, 351
  waste, 334
Plastic degrading enzymes
  carboxylesterases, 346
  chemical structure, 343, 346
  cutinases, 344–346
  lignin, 347
  proteases, 347
Plume, 51
Polybutylene adipate terephthalate, 340
Polybutylene succinate, 339
Polycaprolactone, 339
Polychlorinated biphenyls (PCBs), 23–25
Polycyclic aromatic hydrocarbons (PAHs), 158, 163, 165, 251
Polydimethylsiloxane, 378
Polyether-polyols, 342
Polyhydroxyalkanoates, 338
Polylactic acid, 338
Polymerase chain reaction (PCR), 29
Polypropylene, 341
Polystyrene, 340
Polythionates, 376
Polyurethane, 342
Polyvinyl chloride, 342
POPSO, 372
Prestige, 55, 59
Produced water, 269
Proteases, 347
Protein, 369
Pseudoalteromonas, 52
Pseudomonads, 9
Pseudomonas, 52, 240
Pseudomonas putida
  aromatic hydrocarbons (*see* Aromatic hydrocarbons)
  cell membrane, 292
  genomics, 291–292
  glycolytic Entner-Doudoroff pathway, 288
  3-methylbenzoate catabolism, 293, 294
  origin, 288, 289
  regulatory protein, 289
  TOL system (*see* TOL plasmid)
Pulsing of methane, 235
Pyrene, 143
Pyrolysis, 348

Q

Q10 formulas, 84
qPCR, 37
Quorum sensing, 162

R

Rate limiting nutrient, 95
Reactive oxygen species (ROS), 16
Reasoner's 2A agar, 385
Recycling rates of plastics, 335
Redox potential, 367
Redox state, 204
Reimi sol, 376
Release rates, 92
REMChlor, 210
Remediation, 109, 158, 348
  phytoremediation, 159–160
  rhizoremediation, 160–166
Remediation Technologies Development Forum (RTDF), 35
REMFuel, 210
Reporter genes, 164
Residual fractions, 100
Resins, 46
Respiration rate, 388, 390
Retroactivity, 6, 7, 10
Rhamnolipid, 118
Rhizoremediation, 12, 159
  experiments, 163–164
  monitoring, 164–165
  requirements, 160–163
  research needs, 165–166
Rhizosphere, 125, 159, 162
  adaptation to, 161
  colonization, 164
  diversity of, 162
  effect, 160
  expression of catabolic genes, 165
  induction of catabolic genes, 163

microbial communities, 162
microbial populations in, 162
survival in, 163
Rhodobacter, 398
Rhodospirillum rubrum, 350
Root colonization, 128
Root endophyte, 128

S
S200, 93
Safety-related issues, 166
Sagittula stellata, 397
Saturated hydrocarbons, 46
Saturate fraction, 48
Saturation constant, 391
Sea Empress, 55
Sea of Japan, 56
Secondary metabolites, 163
Seeds, 130
Seeps, 48
cold seeps, 250, 271
hydrocarbon, 270–272
methanotrophic assemblages at, 77
natural oil, 46, 72
Sentinel strategy, 211
Serine esterases, 346
Shale, 217, 250, 265
formations, 270
Sheens, 49
Shoreline, 50
Shotgun metagenomics, 259, 261
Siderophore enterobactin, 134
Singapore, 56
Slow release fertilizers, 92
Smithella, 261
Soil, 158, 166
and (ground)water, 145
farming, 3
Soil Quality Index (SQI), 111
Solid peroxygen materials, 96
Soluble diiron monooxygenases (SDIMOs), 315, 320, 321, 323
Soluble methane monooxygenase (sMMO), 239
Solute stress, 389
SOLVER, 387
Southern Australia, 77
Soxhlet extraction, 102
Sparge arm, 381
Species-specific fluorescent antibodies, 243
Spitsbergen, 56
16S rRNA, 80
16S rRNA gene, 259, 262
16S rRNA sequencing, 222, 223, 225, 226
St. Lawrence River, 56
Stable isotopes, 186–189
probing, 143
Steady state, 385
Structured learning in microbial ecology (SLiME) model, 84, 206
Substrate level phosphorylation, 390
Sulfate-reducing microorganisms, 219
Sulfide-producing lineages, 222
Superbugs, 6, 11
Surface sterilization, 126
Surfactants, 165
Survival in the rhizosphere, 163
Swirling flask' test, 49
Symbiotic diazotrophic bacteria, 132
Synergistic effects, 83
Syngas, 349
fermentation, 349
Synthetic Biology, 8, 9, 12, 17
Syntrophic process, 261

T
Tarballs, 47
Targeted metagenomics, 259, 262–264
TCE-epoxide, 239
Terminal electron acceptors (TEA), 204
Tetrachlorodibenzo-*p*-dioxin, 40
Tetrachloroethene, 35
1,2,3,4-Tetrachloronaphthalene, 40
Texas, 56
Thaumarchaeota, 81
Thermithiobacillus tepidarius, 369, 390
Thermocouple probe, 380
Thermogenic shale formations, 220
Thermophiles, 379, 385
Thermophilic microorganisms, 219
Thermophilic sulfide producers, 223
THF monooxygenase (THFMO), 310, 315, 320, 321
Thiols, 376
Thiomicrospira, 381
Thiomicrospira pelophila, 374
Thiophenes, 394
Threshold concentration, 106
Tiered approach, 106
Tilling, 96
Timor Sea, 77
TNT, 235

TOL plasmid, 298
  catabolic operons, 297–299
  DNA tiling arrays and deep RNA
    sequencing, 295–297
Toluene-2-monooxygenase (T2MO), 307
Toxicity, 50, 95
  effects of micro plastics, 335
  testing, 54
Trace metals solution, 373, 374
Tradeoff, 50
Transcriptional pattern, 161
Transcriptomics with TOL system, 295–297
Transferable plasmids or transposons, 145
Transgenic tag, 10, 14, 15
Translocation, 165
Transport mechanisms, 164
Tree of life, 72
Trichloroethane, 38
Trichloroethene, 35
Trichloroethylene (TCE), 23
2,4,5-Trichlorophenoxyacetic acid, 15
Tricine, 372
Trinidad and Tobago, 79
Tris, 372
Tundish, 378
Turbidostat, 365
Two-step chemo-biotechnological process, 348

U
Ultrasonic extraction method, 102
Urea, 56, 207
Uric acid, 56, 93

V
Valence state, 204
Venezuelan Basin, 79
Vibrio, 78
Vinyl chloride, 35
Viscosity, 49
Vitamin stock solutions, 374, 375
Viton, 378
Volatile compounds, 136
Volutin, 368
Voracotrophy, 394

W
Wall growth, 377–378
Waste management, 351
Waste-to-bioproducts processes, 347
Waxworm, 341
Weathered hydrocarbons
  analysis in soil, 101–106
  bioremediation of, 108–111
  risk assessment for, 106–107
Weathering processes, 47, 90, 100
WebPlotDigitizer, 393
Weimarn sol, 376

X
Xanthobacter tagetidis, 394
Xenobiotic compounds, 2, 160
Xylem, 129, 144