Naser A. Anjum · Sarvajeet Singh Gill
Narendra Tuteja *Editors*

Enhancing Cleanup of Environmental Pollutants

Volume 1: Biological Approaches

Springer

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Preface

Environmental and organismal (flora, fauna, and human) health can be impacted by varied chemical pollutants, continuously increasing in major environmental compartments. Notably, the bioavailability, stabilization, and degradation of pollutants are the major drivers that control the pollutant's exclusion, remediation/accumulation, and/or metabolism, performed by innovative technology involving biological (plants and associated microbes, etc.) and/or non-biological/(electro)chemical strategies.

This two-volume work is an effort to gather information on and get insights into biological and non-biological (chemical) approaches extensively studied and adopted for the speedy cleanup of pollutants from environmental compartments. In *Volume 1*, (a) important concepts such as biological remediation strategies to enhance soil quality at contaminated sites were overviewed; (b) synergistic influences of tolerant plants and rhizospheric microbial strains on the remediation of pesticide-contaminated soil were highlighted; and (c) the role of plant types such as hyperaccumulator plants in the cleanup of polluted soils was discussed. Overall, the literature available on the major mechanisms and underlying natural inherent traits of various plants and microbes for tolerating, excluding, remediating, accumulating, or metabolizing a variety of pollutants were critically appraised and elaborated in *Volume 1*. Non-biological (chemical) approaches for enhancing the cleanup of contaminated soils have been dealt in *Volume 2*. In brief, *Volume 2* (a) highlighted important concepts such as the role of metallic iron in the decontamination of hexavalent chromium polluted waters; (b) discussed nanoscale materials and electrochemical approaches used in water and soil remediation; and (c) elaborated in detail the synthesis and characterization of cation composite exchange material and its application in removing toxic metals.

A good equilibrium between theory and practice without compromising the basic conceptual framework of the concerned topic has been ensured in this treatise.

This work can be a useful asset to students, researchers, and policy makers specializing in the areas of soils/sediments and aquatic pollution, environmental chemistry/microbiology/plant physiology/molecular biology, sustainable development, ecology, soil biology, and related disciplines.

Aveiro, Portugal — Naser A. Anjum
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Biological Approaches for Enhancing the Cleanup of Environmental Pollutants: An Introduction

Naser A. Anjum, Sarvajeet Singh Gill, and Narendra Tuteja

Abstract This chapter aimed to introduce the book *Enhancing Cleanup of Environmental Pollutants: Biological Approaches*. Major approaches based on the use of plants, microorganisms, and their interaction discussed in significant chapters set out in this book are highlighted. Potential readers of this book can be benefitted with the chapter overviews presented herein.

Keywords Environmental pollution • Pollutants • Remediation • Biological approach

Introduction

The pollution of environmental compartments with a myriad of pollutants is inevitable. Also, the cleanup of contaminated environmental compartments including soil and water has been a costly and complicated affair. Strategies for creating a clean environment for healthy life on the planet Earth are being significantly explored. To this end, biological approaches can be a sustainable strategy for combatting and minimizing environmental pollution problems. This book is an effort to gather information on and get insights into biological approaches extensively studied and adopted for the cleanup of pollutants from environmental compartments.

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For the ease of the potential readers of this book, glimpses of burning topics discussed in details in scholarly contributions are penned hereunder.

Chapter Overviews

The dinitrotoluene (DNT) isomers 2,4- and 2,6-DNT are highly toxic compounds, and their occurrence in soils and groundwater has become a widespread problem globally. Notably, these compounds are also known to cause mutagenesis and cancer. Taking into the gravity of the situation, Aburto-Medina et al. (Chap. 2) discuss the significance of microorganisms in the degradation of the dinitrotoluene isomers 2,4- and 2,6-DNT. It was argued that the information related to the degradation of these contaminants could be critical to understanding and predicting their fate in the environment. Though petroleum hydrocarbons are benefitting to human, their improper handling and rapid establishment of industries based on petroleum hydrocarbons have become a major cause of marine and terrestrial pollution. In an effort, Shahsavari et al. (Chap. 3) highlight major petroleum hydrocarbons and their structures and discuss bioremediation strategies for petroleum hydrocarbon pollution from both marine oil spills and terrestrial oil spills. Bioremediation approach using hydrocarbonoclastic bacteria and biopiles (and also windrowing) was thoroughly discussed in context with their role in the bioremediation of petroleum hydrocarbon-contaminated soils. Literature is extensive on the interrelationships between microbes and plants and the potential of utilizing these relationships to improve the dissipation of pollutants. However, information is scanty on the plant-microorganism interrelationship-mediated degradation of environmental pollutants and shaping a sustainable future. To this end, a contribution by Fernández-Luqueño et al. (Chap. 4) aims to provide the cutting-edge knowledge about the different biological interrelationships that are simultaneously taking place on a polluted site, prior, during, and after the bioremediation strategies. This chapter also discusses the experimental findings at the laboratory and field scale by outstanding specialists. Though organic micropollutants are relatively a recent challenge to the environmental and human health, a discussion is imperative on these micropollutants and their fate, occurrence, and ecotoxicological significance. Pathways for human exposure to organic micropollutants, the role of aquatic and terrestrial plants in bioaccumulation, and associated potential risks are discussed in detail by Arslan et al. (Chap. 5). The authors also overview remediation strategies for the abatement of organic micropollutants and their metabolites. Mycorrhizas are symbiotic associations between plant roots and fungus. Mycorrhizas occur in a specialized plant organ, where intimate contact results from synchronized plant-fungus development. In addition to overviewing organic pollutants and their sources, Nwoko (Chap. 6) introduces mycorrhizas and highlights their relationship with plants and role in the degradation of organics providing some experimental evidence. The author also discusses interaction of mycorrhiza with other soil microbes and points resultant effect of degradation on soil health.

Soil contamination by toxic metals-metalloids has been a serious problem since the very beginning of the industrial revolution and nowadays has become a widespread environmental concern. Notably, Chaps. 7, 8, 9, 10, and 11 enlighten various facets of metal-metalloid pollution and its remediation in soils employing sustainable strategies. Depending on the source of coal/ore, coal fly ash and mine tailings may include a conglomerate of toxic heavy metals that cause large-scale ecotoxicity. Indeed, non-reclaimed mine tailing sites and coal fly ash dumpsites have become a worldwide problem. Ilika Ghosh and coauthors (Chap. 7) provide a detailed discussion on the role of Poaceae family members and aromatic grasses for the remediation of mine tailings and fly ash dump sites. It was advocated that various biochemical and molecular mechanisms govern the absorption, distribution, metabolism, and excretion of heavy metal contaminants in grasses growing in mine tailings and fly ash dumpsites. Until recently, the bioremediation phenomena of soils contaminated with heavy metals have not been considered a key sustainability issue for the mining industry. Additionally, a huge amount of mining activities spread out worldwide, and mine wastes accumulated over long periods of time are going to have severe negative impacts on the landscape and may also pose serious threats to environmental and human health. Taking into account the example of the mining district of Sierra de Cartagena-La Unión in Southeast Spain, Cortez et al. (Chap. 8) thoroughly discuss the responses of different soil fractions and highlight their role in the bioremediation of sulfide mine tailings. The problem of metal-metalloid pollution in environmental compartments such as soil is continuously worsening due to a series of human activities. A group of plants known as hyperaccumulators can grow on metalliferous soils and accumulate heavy metals in aboveground organs (such as leaves) at concentrations several 100-folds higher than other plants. Considering hyperaccumulator plants, Srivasta (Chap. 9) dissects adaptive responses of these plants to metal-metalloid exposure and accumulation by highlighting physiological and molecular mechanisms. A number of metals released by tailpipe gases, vehicle parts, and the road infrastructure components are constantly deposited in roadside soils. Hence, traffic-related metal pollution has become a serious worldwide concern. Luís and coauthors (Chap. 10) discuss ins and outs of the metal bioaccumulation by plants in roadside soils and also note major perspectives of roadside plants for bioindication and phytoremediation. The phenomena of co-contamination (multi-metals or organic and inorganic pollutants) can most frequently result into and also aggravate soil pollution and eventually bring negative consequences for soil quality. Hence, exploration of feasible approaches for the reclamation of metal-metalloid-contaminated soils is imperative. Considering a case study, Grifoni et al. (Chap. 11) evaluated both biological and nonbiological approaches used at former manufactured gas plant sites. The authors suggest the applicability of biological strategies, in this case phytoremediation, and advocate the efficiency achieved with maintaining soil quality protection and minimal disturbance of surrounding areas.

Rhizosphere is the narrow region of soil ecosystem, where a close interaction occurs among plant roots, soil, and the soil biota. Notably, the rhizobia, which constitute a fraction of bacteria inhabiting on the root of plants (rhizobacteria; plant growth-promoting rhizobacteria), form endosymbiotic nitrogen-fixing association

with leguminous plants. Discussion focuses on rhizobacteria in context with phytoremediation in Chaps. 12 and 13. Rodríguez-Dorantes et al. (Chap. 12) enlighten insights into "plant-plant growth-promoting rhizobacteria bioassays" and advocate these assays as major tools for comparing the relationships between the in vitro physiological characteristics of rhizobacteria isolated from plant metal accumulators and the plant's physiology response. Insights into leguminious plant root-bacteria symbiotic interaction and the role of rhizobia in phytoremediation are presented by Checcucci et al. (Chap. 13). The authors advocate the exploitation of N2-fixing rhizobial symbionts' genetic resources for improving phytoremediation of contaminated soils. Mobilization, immobilization, or degradation of various pollutants involve many physicochemical processes including chemical precipitation, ion exchange, adsorption, membrane separation, coagulation, flocculation, flotation, electrochemical technologies, etc. However, bioremediation-based processes remain at the top because some of the aforesaid processes, although with fast results in some cases, proved to be less efficient and more expensive. Hlihor and coauthors (Chap. 14) provide a thorough discussion on environmental bioremediation by biosorption and bioaccumulation and also highlight underlying major principles and list their applications.

Degradation of the Dinitrotoluene Isomers 2,4- and 2,6-DNT: Appraising the Role of Microorganisms

Arturo Aburto-Medina, Mohamed Taha, Esmaeil Shahsavari, and Andrew S. Ball

Abstract The dinitrotoluene (DNT) isomers 2,4- and 2,6-DNT are highly toxic compounds, and their occurrence in groundwater and soils is a widespread problem mainly due to the explosive manufacturing industry and from the commercial production of polyurethane foam. Moreover, these compounds have mutagenic and carcinogenic properties making them a great hazard to the public health. Thus, their removal from the environment is paramount, and bioremediation strategies can be applied for the environmental cleanup. Pure cultures of microorganisms able to degrade at least 2,4-DNT have been recently isolated as well as a consortium and different plant species. The pure cultures are an *Arthrobacter* strain isolated from crude oil-contaminated soil, a *Rhodococcus pyridinovorans* NT2, and *Shewanella marisflavi* EP1, while the consortium named UHasselt Sofie 3 (UHS3) was formed by *Burkholderia* HC114, *Variovorax paradoxus* VM685, *Bacillus, Pseudomonas mandelii* HC88, and *Ralstonia* HC90. These microorganisms perform the degradation of the dinitrotoluenes in aerobic conditions except the marine strain *Shewanella marisflavi*. The plant species able to grow in the presence of 2,4-DNT and proposed for phytoremediation are hemp, flax, sunflower, and mustard. The intermediates reported in the majority of successful biodegradation studies are 4-methyl-5-

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nitrocatechol (4M5NC) and 2-hydroxy-5-methylquinone (2H5MQ) under aerobic conditions, while 2,4-diaminotoluene is obtained as an end product via the formation of 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) under anaerobic conditions. These microorganisms add to the growing number of isolates with the ability to degrade these types of compounds since strain DNT was isolated. This mini-review focuses on the microorganisms involved in the degradation of the dinitrotoluene isomers 2,4- and 2,6-DNT in recent years. Information related to the degradation of these contaminants is critical to understanding and predicting their fate in the environment.

Keywords Environmental pollution • Dinitrotoluene isomers • Degradation • Microorganisms

Introduction

2,4-Dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene are major groundwater and soil contaminants derived from the production of polyurethane (foam), but most importantly they are major by-products in the production of the explosive 2,4,6-trinitrotoluene (TNT). DNT-contaminated sites are a wide problem due to the explosive manufacturing and army activities all over the world (Xu and Jing 2012). Both DNT isomers are highly toxic; they have mutagenic and carcinogenic properties and are damaging the reproductive system. They can enter the body by inhalation, skin contact, or consumption of contaminated water.

Because of their abundance, toxicity, mutagenicity, and carcinogenicity, US EPA has classified the DNT isomers as priority pollutants (Dodard et al. 1999; Ellis III et al. 1979; Gong et al. 2003; Rickert et al. 1984). Thus, the removal of these contaminants is imperative in order to prevent public health problems. The bioremediation of these contaminants is a viable alternative since both DNT isomers can be degraded in aerobic (Hudcova et al. 2011; Johnson et al. 2000; Küce et al. 2015; Kundu et al. 2015; Nishino et al. 2000) and anaerobic conditions (Cheng et al. 1996, 1998; Huang et al. 2015; Hughes et al. 1999; Wang et al. 2011). Some of the first reported microorganisms capable of the dinitrotoluene degradation were the *Burkholderia* sp. strain DNT (Suen and Spain 1993), *Burkholderia cepacia* R34 (Johnson and Spain 2003), the fungal strain *Phanerochaete chrysosporium* (Valli et al. 1992), and two consortia containing the key members *Variovorax paradoxus* VM685 and *Pseudomonas* sp. VM908, respectively (Snellinx et al. 2003). Subsequent studies tested several genetically modified strains in order to enhance the degradation of the dinitrotoluene with an emphasis on the 2,4-DNT isomer (Dutta et al. 2003; Fish et al. 2000; Lin et al. 2003; Nasr et al. 2001; Patel et al. 2000). While the degradation genes from strain DNT were transferred to *Sinorhizobium meliloti,* the genes of the bacterial hemoglobin *Vitreoscilla* were added to a few strains (Fish et al. 2000; Lin et al. 2003; Nasr et al. 2001; Patel et al.

2000) achieving higher degradation rates. However, recent studies have unveiled yet more consortia and individual strains capable of the DNT isomers. Some of these strains are the marine isolate *Shewanella marisflavi, Rhodococcus pyridinovorans*, and *Arthrobacter* sp. It is important to mention that the *Rhodococcus* strain was able to degrade both isomers concomitantly and could tolerate an initial concentration of 100 ppm in only 2 days. Also consortia comprised of *Bacillus cereus* NT4, *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, and *Achromobacter* sp. NDT3 (Hudcova et al. 2011) have been reported to degrade 2,4-DNT, the most common of the isomers. The two pairs of intermediates 4-methyl-5-nitrocatechol (4M5NC) and 2-hydroxy-5-methylquinone (2H5MQ) under aerobic conditions and 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) under anaerobic conditions have been reported in most of the biodegradation studies suggesting two main degradation pathways.

Excellent reviews on the degradation of nitroaromatics have been published previously (Anuradha 2015; Gorontzy et al. 1994; Nishino and Spain 2001, 2004; Spain and Hughes 2000). However, this chapter focuses on the microorganisms that have been identified in dinitrotoluene biodegradation studies in the recent years.

Recent Studies on the Degradation of 2,4- and 2,6-DNT

The degradation of 2,4-dinitrotoluene has recently been observed by an *Arthrobacter* strain isolated from crude oil-contaminated soil in Turkey and is plasmid mediated; the plasmid name is pArK1 with an estimated size of 8.1 kb (Küce et al. 2015). The rates of degradation depended mainly on pH and temperature and the optimum conditions were 7 and 30 °C, respectively. Although the degradation of 2,4,6-TNT had been reported previously by an *Arthrobacter* strain, the study by the Turkish group seems to be the first to report the degradation of 2,4-DNT by an *Arthrobacter* strain. Another recent study reported that the strain *Rhodococcus pyridinovorans* NT2 could degrade up to 100 mg L^{-1} of both 2,4- and 2,6-DNT with a complete degradation of the contaminants in 48 h (Kundu et al. 2015). The *Rhodococcus* strain was isolated from soils and effluents in a pesticide industry in Gujarat, India. The growth yield was 0.68 and 0.65 g of cells per $gram^{-1}$, while their degradation rates were 1.38 and 2.08 mg l^{-1} h^{-1} for 2,4 and 2,6-DNT, respectively. Thus, the authors suggest that this *Rhodococcus* strain could be used as the main bioremediation agent of DNT-contaminated sites since it can use both DNT isomers as carbon, nitrogen, and energy source (Kundu et al. 2015).

Phytoremediation has also been proposed as an alternative for the bioremediation of DNT-contaminated sites (Podlipná et al. 2015; Su and Zhu 2007; Susarla et al. 2002). Several plant species such as hemp, flax, sunflower, and mustard were able to grow on up to 1.0 ppm 2,4-DNT-contaminated soil. Moreover germination was not affected when the plants were grown under 200 ppm of 2,4-DNT in the laboratory, and a low concentration of the contaminant (0.252 g kg^{-1}) even had a

stimulatory effect. Furthermore in vitro cultures of *Saponaria officinalis* (soapwort), *Senecio jacobaea* (ragwort), and *Phragmites australis* (reed) demonstrated the 2,4-DNT metabolization with 2-amino-4-nitro compounds and 4-amino-2-nitro compounds as the main intermediates. Soapwort metabolized the contaminant faster than reed, and the authors suggested the presence of specific enzymes in the former plant (Podlipná et al. 2015).

The degradation of 2,4-DNT has also been recently reported under anaerobic conditions. The microorganism *Shewanella marisflavi* EP1was able to degrade 2,4-DNT through anaerobic respiration. Furthermore the degradation occurred at a 7.0–9.0 pH range and at a wide range of temperatures (4–40 °C) and salinity (2–8% NaCl concentration) (Huang et al. 2015). Although several strains had been reported to degrade 2,4-DNT in laboratory studies with diverse strains such as *Lactococcus lactis*, *Clostridium acetobutylicum*, and *Pseudomonas aeruginosa* (Cheng et al. 1998; Hughes et al. 1999; Noguera and Freedman 1996; Shin et al. 2005; VanderLoop et al. 1999), the study involving *Shewanella marisflavi* was conducted in situ, adding information to a previous report on marine sediments (Yang et al. 2009). Strain EP1 was able to completely reduce (100 μM) 2,4-DNT in less than 24 h with lactate as the electron donor. Moreover the authors also proved that lactate oxidation is necessary for electron release to reduce the contaminant and support the growth of EP1 under anaerobic conditions. Two main intermediates are produced during the 2,4-DNT reduction: 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) which in turn become 2,4-diaminotoluene (2,4-DAT). 4A2NT was preferentially formed over 2A4NT, and the authors attribute it to the shielding effect of the methyl group on the ortho-nitro group as explained previously and to the terminal reductases in each of the microorganisms (Huang et al. 2015). Although the complete degradation pathway was not proposed, the authors were able to conclude that dehydrogenase, menaquinone, cytochromes, and flavins play an important role in the electron transport process during the reduction. Furthermore the strain's ability to degrade the 2,4-DNT at a wide range of pH, temperature, and salinity makes it an important potential bioremediation tool in natural environments.

Another recent study reported the successful enhancement of *Arabidopsis* root length under 2,4-DNT stress by the UHasselt Sofie 3 consortium (UHS3) (Thijs et al. 2014). The consortium was formed by *Burkholderia* HC114*, Variovorax paradoxus* VM685*, Bacillus, Pseudomonas mandelii* HC88, and *Ralstonia* HC90 species, and it helped the plant to double the main root length after 9 days when exposed to 1.0 mg l^{-1} of 2,4-DNT (Thijs et al. 2014). Furthermore three consortia UHS1, UHS2, and UHS3 successfully degraded 2,4-DNT, but UHS3 reported the best degradation when compared to UHS1 and UHS2 since no intermediates accumulated, degradation started earlier, and it showed the fastest degradation rate. Thus the authors propose consortium UHS3 to be used in 2,4-DNT-contaminated environments (Thijs et al. 2014). Table 1 summarizes the studies on the degradation of the DNT isomers in the recent years with an emphasis on the microorganisms responsible of the degradation.

Table 1 Recent studies reporting the degradation of at least one of the DNT isomers

Microorganism/study details	Conditions	References
Shewanella marisflavi EP1	Anaerobic	Huang et al. (2015)
Rhodococcus pyridinovorans NT2	Aerobic	Kundu et al. (2015)
Hemp, flax, sunflower, mustard	Anaerobic	Podlipná et al. (2015)
Arthrobacter sp. K1 pArK1 gene	Aerobic	Küce et al. (2015)
Bioreporters	Aerobic	Yagur-Kroll et al. (2014, 2015)
Tolerant strains: *Burkholderia, Variovorax, Bacillus, Pseudomonas, Ralstonia*	Aerobic	Thijs et al. (2014)
Agar absorption	Aerobic	O'Sullivan et al. (2010)
Bacillus cereus NT4, *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, *Achromobacter* sp. NDT3. Individually and as a mixture	Aerobic	Hudcova et al. (2011), Paca et al. (2009)
Suspended (chemostat) and attached (columns) systems. Lowest DNT concentrations to support growth	Aerobic	Han et al. (2011), Fortner et al. (2003)
Closest relatives of DGGE excised bands: Anaerobic reactor: *Pseudomonas* sp SRU_14 & *Chryseobacterium* sp. (FJ 202055) Aerobic reactor: *Riemerella* sp., *Flavobacteriaceae* sp. and *Agrobacterium* sp.	Aerobic/ Anaerobic	Wang et al. (2011)
2,4-DNT degradation with ZVI addition	Aerobic	Oh et al. (2010)
Mixed enrichment cultures in freshwater	Aerobic	Bausum et al. (1992)
Biosensor	Aerobic	Rodríguez et al. (2006)
Lactococcus lactis	Anaerobic	Shin et al. (2005)
Burkholderia strain DNT degrades 2,5 DNT	Aerobic	Leungsakul et al. (2005)
Burkholderia strain YV1 (biodegradation enhancement)	Aerobic	Lin et al. (2003), Nasr et al. (2001), Patel et al. (2000)
Consortium 1 (4 species): *Variovorax paradoxus* VM685 & *Pseudomonas* sp. VM908 Consortium 2 (6 species): *Pseudomonas marginalis* VM683, *P. aeruginosa* VM903, *Stenotrophomonas maltophilia* VM905, *P. viridiflava* VM907	Aerobic	Snellinx et al. (2003)
Sinorhizobium meliloti (GMO) PJS1 biodegradative plasmid introduced from *Burkholderia* strain DNT	Aerobic	Dutta et al. (2003)
Pseudomonas putida UO83	Aerobic	Walia et al. (2002)
Burkholderia cepacia R34	Aerobic	Johnson et al. (2000, 2002), Johnson and Spain (2003)
2,4 & 2,6-DNT *Burkholderia cepacia* JS850 and *Hydrogenophaga paleronii* JS863	Aerobic	Nishino et al. (2000), Nishino and Spain (2001)
Soil slurries	Aerobic	Zhang et al. (2000)
Continuous flow lab fermentor	Anaerobic	Cheng et al. (1996)
Denitrifying conditions bed reactor	Anaerobic	VanderLoop et al. (1999)

(continued)

Table 1 (continued)

Microorganism/study details	Conditions	References
Burkholderia sp. strain DNT	Aerobic	Ortega-Calvo et al. (1999)
Clostridium acetobutylicum	Anaerobic	Hughes et al. (1999)
Ethanol, methanol and acetic acid as substrates	Anaerobic	Cheng et al. (1996, 1998)
Pseudomonas aeruginosa	Anaerobic	Noguera and Freedman (1996)
Burkholderia sp. strain DNT	Aerobic	Haigler et al. (1994, 1996, 1999), Spanggord et al. (1991), Suen et al. (1996), Suen and Spain (1993)
Phanerochaete chrysosporium	Anaerobic	Valli et al. (1992)

Biosensors and Bioreporters for 2,4-DNT Detection

Several studies have described the construction of bioreporters for the detection of 2,4-DNT (Behzadian et al. 2011; Burlage et al. 1998; Lönneborg et al. 2012; Radhika et al. 2007; Rodríguez et al. 2006). One of the studies is centered in the detection of 4-methyl-5-nitrocathecol (4M5NC) and 2,4,5-trihidroxytoluene (2,4,5-THT). These compounds are derivatives in the 2,4-DNT biodegradation and are recognized as polyphenol oxidase (PPO). The authors devised an amperometric biosensor that involved the immobilization of the enzyme polyphenol oxidase into a composite matrix of glossy carbon microspheres and mineral oil (Rodríguez et al. 2006; Yagur-Kroll et al. 2014). The biosensor was able to detect 4M5NC even in the presence of large quantities of 2,4-DNT. The biosensor used the *dntA* (*dntA-aAbAcAd*) genes that encode the 2,4-DNT dioxygenase from *Burkholderia* sp. strain DNT from plasmid pJS37 (Suen et al. 1996). The study confirmed a biosensor lack of response when there are no quinones formed by the PPO from phenols and catechols or in the absence of the enzyme. Thus the authors suggest the biosensor as a great alternative to decentralize environmental testing of 2,4-DNT (Rodríguez et al. 2006).

A couple of more recent studies reported the construction of a genetically engineered *Escherichia coli* bioreporter for the detection of 2,4-DNT and 2,4,6-trinitrotoluene (2,4,6-TNT) (Yagur-Kroll et al. 2014, 2015). The bioreporter employs a genetic fusion between two gene promoters *yqjF* and *ybiJ* to the *Photorhabdus luminescens luxCDABE* genes or the green fluorescent protein gene GFPmut2. Both strains were able to detect 2,4-DNT in vapor form, in aqueous solution, and when buried in soil. The bioreporters are induced by degradation products (not identified) of 2,4-DNT and not the compound itself (Yagur-Kroll et al. 2014). A second study by the same group has reported that the detection threshold, response time, and signal intensity were improved by two rounds of random mutagenesis of the *yqjF* promoter region (Yagur-Kroll et al. 2015).

Biodegradation of 2,4- and 2,6-DNT Under Aerobic Conditions

The degradation of 2,4-DNT has been studied very closely by the Spain group at the Air Force Research Laboratory in Florida, USA. Their early studies reported the degradation of 2,4-DNT by a *Burkholderia* strain DNT (formerly *Pseudomonas*). The degradation is initiated by a dioxygenase attack on the 2,4-DNT to produce 4-methyl-5-nitrocatechol (4M5NC) which is later oxidized by a monooxygenase, and further reactions lead to ring fission (Spanggord et al. 1991). The responsible genes were later characterized; it was revealed that the genes for the DNT pathway are organized in three different operons, and the DNT dioxygenase is a multicomponent enzyme system (Suen and Spain 1993). The open reading frames of the genes were similar to those of naphthalene dioxygenase (NDO) from other *Pseudomonas* strains suggesting that these enzymes share a common ancestor (Suen et al. 1996). The 4-methyl-5-nitrocatechol (4M5NC) enzyme was also purified, sequenced, and elucidated as a flavoprotein also sharing properties with other nitrophenol oxygenases (Haigler et al. 1996). The mechanisms of ring fission reaction performed by the 2,4,5-trihydroxytoluene (THT) oxygenase from strain DNT were elucidated as well as the sequence of the THT oxygenase gene *dntD* (Haigler et al. 1999). Thus, it was revealed that it is similar to the catechol 2,3-dioxygenase gene family I and that the native proteins consist of two identical subunits.

Additional strains to the *Burkholderia* sp. strain DNT with the ability to degrade 2,4- and 2,6-DNT were isolated in a later study (Nishino et al. 2000). Burkholderia cepacia JS850 and *Hydrogenophaga paleronii* JS863 were able to degrade 2,6-DNT by a different pathway; 3-methyl-4 catechol was the product of a dioxygenation reaction to 2,6-DNT, which in turn was converted to 2-hydroxy-5-nitro-6-oxohepta-2, 4-dienoic acid, and 2-hidroxy-5-nitropenta-2,4-dienoic acid by an extradiol ring cleavage dioxygenase. The other isolated strains able to degrade 2,4-DNT were designated *Burkholderia cepacia* R34 and P37, *Alcaligenes denitrificans* JS867 and JS871, *Alcaligenes xylosoxidans*, and *Burkholderia cepacia* JS872 (Nishino et al. 1999), and they all used the same pathway as the original isolate but were not able to degrade 2,6-DNT (Nishino et al. 2000). Furthermore it was also shown that the fastest-growing strain JS863 in 2,6-DNT was very similar to *Hydrogenophaga palleroni*; the genus *Hydrogenophaga* is quite versatile since it contains other species capable of hydrocarbon degradation such as benzene (Aburto et al. 2009; Aburto and Ball 2009; Fahy et al. 2006, 2008) and 4-aminobenzenesulfonate (Gan et al. 2011).

The degradation of 2,4-DNT was inhibited by the presence of 2,6-DNT and high concentrations (>100 μM) of either DNT isomer inhibit growth of DNT-degrading strains on simple substrates although concomitant degradation but at lower concentrations has been observed before (Lendenmann et al. 1998). Moreover the degradation pathway for the 2,6-DNT was proposed for the first time: either of the nitro groups is attacked by a dioxygenase to convert 2,6-DNT to 3M4NC with nitrate elimination. This is followed by opening the ring of an extradiol ring cleavage dioxygenase producing 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid, and a

hydrolytic attack would produce 2-hidroxy-5-nitropenta-2,4-dienoic acid with the loss of acetate (Nishino et al. 2000). Further studies by the same group characterized the subsequent steps in the 2,4-DNT degradation pathway in strain R34 (Johnson et al. 2002; Johnson and Spain 2003). The genes located downstream *dntD* include a CoA methylmalonate semialdehyde dehydrogenase (*dntE*), a putative NADH-dependant dehydrogenase (ORF13), and a bifunctional isomerase/hydrolase (*dntG*); other genes within the pathway were also uncovered such as *dntAaAbAcAd*, *dntB*, ORF12, ORF3, ORF10, ORF5 to ORF8, and ORF11. The authors suggest that the pathway evolved recently, since there are still extraneous elements found in the region (Johnson et al. 2002; Johnson and Spain 2003).

Other studies focused to enhance the 2,4-DNT degradation by engineering strains to produce *Vitreoscilla* with the bacterial hemoglobin gene *vgb* (Fish et al. 2000; Lin et al. 2003; Nasr et al. 2001; Patel et al. 2000) since it promotes cell growth, protein synthesis, fermentation, and biodegradation, among other applications (Stark et al. 2011). In all cases, 2,4-DNT degradation was enhanced with the strains containing the *vgb* gene. A three- to fourfold increase in the contaminant degradation was observed for the engineered strain PF6 (Fish et al. 2000), while complete degradation was registered in 3 days when strain YV1 was exposed to an initial concentration of 200 ppm 2,4-DNT (Patel et al. 2000). Also strain YV1 increased the 2,4-DNT degradation 3.5-fold compared to strain DNT when it was grown with co-substrates and limited aeration, and a further strain YV1m also increased 1.3-fold the contaminant degradation compared to YV1 (Nasr et al. 2001). Moreover increased degradation rates were registered in a fed-batch reactor (Lin et al. 2003). Similarly, the transconjugant strain DHK1 was able to degrade 94% of 2,4-DNT (0.55 mM initial concentration) in contaminated soil and allowed alfalfa plants to grow twofold higher than the parent strain in 0.14 mM 2,4-DNT-impacted soil. The strain DHK1 resulted from the addition of the pJS1 DNT-biodegradative plasmid to *Sinorhizobium meliloti* USDA1936 (Dutta et al. 2003).

Another study isolated two consortia capable of 2,4-DNT degradation and with the ability to use the contaminant as sole nitrogen and carbon source from a nitroaromatic-contaminated site (Snellinx et al. 2003). Although the consortia did not share common members, both had two species capable of the contaminant degradation *Variovorax paradoxus* VM685 (consortia 1) and *Pseudomonas* sp. VM908 (consortia 2). The consortia were formed of four and six bacterial species, respectively, and both start the degradation pathway with an oxidation that releases nitrite and forms 4-methyl-5-nitrocatechol (4M5NC) with a gene similar to the *dntAa*; 4M5NC is later metabolized to 2-hydroxy-5-methylquinone (2H5MQ) presumably by a monooxygenase as seen previously for strain DNT. *Pseudomonas marginalis* VM683 in the first consortium and *P. aeruginosa* VM903, *Sphingomonas* sp. VM904, *Stenotrophomonas maltophilia* VM905, or *P. viridiflava* VM907 in the second consortium were crucial for the intermediate metabolization, and a similar gene to the *dntD* was also found in these strains. The authors propose an interspecies metabolic interaction for the consortia and suggest that the released nitrite by *Variovorax paradoxus* VM685 may allow nitrogen assimilation by other consortia members (Snellinx et al. 2003).

A later study reported for the first time the degradation of 2,3- and 2,5-DNT by performing a saturation mutagenesis on codon I204 of the *Burkholderia* sp. strain DNT alpha subunit (DntAc). Oxidation of the other isomers 2,4- and 2,6-DNT was also enhanced, and their transformation was twofold faster than the wild type (Leungsakul et al. 2005). Moreover, it was revealed that the dioxygenases from *Burkholderia* sp. strain DNT and *B. cepacia* R34 are more closely related than originally reported.

Bioremediation with bacteria-laden agar, physical absorption of DNT by agar, and photocatalysis by UV light were evaluated as methods for the 2,4-DNT removal from concrete (Phutane et al. 2007). While photocatalysis only achieved 50% removal, 80% removal and desorption were observed with the sterile agar permitting separate further biodegradation; the bioremediation method was able to reach efficiency above 95% at optimum conditions but is also rate limiting. Another study tested photocatalysis on both isomers 2,4-DNT and 2,6-DNT. They reported a photolysis half-life of 15 and 100 h in salt and freshwater, respectively, for 2,4-DNT, while the photolysis half-life in fresh and seawater was 20 and 5 h, respectively (O'Sullivan et al. 2010).

The aerobic degradation of both DNT isomers 2,4 and 2,6 with concentrations up to 45 mg l^{-1} was evaluated in continuous packed bioreactors (poraver and fireclay). Higher removal efficiencies (above 90%) were observed in the poraver reactor than the fireclay (65%); a more efficient degradation of 2,4-DNT than 2,6-DNT was also observed in both reactors. The reactors were inoculated with adapted microorganisms that included eight Gram-negative and one Gram-positive bacterial strains that had been isolated previously (Páca et al. 2008). The strains were *Pseudomonas putida* A1, *Pseudomonas veronii* B1, *Pseudomonas* sp. C1, *Chryseobacterium* sp. D1, *Stenotrophomonas* maltophilia D2, *Sphingobacterium multivorum*, *Sphingomonas* sp. PCN3, and *Paenibacillus glucanolyticus* D1/B8. After 8 months of operation of the bioreactors, the microbial communities changed to *Brevundimonas* sp., *Achromobacter xylosoxidans* ssp., *A. denitrificans*, *Pseudomonas aeruginosa*, and *Bacillus cereus* in the poraver reactor, while a wider diversity was observed in the fireclay reactor since non-degrading strains as well as fungal strains were also registered such as *Cryptococcus humicolus*, *Pichia guilliermondii*, *Haplosporangium*, and *Stachybotrys*. The bacterial strains were *Sphingomonas* sp., *Chryseobacterium indologenes*, and *Pseudomonas* sp. (Paca et al. 2009).

The addition of persulfate activated with zerovalent iron was successful for the degradation of 2,4-DNT (50 mg l^{-1} initial concentration). The rate of DNT degradation is increased by the addition of zerovalent iron but not with Fe^{2+}, and the reduction products of DNT were preferentially oxidized by persulfate than DNT, which suggests that a sequential zerovalent reduction and persulfate oxidation may be highly effective in the degradation of 2,4-DNT and other nitroaromatics (Oh et al. 2010). A study established the lowest concentrations of the dinitrotoluene isomers to allow sustained growth of DNT-degrading microbes. These values were 0.054 and 0.057 μM in chemostat and column systems, respectively, for 2,4-DNT, while for 4,6-DNT they were 0.039 and 0.026 μM for chemostat and columns systems, respectively (Han et al. 2011). The authors state that these limits are below the regulatory requirements, and bioremediation strategies such as monitored natural attenuation could be used. Further bacterial strains capable of dinitrotoluene degradation

were isolated from a nitroaromatic-contaminated soil from an ammunition plant in the Czech Republic. The strains were *Pseudomonas putida* NDT1, *Pseudomonas fluorescens* NDT2, *Achromobacter* sp. NTD3, and *Bacillus cereus* NTD4, and they were tested as individual strains and as a mixture in the degradation of 2,4-DNT. The strain mixture had shorter lag periods and degraded the contaminant nearly 50 times faster than any individual strain; moreover it could degrade a wide spectrum of nitrotoluenes over a wider concentration range (Hudcova et al. 2011).

Degradation of 2,4-DNT Under Anaerobic Conditions

The degradation of 2,4-DNT has been observed in anaerobic conditions in several studies (Cheng et al. 1996, 1998; VanderLoop et al. 1999; Wang et al. 2011; Yang et al. 2009) and by individual strains such as *Pseudomonas aeruginosa* (Noguera and Freedman 1996), *Lactococcus lactis* (Shin et al. 2005), *Clostridium acetobutylicum* (Hughes et al. 1999) by the lignin-degrading fungus *Phanerochaete chrysosporium* (Valli et al. 1992), and more recently marine *Shewanella marisflavi* (Huang et al. 2015). The white rot basidiomycete *Phanerochaete chrysosporium* was able to degrade 2,4-DNT under ligninolytic conditions. The enzymes involved are lignin peroxidase (LiP), manganese peroxidase (MnP), and crude extracellular extracts, also the first intermediates in the degradation pathway include 2-amino-4-nitrotoluene and 4-nitro-1,2-benzoquinone, and the authors proposed the removal of both aromatic groups before ring cleavage (Valli et al. 1992). A later study reported the isolation of a *Pseudomonas aeruginosa* strain from a propellant manufacturing wastewater treatment plant capable of degrading 2,4-DNT. The strain reduced both DNT nitro groups, and the main metabolites 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, and 2,4-diaminotoluene were formed among others (Noguera and Freedman 1996). Further studies revealed the degradation of 2,4-DNT with different substrates such as ethanol, methanol, and acetic acid (Cheng et al. 1996, 1998). Moreover a *Clostridium acetobutylicum* strain was exposed to both isomers since it had been shown to transform nitroaromatics (Hughes et al. 1998), and they were biotransformed to dihydroxylamino intermediates not previously seen before (Hughes et al. 1999). *Lactococcus lactis* strain 27 was isolated from the intestines of earthworms and could also biotransform the four dinitrotoluene isomers – 2,3-, 2,4-, 2,6-, and 3,4-DNT – into the corresponding aminonitrotoluenes. However, no simultaneous or sequential reduction of two nitro groups of the dinitrotoluene was observed; furthermore the authors warn that production of dinitroazoxytoluenes may increase the environmental risk (Shin et al. 2005).

Another study reported a 99% removal efficiency of 2,4-DNT with an initial concentration of 95.5 mg l^{-1} in a treatment combining aerobic and anaerobic filters. The microorganisms recovered from the anaerobic filters by DGGE were highly similar to *Pseudomonas* sp. SRU_14, *Riemerella* sp. clone GI7-5-G11, *Chryseobacterium* sp. (FJ 202055), Flavobacteriaceae family (EU 839047), and

Agrobacterium sp. (CCAM 010004), and the main metabolites were also 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, and 2,4-diaminotoluene (Wang et al. 2011). In anaerobic conditions, the obligate marine *Shewanella marisflavi* EP1 has been shown to degrade 2,4-dinitrotoluene in 24 h with the transformation product 2,4-diaminotoluene via 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene as intermediates. Flavins, dicumarol, and Cu^{2+} enhanced the degradation of DNT, suggesting they are involved in the electron transfer process. EP1 oxidize lactate to reduce 2,4-DNT as electron acceptor via a respiration process that supports anaerobic growth (Huang et al. 2015).

The majority of the studies conducted in anaerobic conditions reported 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), and 2,4-diaminotoluene (2,4-DAT) as the main intermediates in the degradation process, suggesting the presence of a common degradation pathway.

DNT Isomer Degradation Pathway

Aerobic Conditions

2,4-DNT Degradation Pathway

When there is oxygen available, the degradation follows an oxidative pathway, which involves a dioxygenase, a reductase, a monooxygenase, a dehydrogenase, and a hydrolase. The degradation starts with a dioxygenase (*dntA*) catalyzing the oxidation of 2,4-DNT to 4-methyl-5-nitrocatechol (2M5NC) and the release of nitrite. A monooxygenase (*dntB*) removes the second nitro group producing 2-hydroxy-5-methylquinone (2H5MQ) that is later reduced to 2,4,5-trihydroxytoluene (2,4,5-THT) by the reductase *dntC*. Another dioxygenase (*dntD*) catalyzes the meta cleavage of 2,4,5-THT producing 2,4-dihydroxy-5-methyl-6-oxo-2,4-hexadienoic acid (DMOHA). The degradation pathway continues with a CoA-dependant methylmalonate semialdehyde dehydrogenase (*dntE*) and a bifunctional isomerase/hydrolase (*dntG*) catalyzing the further reactions to produce propionyl CoA and pyruvate; these compounds enter the central metabolic pathways of the cell. This pathway has been extensively studied by the research group at the Air Force Research Laboratory in Florida, USA (Haigler et al. 1994, 1996, 1999; Johnson et al. 2000, 2002; Johnson and Spain 2003; Nishino et al. 2000; Spanggord et al. 1991; Suen et al. 1996; Suen and Spain 1993), and it has also been revealed that it evolved from those of naphthalene biodegradation routes (de las Heras et al. 2011).

The degradation of the 2,6-DNT isomer has been reported for several studies, and a degradation pathway was proposed by Nishino and collaborators (Nishino et al. 2000). The pathway also initiates with a dioxygenase attack converting the 2,6-DNT to 3-methyl-4-nitrocatechol and nitrite is released. An extradiol ring cleavage attacks the 3-methyl-4-nitrocatechol yielding 2-hydroxy-5-nitro-6-oxohepta-2,4-dienoic acid which in turn is converted to 2-hydroxy-5-nitropenta-2,3-dienoic

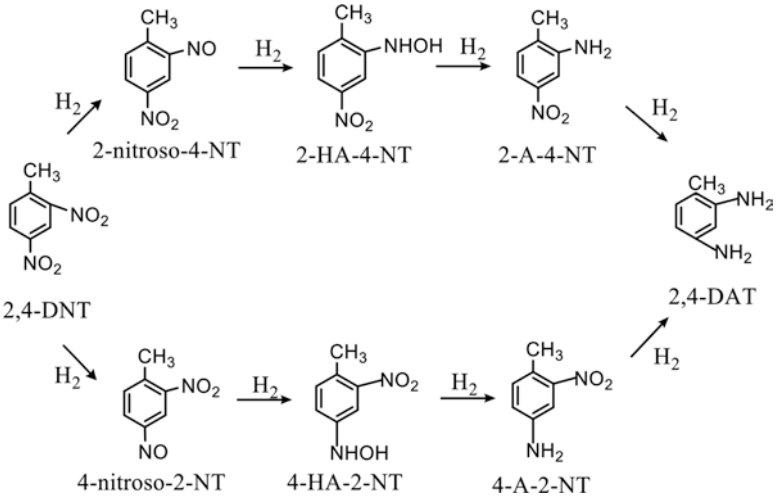

Fig. 1 Anaerobic DNT degradation pathway (Redrawn from Wang et al. (2011))

acid. The strains *Hydrogenophaga palleroni* JS863, Burkholderia cepacia JS850, and *Pseudomonas putida* JS881 were able to degrade 2,6-DNT, and the main intermediates were 3M4NC and HNOHA (Nishino et al. 2000).

Anaerobic Conditions

During anaerobic conditions a reduction occurs; the end product is 2,4-diaminotoluene via the formation of two metabolites: 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene. These intermediates have been observed in all the degradation studies with the lack of oxygen (Cheng et al. 1996, 1998; Huang et al. 2015; Wang et al. 2011) (Fig. 1).

Future Works

The number of individual strains and consortia able to degrade the dinitrotoluene has increased in the recent years. Furthermore engineered strains have enhanced the contaminant removal, and the degradation pathways in the presence and absence of oxygen have been elucidated. Thus, future efforts could focus on metagenomics studies performed in dinitrotoluene-degrading consortia in order to understand the function of each of the consortium members.

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Bioremediation Approaches for Petroleum Hydrocarbon-Contaminated Environments

Esmaeil Shahsavari, Gregory Poi, Arturo Aburto-Medina, Nagalakshmi Haleyur, and Andrew S. Ball

Abstract Despite the many positive impacts of petroleum hydrocarbons to human industrialization and activity, environmental contamination by petroleum hydrocarbons represents a major cause of marine and terrestrial pollution. Petroleum hydrocarbons contain various compounds such as alkanes, light aromatics (MAHs), cycloalkanes, heavy aromatics (PAHs) and asphaltenes, among others. A number of these compounds are potentially carcinogenic and mutagenic. Among the various remediation technologies, bioremediation or the use of microorganisms to degrade the hydrocarbons is considered a clean, cost-effective and environmentally friendly approach. Unlike other physical and chemical methods, it does not lead to secondary contamination, generally resulting in the complete mineralization of hydrocarbons. Several reports have now confirmed bioremediation as a promising technology to clean up the environments. This chapter presents an overview of current bioremediation approaches for the treatment of petroleum hydrocarbons.

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Introduction

Petroleum hydrocarbon (PHC) pollution is commonly associated with operations at oil refineries, chemical plants and shipyards. Industrial activities and contingency situations such as tanker spills or leakage from storage tanks in aquatic and terrestrial environments pose significant hazards. The problem is compounded when petrol, diesel, gasoline and other petrochemical products contaminate groundwater (Andreoni and Gianfreda 2007). As a result, the release of petroleum hydrocarbon (e.g., crude oil) into the environment is a major cause of marine and terrestrial pollution (Kingston 2002; Macaulay and Rees 2014). The composition of crude oil varies, but on average there is a rough parity between paraffins, naphthenes and aromatic hydrocarbons. Paraffins are saturated linear and branched hydrocarbons, while naphthenes are cyclic saturated hydrocarbons (Fig. 1). Hydrocarbons are not all biodegraded at similar rates and not all hydrocarbons are readily degradable, but estimates

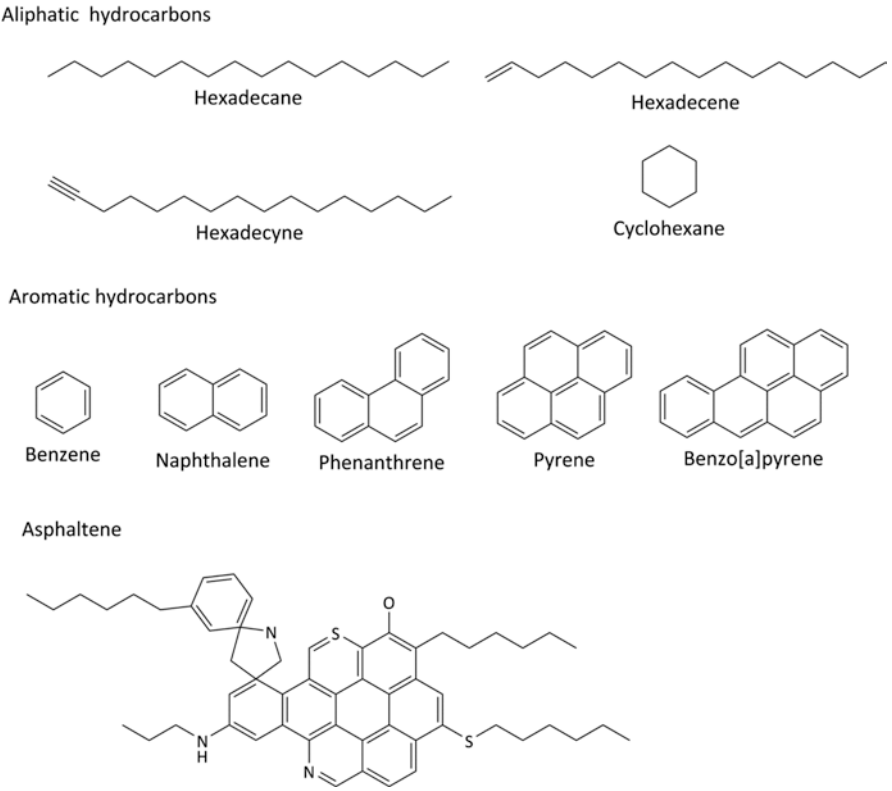

Fig. 1 Molecular structure of different petroleum hydrocarbon representatives

for different crude oils range from 70 to 90% degradability, with the remaining hydrocarbons being primarily the asphaltenes and resins (Prince et al. 2003).

The susceptibility of crude oil components to microbial degradation has been described as follows: alkanes > light aromatics (MAHs) > cycloalkanes > heavy aromatics (PAHs) > asphaltenes (van Hamme et al. 2003). PAHs may contain one or more benzene rings, and they include naphthalene (two-ringed), phenanthrene (three-ringed) and anthracene (three-ringed) which are considered low molecular weight or light PAHs, while those with four or more rings such as pyrene (four-ringed), chrysenes (four-ringed), fluorenthene (five-ringed), benzo[a]pyrene (five-ringed) and coronenes (seven-ringed) are referred to as heavy PAHs. These common petroleum pollutants are considered to be potentially mutagenic and carcinogenic (Boonchan 2000; Mao et al. 2012). Consequently, the contamination of marine environments by hydrocarbons represents a global concern with potential consequences for both ecosystem and human health (Andersson et al. 2006). It is estimated that between 1.7 and 1.8 million metric tonnes of crude oil find their way into the world's water every year, of which more than 90% is directly related to human activities (Nikolopoulou et al. 2007). Therefore, the bioremediation of contaminated environments is of great public concern. Petroleum hydrocarbons are only eliminated from the environment when converted to carbon dioxide and water by two processes, combustion and biodegradation. The remediation techniques used include are physical, chemical and biological (bioremediation) methods. Amongst these, bioremediation approaches or the use of microbes for the degradation of hydrocarbons are considered as clean and cost-effective technologies. In this book chapter, fundamental knowledge regarding the bioremediation of petroleum hydrocarbons in contaminated environments is presented.

Bioremediation Approaches

Bioremediation of contaminated environments relies on breaking down target pollutant compounds by microbial degradation. While biostimulation (BS) typically involves the addition of nutrients or substrates in the form of nitrogen and phosphate, bioaugmentation (BA) requires the addition of microbial cultures to the contaminated matrix, usually in combination with biostimulation (Boopathy 2000). This may be implemented as an in situ process that includes strategies such as soil amendment, bioventing or biosparging, bioslurping, phyto-/rhizoremediation and monitored natural attenuation. Ex situ processes require the soil materials to be excavated and loaded into a bioreactor pit or a treatment facility, and they include biopiling, composting, bioreactors and land farming (Macaulay and Rees 2014). An overview of bioremediation methods is presented in Fig. 2.

The treatment of waste solids, including soil containing hydrocarbon pollutants, can be relatively expensive making economic drivers the primary determinant behind the chosen options (Makadia et al. 2011). However, there are other considerations such as opportunity costs and the demands of the locality which pose practical restrictions on the application of certain technologies.

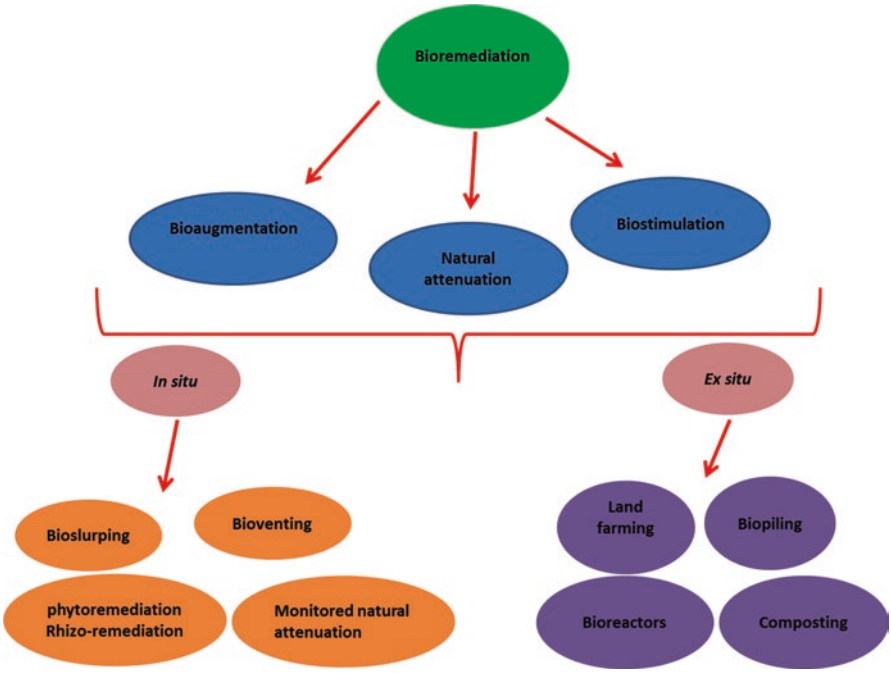

Fig. 2 An overview of bioremediation technologies

Bioremediation of Petroleum Hydrocarbon Pollution from Marine Oil Spills

In a review on the efficacy of bioremediation on marine oil spills (on the surface and on shorelines), bioremediation was determined to be effective, but no advantage was found with bioaugmentation with commercial microbial preparations over biostimulation of indigenous organisms. However, this was viewed from a perspective of a last resort technology following a marine oil spill that could not be collected or burnt and had to be dealt with in situ within a hostile environment either still floating on the surface at sea or on a shoreline (Prince et al. 2003).

The use of physical and chemical methods for petrogenic hydrocarbon remediation is inadequate, in that these methods do not completely remediate the hydrocarbons in the environment (Gavrilescu et al. 2014). Reports of large oil spills, marine or otherwise, often capture public attention followed by a demand for a prompt and environmentally sensitive response. In situations where the containment of the oil spill with booms or collection with skimmers is impractical, stimulating the natural biodegradation of oil offers the next best alternative. Such an approach would include strategies such as the spraying of dispersants to enhance the surface area for microbial colonization, as well as nutrient supplementation without the addition of cultures. In contrast to biostimulation, bioaugmentation involves the addition of

exogenous cultures to initiate and accelerate the process of bioremediation but lacks effective, quantitative demonstration (Prince 2010).

It has been reported that hydrocarbon-degrading bacteria are ubiquitous in the sea, and thus biostimulation would suffice. The problem lies in the slow-acting nature of the process. It may be too slow to prevent oil from reaching the shore and causing environmental damage as documented in the Exxon Valdez and Gulf of Mexico disasters. The introduction of uric acid has been recommended as a means to accelerate the process by providing the supply of nitrogen and phosphorus required, given that these constitute the rate-limiting factor for petroleum degradation at sea (Ron and Rosenberg 2014). Others suggest the addition of rhamnolipids as biosurfactants to enhance the rate of marine oil spill bioremediation (Chen et al. 2013). From a different perspective, it has been demonstrated that the process of bioaugmentation could be enhanced by using autochthonous bioaugmentation (ABA), defined as the exclusive use of adapted indigenous microorganisms for decontamination. The rate of hydrocarbon degradation was enhanced by the addition of lipophilic fertilizers (uric acid and lecithin) in combination with rhamnolipids acting as biosurfactants along with the addition of adapted indigenous microorganisms (Nikolopoulou et al. 2013).

The lack of quantitative demonstration of the efficacy on bioaugmentation in the field has been a considerable obstacle to the adoption of this technique as a tool for the biodegradation of petroleum hydrocarbon spills at sea (Prince 2010). This has been compounded by a gap between the availability of peer-reviewed documentation for laboratory-based work and that for field-based work to demonstrate effective translation and scale-up (Macaulay and Rees 2014). There has been an increase in attempts to bridge this gap as demonstrated by more recent work that follow up from bench-scale experimentation using shake flask to laboratory-based mesocosm experiments at pilot scale using large volumes such as 840 L (Bao et al. 2012) and 10,000 L (Hassanshahian et al. 2014). In the former, preliminary shake flask trials conducted on a mixed-species consortium containing four strains of marine bacterial isolates were found to be suitable candidates for the degradation of crude oil in a simulated marine environment and subsequently scaled up in a mesocosm experiment using a tank (1.5 m × 0.8 m × 0.7 m) with a volume of approximately 840 L. These four strains which included *Ochrobactrum* sp. (N1), *Brevibacillus parabrevis* (N2), *B. parabrevis* (N3) and *B. parabrevis* (N4) removed over 51.1% of crude oil from the simulated water body (Bao et al. 2012). In the latter, three different series of experiments were performed in a 'mesocosm facility' (10,000 L) where natural seawater was artificially polluted with crude oil (1000 ppm) and was amended with inorganic nutrients (Mesocosm 1, M1), inorganic nutrient plus an inoculum of *Alcanivorax borkumensis* SK2T (Mesocosm 2, M2) and inorganic nutrient plus an inoculum of *A. borkumensis* SK2T and *Thalassolituus oleivorans* MIL-1 T (Mesocosm 3, M3), respectively.

Experimental analyses performed in the mesocosms showed that the load of crude oil increased the total microbial abundance but inhibited the activity of some enzymes while stimulating some others. Bioaugmentation with only *A. borkumensis* SK2T produced the highest percentage of degradation (95%) in comparison with

the biostimulation treatment (80%) and bioaugmentation using an *Alcanivorax-Thalassolituus* bacterial consortium (70%), which indicated an unfavourable interaction between the two bacterial genera used (Hassanshahian et al. 2014). This suggests that simply combining different species of hydrocarbonoclastic bacteria is not necessarily an advantage in the design of suitable consortia of biodegradation of hydrocarbons.

It was reported that *Acinetobacter* and *Cloacibacterium* were the dominant genera in freshwater microcosms, while the *Oceanospirillales* order and the *Marinobacter*, *Pseudomonas* and *Cycloclasticus* genera predominated in marine microcosms. It was also found that the *Oceanospirillales* order and the *Marinobacter* genus were selected in the different hydrocarbon-containing microcosms in hypersaline water. *Pseudomonas* appears to be the only genus of hydrocarbonoclastic bacteria present in freshwater, seawater and terrestrial systems (Afzal et al. 2007; Felföldi et al. 2010; Kadali et al. 2012; Mirdamadian et al. 2010; Zhang et al. 2011; Zhao et al. 2011). Nevertheless, the development of bioremediation as a technology for cleaning up oil spills is ongoing and has been driven by the relative low costs involved and the favourable impact it has on the environment as compared to alternative technologies (Macaulay and Rees 2014).

Bioremediation of Terrestrial Oil Spills

Growing industrialization and demands for energy have led to soil contamination by crude oil and refined products. If not mitigated, these petroleum hydrocarbon (PHC) pollutants pose a threat to both the environment and human health (Sanscartier et al. 2011). Diesel oil is a complex mixture of alkanes and aromatic compounds which is frequently reported in terrestrial hydrocarbon spills, often found leaking from storage tanks and pipelines or released in accidental spills and has been the subject of several pilot-scale to field-scale clean-up projects (Chemlal et al. 2013; Łebkowska et al. 2011).

Bioremediation Strategies to Treat PHC-Contaminated Soil

Landfarming is essentially a low-cost and low-technology method of ex situ biostimulation that has been successful in degrading PHC-contaminated soil, mainly in the superficial layer of soils since most oleophilic microbes are confined to the 15–30 cm region (Zouboulis and Moussas 2011). While reportedly effective for the degradation of low molecular weight PAHs (Picado et al. 2001), it has been shown to be unsuccessful in the degradation of heavy PAHs and requires a long residence time (Macaulay and Rees 2014). Composting is another simple ex situ aerobic biostimulation technology that uses organic amendments such as manure (Akinde and Obire 2008; Groudeva et al. 2001) and biowaste (Van Gestel et al. 2003) to provide

both the microbial consortia and nutrients. The use of bioreactors for soil bioremediation overcomes some of the problems associated with the supply of oxygen and delivery of nutrients to the aerobic microorganisms, offering some degree of control over the environmental factors that influence biodegradation (Zouboulis and Moussas 2011).

Bioremediation Strategies to Treat PHC-Contaminated Soil and Groundwater

PHC contamination of soil and groundwater poses a major concern for human health and the environment (Andreoni and Gianfreda 2007; Paul et al. 2005; Dorn and Salanitro 2000). The release of fugitive PHC materials into the environment makes in situ bioremediation the only option where biostimulation or slow monitored natural attenuation is acceptable where no other options exist. In this context, the efficacy of bioremediation of groundwater (GW) in situ has been by most accounts attributed to natural managed attenuation and monitored natural attenuation (Aburto 2007; Aburto and Ball 2009; Aburto et al. 2009; Aburto and Peimbert 2011) as opposed to biodegradation and bioaugmentation (Chapelle 1999), with reports of effective bioremediation taking relatively long periods of 1–2 years when biostimulation was applied (Kao et al. 2008; Chen et al. 2010).

The most successful cases of bioaugmentation that have been documented are those using bioreactors to optimize the growth and activity of the microbial population to bioremediate the contaminated groundwater (El Fantroussi and Agathos 2005). This ex situ bioremediation strategy involved the pumping of the polluted groundwater for biotreatment followed by the injection of the treated groundwater back into the polluted site as part of a 'pump and treat' system. The integration of fixed-film microbial growth with such a system had been shown to be effective in the treatment of contaminated groundwater (Rodríguez-Martínez et al. 2006). Although effective, the costs associated with the building of the wells and the treatment process have been reported to be relatively high compared to other strategies (Macaulay and Rees 2014). However, these costs can be effectively mitigated using the same monitoring wells that would have already been in place for routine sample analyses for the 'pump-out' and recharge injection. The costs can be further reduced when coupled with a simplified modular bioreactor that has been designed for low operational costs that can be transported from site to site.

While the observation for bioremediation of groundwater has been attributed primarily to natural managed attenuation excluding the ex situ 'pump and treat' method (Chapelle 1999), this observation does not appear to be reflected in the literature in the case of land-based bioremediation of PHC-contaminated soil where the bioremediation is often carried both in situ and ex situ. An earlier study compared different approaches on the bioremediation of diesel-contaminated soil (Bento et al. 2005) using natural attenuation, biostimulation and bioaugmentation.

The laboratory-scale study used a microbial consortia derived from hydrocarbonoclastic isolates sourced from Long Beach, California, USA, using 450 g soil taken from a beach in Hong Kong. The consortium had been shown to be effective for the degradation of TPH in diesel-contaminated Long Beach soil (California), where it was more effective than natural attenuation or biostimulation after 12 weeks. However, when the same consortium was applied to the diesel-contaminated soil from Hong Kong, Bento et al. (2005) reported that biostimulation (addition of nutrients) was less effective than natural attenuation or bioaugmentation, with natural attenuation being most effective at the degradation of diesel as measured by the reduction in light oil fraction for C_{12}–C_{23}. However, bioaugmentation for the degradation of the heavy oil fraction for C_{23}–C_{40} was more effective than biostimulation, followed by natural attenuation in Hong Kong soil. It was in this context that Bento et al. (2005) reported that 'the consortium degraded 73–75% of the light and heavy oil fraction of the TPH present in the Long Beach soil contaminated with diesel oil but had no effect on the Hong Kong soil". Overall, optimum bioaugmentation performance occurs when the exogenous organisms are capable of competing with the indigenous microbes for nutrients resulting in increased abundance. This is consistent with the enrichment of indigenous microorganisms from a given microcosm to be used for inoculation for bioaugmentation in hydrocarbon-contaminated soil (Łebkowska et al. 2011), also referred to autochthonous bioaugmentation (Nikolopoulou et al. 2013). There have recently been several studies comparing the efficacy of different bioremediation approaches including natural attenuation, biostimulation and bioaugmentation of PHC-contaminated environments. The general finding has been that bioaugmentation in combination with biostimulation usually provides a faster rate of bioremediation than biostimulation on its own with a variety of hydrocarbon pollutants across a wide range of conditions (Calvo et al. 2009; Coulon et al. 2010; Kauppi et al. 2011; Łebkowska et al. 2011; Grace Liu et al. 2011; Sheppard et al. 2011; Zhao et al. 2011).

Translation and Scale-Up

Laboratory experiments have to be effectively extrapolated to the field scale (Diplock et al. 2009). The optimization of operational parameters is an important part of the process to evaluate the strategies in the implementation of a bioremediation process. While laboratory-scale experiments provide an opportunity to gain insights into the conditions for effective translation and scale-up for large-scale operations, it is with the caveat that the biotreatment can be accurately reproduced at laboratory scale (Lors et al. 2012). This is because it is not always possible to replicate field conditions in the lab, a key example being the absence of ecological considerations in most laboratory experiments, where the presence of predators and antagonistic microbes are capable of impacting on the efficacy of a given process (Macaulay and Rees 2014).

Hydrocarbonoclastic Bacteria in the Bioremediation of PHC-Contaminated Soil

TPH has been used to evaluate the efficacy of bioremediation as a means to bioremediate PHC-contaminated soil in bench-scale experiments (Aleer et al. 2010; Sheppard et al. 2011; Shahsavari et al. 2013; Adetutu et al. 2013) as well as field-scale experiments (Coulon et al. 2010; Gogoi et al. 2003; Mishra et al. 2001; Compeau et al. 1991). Field samples of soil contaminated with diesel oil collected from California, USA, and Hong Kong, China, showed that bioaugmentation showed the greatest degradation of the light (72.7%) and heavy (75.2%) fractions of TPH. The microbial consortium used for the bioaugmentation included *Bacillus cereus*, *Bacillus sphaericus*, *Bacillus fusiformis*, *Bacillus pumilus*, *Acinetobacter junii* and *Pseudomonas* sp. While the number of diesel-degrading microorganisms and heterotrophic population was not influenced by the bioremediation treatments, it was found that soil properties and the indigenous soil microbial population affected the degree of biodegradation (Bento et al. 2005). Contaminated soil sourced from a petroleum refinery in Portugal showed that factors such as exposure to the elements (air and sunlight) enhanced natural attenuation resulted in 30% TPH degradation as compared to bioaugmentation combined with nutrient and surfactant amendments which reached about 50% TPH degradation (Couto et al. 2010). The ability of bacterial groups such as *Pseudomonas*, *Acinetobacter* and *Rhodococcus* (Lin et al. 2010; Lee et al. 2012) as well as those from the *Bacillus* group has been identified as being important hydrocarbon degraders (Bento et al. 2005; Das and Mukherjee 2007; Łebkowska et al. 2011). These results have been translated and scaled up in the bioremediation of PHC- and oil-contaminated soil in the field with varying degrees of success (Menendez-Vega et al. 2007; Kauppi et al. 2011; Lee et al. 2012).

Bioremediation of PHC-Contaminated Soil Using Biopiles and Windrowing

An earlier ex situ treatment of diesel-contaminated soil using 375 kg batches was performed to compare the efficacy of biopiles and windrows (1.5 m × 0.5 m × 0.5 m). Coarse wood chips and horse manure were used as a bulking agent for the contaminated soil, and they were compared with NPK fertilizer (7% each of N, P and K). Results provided evidence for the efficacy of bioaugmentation over biostimulation as a remediation strategy where rapid mineralization was achieved using static biopiles in contrast to windrow systems. The former was less labour intensive and did not require specialist soil-turning equipment and associated staff on-site to carry out translation to a full-scale remediation project (Cunningham and Philp 2000). It has been demonstrated that the process of bioaugmentation could be enhanced by using autochthonous bioaugmentation (Nikolopoulou et al. 2013).

Łebkowska et al. (2011) reported that there was a lack of data in the literature concerning the efficiency of bioremediation of PHC-contaminated soil in relation to inoculation frequency, usually with reports of only a single application of (autochthonous) bioaugmentation and biostimulation. In one study, indigenous bacterial strains isolated from polluted soils were applied ex situ in high concentrations of 10^7–10^8 CFU g^{-1} dry weight to bioaugment soil contaminated by diesel oil, engine oil and aircraft fuel, with the inoculation performed every 3 days. Although the indigenous bacterial strains appeared to share some commonality for each mesocosm with *Bacillus* sp. and *Pseudomonas* sp. being dominant for all three mesocosms, there were significant differences in some of the key microorganisms in terms of distribution, with some microorganisms such as *Pseudomonas alcaligenes*, *Sphingomonas paucimobilis*, *Alcaligenes xylosoxidans*, and *Comamonas testosteroni* present only in the aircraft fuel-contaminated soil. The diesel-contaminated soil had an initial value of only 2509 mg kg^{-1} compared to 5568 mg kg^{-1} for the soil contaminated with aircraft fuel yet required more than double the residence time to achieve approximately 80% degradation. In contrast the aircraft fuel was degraded by 97.57% within only 22 days. This technology which had been previously patented was successfully scaled up to treat over 150 MT of soil (Łebkowska et al. 2011).

There have been several small-scale laboratory-based microcosm studies (less than 2.5 kg) conducted on PHC-contaminated soil to compare the efficacy of natural attenuation, biostimulation, bioaugmentation and biostimulation/bioaugmentation for bioremediation (Aburto-Medina et al. 2012; Aleer et al. 2010; Dandie et al. 2010; Sheppard et al. 2011; Makadia et al. 2011). While most of the studies have focused on comparing the different bioremediation approaches, Makadia et al. (2011) adopted the approach of recycling soil from an old biopile that was previously bioremediated to below 10,000 mg kg^{-1} to harness the hydrocarbon catabolic ability of the residual microbial population in lieu of BA using laboratory-cultured organisms to treat waste oil sludge sourced from crude oil tank bottom.

The advantage was twofold: firstly, the treated soil could be reused to reduce the landfill space required and, secondly, to exploit the hydrocarbon-degrading potential of the treated soil to reduce the cost of subsequent bioremediation projects. Four treatment strategies were employed: biostimulation (BS), bioaugmentation (BA), natural attenuation (NA) and a combination of BS and BA to assess the degradation of spiked waste oil sludge present in contaminated soil for a period of 12 weeks. Initial results in weeks 2 and 3 showed that both BS and the BA/BS samples had substantially higher rates of hydrocarbon reduction than BA or NA samples. However, this trend had changed by week 12; although there was substantial reduction in the TPH content of the soil microcosms, the percentage reduction for NA (86% reduction) was not significantly different (ANOVA, $P > 0.05$) to the reductions observed in the amended soil microcosms: BS (91%), BA (91%) and BS/BA (92%). Aleer et al. (2010) conducted work on 200 g lots of petroleum hydrocarbon-contaminated soils obtained from old hydrocarbon biopiles that were spiked with waste engine oil and monitored for 3 months. These were done to compare the efficacy of different types of treatment that included NA, BS, BS and combined

treatment of BS/BA. TPH analyses showed that BS and BS/BA accelerated hydrocarbon degradation. Moreover, it was an effective treatment, with over 84% reduction to less than 10,000 mg kg^{-1} at week 8. However, a further 2 weeks of treatment was required for other microcosms to obtain the same level at week 10. The BS/BA microcosms yielded the highest degradation yield of 92% by week 10. It was determined that there were no significant differences in hydrocarbon levels in naturally attenuated and treated microcosms at week 12. The results for the 16S rRNA- and ITS-based denaturing gradient gel electrophoresis profiling showed diverse bacterial and fungal communities with some dominant members belonging to hydrocarbon-degrading *Proteobacteria* spp., *Ascomycetes* spp. and *Basidiomycetes* spp. The study showed that hydrocarbon-polluted soils possessed microbial hydrocarbon-degrading potential that could be recycled and harnessed for further application to the degradation of engine oil, with the combination of BS/BA microcosms giving the highest degradation yield, better than BS or BA. However, the results for NAT were better than BS or BA as a single treatment on its own (Aleer et al. 2010).

In another study by Sheppard et al. (2011), BS, using the addition of nutrients for fungi was compared with BA with the fungus *Scedosporium apiospermum*. The primary focus of this study was to use ecological toxicity as a means to complement chemical analyses to meet legislated guidelines for the disposal of bioremediated soil. This was performed in combination with biostimulation in the form of providing nutrients for fungi with soil maintained at approximately 50% water holding capacity, incubated at 30 °C. The results for NAT gave the highest degradation yield (43.42%) making the soil suitable for disposal as waste under current guidelines (as both pesticide and metal contents were within safe limits). This result was in contrast to the lower degradation values for BS (32.75%) and BA (31.98%). The BS/BA degradation value (37.20%) was lower than that for BS without BA (Sheppard et al. 2011). This would suggest that NA by the indigenous microorganisms plus BS performed better than BA with the fungi.

While the above studies relied on TPH analyses as the primary method to assess the end point of PHC-contaminated soil bioremediation, a separate study was done by Soleimani et al. (2013) to compare TPH concentrations and CHEMometric™ analysis of selected ion chromatograms (SIC) to assess the end point of biodegradation. The latter, termed the CHEMSIC method of petroleum biomarkers included terpanes and regular, diaromatic and triaromatic steranes used for determining the level and type of hydrocarbon contamination. Six methods for enhancing bioremediation were tested on oil-contaminated soils from three refinery areas in Iran (Isfahan, Arak and Tehran), including bacterial enrichment and planting and addition of nitrogen and phosphorus, molasses, hydrogen peroxide and a surfactant (Tween 80) at an incubation temperature of 28 ± 2 °C. Results demonstrated that bacterial enrichment (BA) and addition of nutrients (BS) were most efficient with 50–62% removal of TPH after 60 days. BA was performed using an inoculum based on a consortium containing five organisms: *Bacillus*, *Listeria*, *Pseudomonas*, *Rothia* and *Corynebacterium* spp. (Soleimani et al. 2013).

The CHEMSIC results demonstrated that the bacterial enrichment was more efficient in the degradation of n-alkanes and low molecular weight PACs as well as alkylated PACs (e.g. naphthalenes, phenanthrenes and dibenzothiophenes), while nutrient addition led to a larger relative removal of isoprenoids (e.g. norpristane, pristane and phytane), with the conclusion that the CHEMSIC method could be used as a suitable tool for assessing bioremediation efficiency (Soleimani et al. 2013). However, while the study did not differentiate between the different BS and BA approaches used, the study did establish that bioremediation using the five-strain bacterial consortium was effective in the degradation of PHC contaminants as measured by TPH degradation. Table 1 summarizes laboratory-scale investigations on PHC-contaminated soil with volumes ranging from 1.0 to 149 kg for a variety of pollutants including diesel (Chemlal et al. 2012, 2013), PHC-contaminated soil from oil storage site (Grace Liu et al. 2011) and crude oil-spiked soil (Zhao et al. 2011). The small-scale study by Chemlal et al. (2012) on 2.0 kg of diesel-contaminated soil with an initial concentration of 5800 mg kg^{-1} showed 70.69% degradation within 40 days and was followed up with a scale-up to 149 kg at almost twice the concentration, 13,000 mg kg^{-1}. This resulted in 85.38% degradation but with a longer residence time of 76 days, with the observation that alkanes were degraded before aromatics.

Liu et al. (2011) performed a series of experiments using 2.5 kg soil to compare various bioremediation combinations using BS, BA, BS/BA, and other additions including biosurfactants (BSF) and even kitchen waste (KW) as treatments for the bioremediation of PHC-contaminated soil from an oil storage site in Taiwan over 140 days. BA was performed using a microbial consortium which consisted of five strains of microorganisms including *Gordonia alkanivorans* (CC-JG39), *Rhodococcus erythropolis* (CC-BC11), *Acinetobacter junii (*CC- FH2), *Exiguobacterium aurantiacum* (CC-LSH4-1) and *Serratia marcescens*. The treatment using NA gave the lowest degradation yield at 15.6%, in sharp contrast to the highest degradation for KW at 81.9%. The next best yield was for BS using the lower concentrations of nitrogen and phosphate at 79.7%, while that using higher concentrations was lower, 58.9% suggesting that greater nutrient biostimulation did not correspond to improved yields (Zhao et al. 2011). A similar degradation of 61.90% was observed for crude oil-spiked soil with an initial concentration of 10,000 mg kg^{-1} using a consortium of five strains including *Pseudomonas* spp., *Brucella* spp., *Bacillus* spp., *Rhodococcus* spp., *Microbacterium* spp., *Roseomonas* spp. and *Rhizobiales* spp., at a shorter residence time of 60 days but with a smaller volume of only 1.0 kg of soil (Zhao et al. 2011). A summary of selected pilot-scale experiments that have been conducted on PHC-contaminated soil of up to 20 MT in mass per batch is shown in Table 2. The soils contained a variety of pollutants ranging from bunker fuel (Coulon et al. 2010), diesel (Lin et al. 2010) and PAHs (Sun et al. 2012). Coulon et al. (2010) performed a comparison of biopiled and windrowed soils in a full-scale trail where the end point of assessment targets was defined by human risk assessment and ecotoxicological hazard assessment approaches to compliment chemical analyses using TPH. The study reported that the amendment of nutrients significantly increased hydrocarbon degradation at the initial stages of

Table 1 Examples of bench-scale laboratory-based experiments, up to 2.5 kg in mass per batch

Treatment/cultures	Sources	TPH (mg kg^{-1})		Mass (kg)	Percentage degraded (%)	Residence time (days)	References
		Initial	Final				
BS/BA with unknown cultures/ unspecified	Diesel oil-contaminated soil	5,800	1,700	2	70.69	40	Chemlal et al. (2012)
Not specified	Diesel oil	13,000	1,900	149	85.38	76	Chemlal et al. (2013)
BS/BA 1. *Gordonia alkanivorans CC-JG39*, 2. *Rhodococcus erythropolis CC-BC11*, 3. *Acinetobacter junii CC-FH2*, 4. *Exiguobacterium aurantiacum CC-LSH4-1*, 5. Serratia marcescens	PHC soil from an oil storage site	14,032.3	11,847.8	NA 2.5	15.6%	141	Liu et al. (2011)
		13,724.0	5,250.8	CT 2.5	61.7%		
		14,032.5	3770.0	BSF 2.5	73.1%		
		13,724.0	3,783.3	BSF 2.5	72.4%		
		13,724.0	5,644.4	NPH 2.5	58.9%		
		13,724.0	2,780.1	NPL 2.5	79.7%		
		13,724.0	4,018.6	BA 2.5	70.7%		
		13,724.0	2,481.4	KW 2.5	81.9%		
BS/BA 1. *Pseudomonas* sp., 2. *Brucella* sp., 3. *Bacillus* sp., 4. *Rhodococcus* sp., 5. *Microbacterium* sp., 6. *Roseomonas* sp., 7. *Rhizobiales* sp.	Crude oil-spiked soil	10,000	3,810	1	61.90	60	Zhao et al. (2011)

NA natural attenuation, *BS* biostimulation, *BA* bioaugmentation, *CT* control batch, *BSF* biosurfactants, *NPH* low-level nutrient addition, *NPL* high-level nutrient addition, *KW* kitchen waste

Table 2 Examples of pilot-scale and field-scale experiments for contaminated soil up to 20 MT in mass per batch

Treatment/cultures	Sources	TPH (mg kg^{-1})		Volume (MT)	Percentage degraded	Residence time (days)	References
		Initial	Final				
BS/BA; cultures were not specified	Bunker fuel-contaminated soil, 40 MT soil	13,009	1474	3 MT per batch, soil, windrowed + nutrient broth + Inoculum	88.67	28	Coulon et al. (2010)
BA; cultures were not specified		13,009	2604	3 MT per batch, soil, biopile + nutrient broth + inoculum	79.98		
BS/BA *Gordonia alkanivorans CC-JG39*, *Rhodococcus erythropolis CC-BC11*, *Acinetobacter junii CC-FH2* and *Exiguobacterium aurantiacum CC-LSH4-1*	Diesel- and fuel oil-contaminated 'S' soil	3,427	1757	20	48.73	28	Lin et al. (2010)
	Diesel- and fuel oil-contaminated 'T' soil	6,780	5215		23.08		
As above	'S' soil	3,427	<500	20	≈85	240	Lin et al. (2010)
BS/BA with *Rhodococcus ruber Em1*	PAH-contaminated soil, BA	364	266	5	26.82	175	Sun et al. (2012)
	PAH-contaminated soil, BS	364	240	5	33.90	175	
	PAH-contaminated soil, BA/BS	364	204	5	43.90	175	

NAT natural attenuation, *BS* biostimulation, *BA* bioaugmentation, *NB* nutrient broth/biostimulation

the experiment, which was further enhanced by BA. Coulon et al. (2010) inferred that while the microbial population in the control soils was nutrient limited, there was already a capable microbial population present (Atlas 1981; Coulon et al. 2004; Bamforth and Singleton 2005; Delille and Coulon 2008) as the control soil with only an indigenous population was capable of degrading the hydrocarbon without any further treatment, but at a slower rate. The application of BS/BA to the bunker fuel-contaminated soil at field scale showed an increased rate of biodegradation, with windrow turning shown to be more effective than biopiling. Windrowing was effective for contaminated soil, which was more friable, in comparison with coarser soil, which may be more amenable to biopiling (Coulon et al. 2010).

A comparative pilot-scale study was conducted on the bioremediation of soil heavily contaminated by PAH soil in outdoor pot trials using three approaches: BA with bioemulsifier-producing microbial strain, BS and a combined BS/BA approach. The results for the BA approach showed that the concentration of total PAHs and 4–6 ring PAHs was reduced by 26.82% and 35.36%, respectively; BS at 33.9% and 11.0%, respectively; and BS/BA at 43.9% and 55.0%, respectively. The results showed that the combination of BS and BA had the highest percentage removal of PAHs in the contaminated soil.

The batch volumes of the PHC contamination for those references in Table 3 ranged from 50 MT to 990 MT in translation and scale-up experiments. The composition of the PHC contamination in the soil was varied and included mixtures of diesel, engine oil and aircraft fuel (Łebkowska et al. 2011), PAH (Lors et al. 2012), heavy residual fuel oil 'mazut' (Beskoski et al. 2011). An ex situ field-scale bioremediation was conducted on 600 MT of heavy residual fuel oil (mazut)-polluted soil from an energy power plant using BA, BS and a combination of BS/BA with multiple reinoculation of microbial consortia isolated from the mazut-contaminated soil compared with biostimulation using added nutritional elements (N, P and K). The biopile was comprised of mechanically mixed polluted soil with softwood sawdust and crude river sand with aeration aided by systematic mixing and protected from direct external influences by a polyethylene cover. Part (10 m^3) of the material prepared for bioremediation was set aside uninoculated and maintained as an untreated control pile (CP). Biostimulation and reinoculation with zymogenous microorganisms increased the number of hydrocarbon degraders after 50 days by more than 20 times in the treated soil. During the 5 months, the TPH content of the contaminated soil was reduced to 6% of the initial value, from 5.2 to 0.3 g kg^{-1} dry matter, while TPH reduced to only 90% of the initial value in the CP. After 150 days there were 96%, 97% and 83% reductions for the aliphatic, aromatic and nitrogen-sulphur-oxygen and asphaltene fractions, respectively. The isoprenoids, pristane and phytane fractions were more than 55% biodegraded, which indicated that they were not suitable biomarkers for following bioremediation. (Beskoski et al. 2011). An extended large-scale biopile comparing multiple inoculations to single inoculation of soil with indigenous microorganisms with suitable controls, to different lots of soil contaminated with diesel oil and aircraft fuel, respectively, was performed in Poland (Łebkowska et al. 2011). It was concluded that bioremediation was 50% more effective than the non-inoculated controls and 30% more effective than soil

Table 3 Examples of pilot-scale and field-based experiments; up to 990 MT in mass per batch

Treatment/cultures	Sources	TPH (mg kg^{-1})		Volume (MT)	Percentage degradation	Residence time (days)	References
		Initial	Final				
BS/BA— cultures not specified (270 MT oily soil was mixed with 360 MT filler)	Heavy fuel oil (Mazut)	5200	300	613 (270 oily soil 360 filler)	94.23	150	Beškoski et al. (2011)
Bacillus sp. (two species including one producing BSF), *Pseudomonas mendocina*, *Pseudomonas putida*	Diesel oil- and engine oil-contaminated aged soil	2509	452	ABA 198 soil A	81.98	25	Łebkowska et al. (2011)
Bacillus sp. (two species), *Pseudomonas mendocina*, *Acinetobacter lwoffii*	Diesel oil-contaminated aged soil	5568	1084	ABA 198 soil B	80.53	65	
Bacillus sp. (two species), *Pseudomonas alcaligenes*, *Sphingomonas paucimobilis*, *Alcaligenes xylosoxidans*, *Comamonas testosteroni*	Aircraft fuel-contaminated aged soil	3336	81	ABA 198 soil C	97.57	22	
Pseudomonas Enterobacter	PAH-contaminated soil	2895	440	990	84.80	183	Lors et al. (2012)

BSF biosurfactants, *ABA* autochthonous bioaugmentation

that had only a single inoculation. As part of the soil preparation procedure, stones and bigger solid particles were removed with the soil particle size reduced to about 5 cm, with the bioremediation conducted ex situ in biopiles at average temperatures of 15–30 °C (Łebkowska et al. 2011). Bioremediation depends not just on the intrinsic biodegradability of PHC fractions but also the availability of hydrocarbons, the weathered state of the hydrocarbons and the properties of the soil which support the biodegradation of the hydrocarbon contaminants (Gallego et al. 2011). Another large-scale study compared 3 MT biopiles with winnowing for the bioremediation of soil contaminated with bunker C fuel oil and found that soil which had a heavy texture was effectively remediated by windrowing and that coarser textures may be more amendable to biopiling. The amendment of treatments with nutrients was found to have significantly increased the rate of degradation at the initial stages, which was further increased, with the addition of inocula (Coulon et al. 2010).

Controlled field trials of the bioremediation of soils contaminated with petroleum hydrocarbons found bioremediation to be ecologically sound with a quantifiable reduction in the ecotoxicity observed as the measured TPH decreased. Ecotoxicological analysis of field work on biopiles in an oil refinery in Poland showed an 81% reduction in TPH versus 30% in the untreated biopile accompanied by a marked reduction in toxicity in the former, based on toxicity analysis including Microtox and phytotoxicity bioassays (Płaza et al. 2005). This approach was also used to assess the extent to which soil contaminated with bunker C fuel bioaugmentated in biopiles was remediated. In this case, a combination of chemical analysis and bioassays, including phytotoxicity assays as well as ecotoxicity evaluation with earthworms, was employed (Coulon et al. 2010). A key finding of the study was that although the bioremediated soil showed a significant ecological recovery, it was still relatively impaired with respect to human risk criteria with a need to perform further comparative studies to better assess the relationship and relative sensitivity of receptor-based end points (Coulon et al. 2010).

Conclusions

This chapter discussed recent literature in regard to the bioremediation of petroleum hydrocarbons. The bioremediation of PHC-contaminated soil has been extensively investigated at bench scale under controlled laboratory conditions. While the general finding has been that the degradation of PHC-contaminated soil has been more amenable to bioaugmentation than to either biostimulation or natural attenuation, there are a number of key exceptions, confirming the need to laboratory trials to be used to optimize treatment. However, the laboratory trials must try to emulate filed conditions and the results reviewed with caution. The translation and scale-up of bioremediation operations in the field have, on occasion, failed to measure up to expectations. There have also been more recent instances where the fieldwork has shown bioremediation to be effective, with bioaugmentation combined with some form of biostimulation showing the most biodegradation, particularly when

performed ex situ. Recent developments in environmental microbiology, particularly next-generation sequencing, should play a key role in ensuring the commercial future of bioremediation technologies.

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Bioremediation of Polycyclic Aromatic Hydrocarbons-Polluted Soils at Laboratory and Field Scale: A Review of the Literature on Plants and Microorganisms

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Abstract The interrelationships between microbes and plants and the potential of utilizing these relationships to improve the dissipation of pollutants have been widely discussed during the last decades. However, to the best of our knowledge, there has been no prior study on the interrelationships between plants and microorganisms to degrade pollutants and shape a sustainable future. The characterization, identification, culturing, and management of plants and microorganisms suited for remediation techniques should be clearly defined, with the intention that the bioremediation techniques not only recover contaminated sites but also contribute to sustainable development and increasing social welfare. This chapter aims to provide the cutting-edge knowledge about the different biological interrelationships that are simultaneously taking place on a polluted site, prior, during, and after of the bioremediation strategies, taking into account and at the same time discussing the experimental findings at the laboratory and field scale by outstanding specialists.

Keywords Bioaugmentation • Biostimulation • Decontamination • Environmental Pollution • Phytoremediation • Sustainable Development

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Introduction

Living organisms such as plants, earthworms, and microorganisms have been recognized by their capacity to dissipate pollutants (Hong et al. 2015; Lu and Lu 2015; Xue et al. 2015). Some biochemical and physiological properties of these organisms are used to increase the dissipation of polycyclic aromatic hydrocarbons (PAHs) through biodegradation and bioremediation processes (Abbasian et al. 2015; Haritash and Kaushik 2009). Biodegradation is a natural way of recycling wastes or pollutants, which are usually used in relation to ecology, waste management, and mostly associated with bioremediation, a technology for environmental remediation. Bioremediation is defined as the treatment of pollutants or waste by the use of living organisms in order to eliminate, attenuate, degrade, transform, or break down (through metabolic or enzymatic action) the undesirable substances to inorganic components, such as CO_2, H_2O, and NO_3^- (Fernández-Luqueño et al. 2011; Pistelok and Jureczko 2014).

Organic pollution by PAHs is an increasing concern by the environmental scientists nowadays. Increasing concern for the environment has recently highlighted three major problems to resolved, namely, pollution, scarcity of resources, and unsustainable development of our societies. Pollution is defined as the introduction of elements, compounds, substances, or energy into the environment at concentrations that adversely alter its biological functioning or that present an unacceptable risk to humans or other targets that use or are linked to the environment (Fernández-Luqueño et al. 2011; Okparanma and Mouazen 2013; Berezina et al. 2015). In addition, PAHs pollution is a cause of many human and environmental health-related problems.

PAHs are organic molecules that often contaminate water (Fernández-Luqueño et al. 2013a; Leonov and Nemirovskaya 2011; Vodyanitskii 2014), soil (Alagic et al. 2015; Chen et al. 2015; Ibrahim et al. 2015; Wloka et al. 2015), sediments (Hall et al. 2011; Meng et al. 2015), and air (Ma and Harrad 2015; Szulejko et al. 2014). Although several hundred PAHs exist, most studies have been focused on a limited number of them, the so-called 16 EPA priority PAHs, seven of them might be mutagenic, carcinogenic, and teratogenic (Keith 2015). In the natural environment, the PAHs undergo transformations involving both biotic and abiotic processes such as volatilization, adsorption, photolysis, chemical oxidation, and the microbial degradation, among others. However, plants and microbial activities make up the primary pathway for PAHs removal from the environment (Fig. 1).

Recently, different papers have reviewed the biodegradation and bioremediation of soil, water, and air polluted with PAHs, e.g., Fernández-Luqueño et al. (2011), Abbasian et al. (2015), Alagic et al. (2015), and Xue et al. (2015). However, until now, there have been no reviews summarizing the relationship between microbial and vegetal populations under different PAHs-polluted ecosystems in order to enhance the degradation of PAHs, while the main biotechnological challenges to increase the biodegradation of PAHs at laboratory and field scale have neither been published. The objective of this chapter is to provide

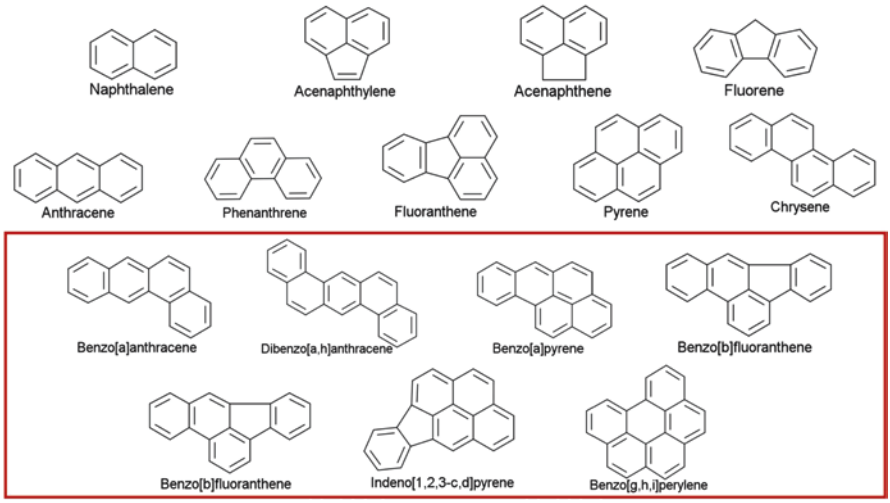

Fig. 1 List of 16 EPA priority polycyclic aromatic hydrocarbons (in the *red* box, there are seven PAHs that might be mutagenic, carcinogenic, and/or teratogenic)

the cutting-edge knowledge about the different biological interrelationships that are simultaneously taking place on a polluted site, prior, during, and after of the bioremediation strategies, taking into account and at the same time discussing the experimental findings at the laboratory and field scale by outstanding specialists.

Plants and Microorganisms Suited for Remediation Techniques

Pollution of soil, water, sediments, and air by PAHs is a common phenomenon across the globe, which may pose a great threat to the environment and human being at large. Different treatment methods have been employed to reclaim contaminated soils, water bodies, or air nowadays. However, plants and microorganisms have been recognized by their potential to dissipate PAHs within a very narrow range of climates and physical and biochemical characteristics of polluted substrates (soil, sediments, water, and air), e.g., Fernández-Luqueño et al. (2011) and Yavari et al. (2015).

Phytoremediation is a strategy that employs plants to degrade, stabilize, and/or remove PAHs, which can be an alternative green technology method for remediation of PAHs-polluted soils, water, and air. Phytoremediation, as a green technology option, is defined as the use of plants to remove pollutants from the environment or to render them harmless. This technique includes seven main strategies such as (Fig. 2):

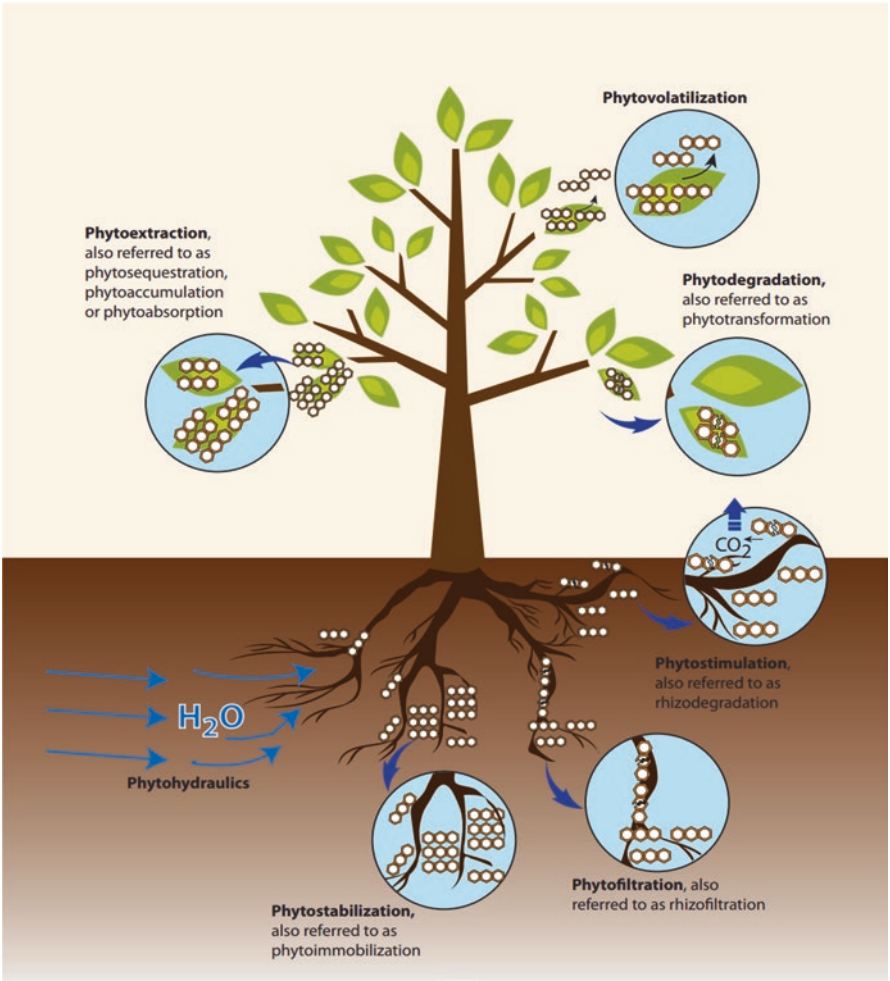

Fig. 2 Main strategies used to remediate contaminated soils, sediments, air, and water bodies

- Phytoextraction, also referred to as phytosequestration, phytoaccumulation, or phytoabsorption: plants remove PAHs from the soil and concentrate them in the harvestable parts of plants (Jiao et al. 2015).
- Phytodegradation, also referred to as phytotransformation: plants break down PAHs into simpler compounds that are integrated with plant tissue, which in turn, foster plant growth (Al-Baldawi et al. 2015).
- Phytofiltration, also referred to as rhizofiltration: plants and/or roots absorb, adsorb, concentrate, and/or precipitate PAHs. It involves filtering water through a mass of tissues to remove toxic substances or nutrients (Lee 2012).

- Phytohydraulics: this process is used to limit the movement of contaminants with water. Plants are used to increase evapotranspiration, thereby controlling soil water and contaminant movement (Hong et al. 2001).
- Phytostabilization, also referred to as phytoimmobilization: plants reduce the mobility and bioavailability of pollutants in the environment either by immobilization or by prevention of migration (Pulford and Watson 2003; Masu et al. 2014).
- Phytostimulation, also referred to as rhizodegradation: process where roots release compounds in order to enhance microbial activity in the rhizosphere through the rhizospheric associations among plants and symbiotic soil microorganisms (Gartler et al. 2014).
- Phytovolatilization: plants increase the volatilization of pollutants into the atmosphere via themselves through its ability to take up, translocate, and subsequently transpire volatile contaminants (Shiri et al. 2015).

It is well known that the plants may use more than one strategy of the abovementioned simultaneously during a common phytoremediation process. In addition, there are other strategies to improve the environmental quality and remove pollutants using plants, which are categories or variations of the abovementioned strategies. These include constructed wetlands, hydraulic barriers, phytodesalination, and vegetation covers.

Phytoremediation has now emerged as a promising strategy for in situ removal of many contaminants, while microbe-assisted phytoremediation including rhizoremediation appears to be particularly effective for the removal and/or degradation of organic contaminants from PAHs-polluted substrates (Zawierucha et al. 2014; Chen et al. 2016). Furthermore, root exudates from plants do help to dissipate PAHs and act as substrates for soil microorganisms, which result in increased rate of PAHs biodegradation. It has to be remembered that the strategies chosen for a phytoremediation project depend on the contaminant level, contaminant properties, and the contaminated matrix (Fig. 3).

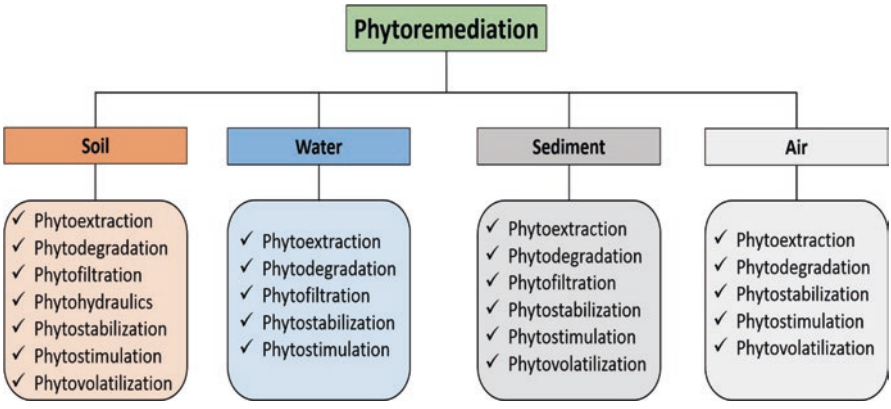

Fig. 3 Application of phytoremediation strategies as a function of the matrixes (such as soil, water, sediments, and air)

Different plants and crops have been found useful for phytoremediation of PAHs-polluted substrates (Table 1). Phytoremediation is a particularly useful in wetland environments because it uses plants and their associated microorganisms to recover PAHs-polluted soil and water (Table 2). Plant-associated rhizobacteria are involved in the PAHs degradation in contaminated substrates, while the plants themselves have the potential to enhance the rhizobacteria population. It is well known that many studies have been concentrating on the plant-microorganism interaction in phytoremediation, where the presence of autochthonous microorganisms can enhance the remediation efficiency of plants.

It has to be remembered that Macek et al. (2000) stated some advantages and disadvantages of phytoremediation. The main advantages of phytoremediation in comparison with classical remediation methods can be summarized as follows: (i) it is far less disruptive to the environment, (ii) there is no need for disposal sites, (iii) it has a high probability of public acceptance, (iv) it avoids excavation and heavy traffic, (v) it has potential versatility to treat a diverse range of hazardous materials, and (vi) it is cheaper than other techniques. However, the use of phytoremediation is also limited by the climatic and geological conditions of the site to be cleaned, temperature, altitude, soil type, and accessibility by agricultural equipment.

According to Macek et al. (2000), phytoremediation also has some disadvantages such as:

1. Formation of vegetation may be limited by extremes of environmental toxicity.
2. Contaminants collected in leaves can be released again to the environment during litter fall.
3. Contaminants can be accumulated in fuel woods.
4. The solubility of some contaminants may be increased, resulting in greater environmental damage and/or pollutant migration.
5. It may take longer than other technologies.
6. The plant biomass may require additional management prior to final disposition.
7. It may need the use of plants or microorganisms transgenic.
8. It requires technicians with strong academic skills about phytoremediation and about their economic, social, and environmental implications.

In addition, according to Eapen and D'Souza (2005), a plant suitable for phytoremediation should possess the following characteristics: (i) ability to tolerate, accumulate, or degrade pollutants in their aboveground parts, (ii) tolerance to pollutants concentration accumulated, (iii) fast growth and high biomass, (iv) widespread highly branched root system, and (v) easy harvestability.

Regarding the interactions among plants and indigenous rhizobacteria, Fernández-Luqueño et al. (2011) and Chen et al. (2016) stated that microbe-assisted phytoremediation has been well documented in scientific literature so that there is enough evidence to state that microbe-assisted phytoremediation has potential as an effective and inexpensive technique for removal, degradation, or dissipation of organic pollutants from polluted systems such as soils, water bodies, or air.

Table 1 Plants and/or crops used to phytoremediation, their rates of degradation, and the additional benefits

Plant	Degraded pollutant	Rate of PAHs degradation/ dissipation	Additional benefits	Reference(s)
Azolla caroliniana Willd.	Phenanthrene	80% in 49 days	Bioenergy production	Castro-Carrillo et al. (2008)
Bassia scoparia (L.) A.J.Scott	Crude oil (TPHs)	31.2–57.7% for natural soil and 28.7–51.1% for pre-sterilized soil after 5 months	Soil erosion control	Moubasher et al. (2015)
Bidens maximowicziana Oett.	Pyrene	79% in 50 days	Soil erosion control	Lu et al. (2010a)
Bidens pilosa L.	Crude oil (TPHs)	9% in 64 days	Soil erosion control	Kuo et al. (2014)
Brassica juncea (L.) Czern.	Pyrene	67% in 60 days	Can accumulate Cu	Chigbo and Batty (2013)
Brassica napus L.	Pyrene	30% in 90 days	Forage	D'Orazio et al. (2013)
Chromolaena odorata (L.) R.M. King & H. Rob.	Crude oil	80% in 180 days	It can degrade to 65% of HM in 180 days	Atagana (2011)
Cyperus brevifolius L.	Crude oil (TPHs)	61.2–86.2 in 360 days	Soil erosion control	Basumatary et al. (2012)
Echinacea purpurea (L.) Moench	Fluoranthene, pyrene, benzo(a) anthracene, chrysene, benzo(b) fluoranthene, benzo(k)fluoranthene, benz(a)pyrene, and dibenzo(a,h) anthracene	The removal rate of $\sum 8$ PAHs was 92.92% in 50 days	Soil erosion control	Liu et al. (2014a)
Eichhornia crassipes (Mart.) Solms	Naphthalene	66% after 5 h of experimental setup	Soil erosion control	Nesterenko-Malkovskaya et al. (2012)
Eleusine indica (L.) Gaerth.	PAHs	32% in 5 months	Soil erosion control	Lu et al. (2010b)
Festuca arundinacea Schreb.	PAHs	84% in 7 months	Soil erosion control	Soleimani et al. (2010)
Festuca pratensis Huds.	PAHs	64–72% in 7 months	Soil erosion control	Soleimani et al. (2010)

(continued)

Table 1 (continued)

Plant	Degraded pollutant	Rate of PAHs degradation/ dissipation	Additional benefits	Reference(s)
Fimbristylis littoralis Gaudich.	PAHs	92% in 90 days	Degrade 96% of as in 90 days	Oluchi-Nwaichi et al. (2015)
Fire Phoenix (is a combined Poaceae species including *Festuca arundinacea* Schreb., *Festuca elata* Keng ex E. Alexeev, and *Festuca gigantea* (L.) Vill.)	Fluoranthene, pyrene, benzo(a) anthracene, chrysene, benzo(b) fluoranthene, benzo(k)fluoranthene, benz(a)pyrene, and dibenzo(a,h) anthracene	The degradation rate of Σ8PAHs was 99.40% after 150 days	Soil erosion control	Liu et al. (2014b), Xiao et al. (2015)
Impatiens balsamina L.	TPHs	18.13–65.03% after 4 months	Soil erosion control	Cai et al. (2010)
Jatropha curcas L.	Lubricating oil (TPHs)	89.6–96.6% in 180 days	Bioenergy production	Agamuthu et al. (2010)
Juncus roemerianus Scheele	PAHs	84–100% in 1 year	Can degrade *n*-alkanes	Lin and Mendelssohn (2009)
Juncus subsecundus N.A.Wakef.	Phenanthrene and pyrene	97% and 43–63% after 10 weeks, respectively	Can accumulate Cd	Zhang et al. (2012)
Kandelia candel (L.) Druce	Phenanthrene and pyrene	56.8 and 47.7% after 60 days, respectively	Soil erosion control	Lu et al. (2011)
Lolium multiflorum Lam.	TPHs	59% in 80 days	Forage	Alarcon et al. (2008)
Lolium perenne L.	Pyrene	28% in 90 days	Livestock feed (forage)	D'Orazio et al. (2013)
Luffa acutangula (L.) Roxb.	Anthracene and fluoranthrene	98.2–98.9% and 85.9–96.9% in 45 days, respectively	Due to its fibrous characteristics, (the fruit) is used as exfoliating	Somtrakoon et al. (2014)
Medicago sativa L.	Pyrene, fluoranthene, benzo(a) anthracene, chrysene, benzo(b) fluoranthene, benzo(k)fluoranthene, benz(a)pyrene, and dibenzo(a,h) anthracene	32% removal of pyrene in 90 days and a removal rate of 98.11% for the ∑8PAHs in 150 days	Livestock feed (forage)	D'Orazio et al. (2013), Xiao et al. (2015)

Mirabilis jalapa L.	TPHs	41.61–63.20% in 127 days	Soil erosion control	Peng et al. (2009)
Onobrychis viciifolia Scop.	Pyrene and phenanthrene	74.1% and 85.02%, respectively, in 120 days	Forage	Baneshi et al. (2014)
Potamogeton crispus L.	Phenanthrene and pyrene	18.3–34.1% and 14.1–27.8%, respectively, in 54 days	Soil erosion control	Meng and Chi (2015)
Sagittaria trifolia L.	Diesel	54–85% in 50 days	Soil erosion control	Zhang et al. (2015)
Salix rubens Schrank	PAHs	98.56% in 36 months	Soil erosion control	Da Cunha et al. (2012)
Salix triandra L.	PAHs	98.65% in 36 months	Soil erosion control	Da Cunha et al. (2012)
Scirpus grossus L.f.	TPHs	66.6–81.5% in 72 days	Soil erosion control	Al-Baldawi et al. (2015)
Sorghum sp.	Pyrene and phenanthrene	73.84% and 85.2%, respectively, in 120 days	It used for human consumption and forage	Baneshi et al. (2014)
Tagetes patula L.	Benzo(a)pyrene	78.2–92.9% after 92 days	Can accumulate heavy metals	Sun et al. (2011)
Trifolium repens L.	Pyrene	77% after 60 days	Forage	Xu et al. (2009)
Triticum sp.	Pyrene and phenanthrene	65–70% and 98–100% in 90 days, respectively	Use of food	Shahsavari et al. (2015)
Vallisneria spiralis L.	Phenanthrene and pyrene	53.3–59.6% and 50–53.6% in 54 days, respectively	Soil erosion control	Liu et al. (2014c)
Zea mays L.	PAHs (crude oil)	52.21–72.84% in 60 days	Use of food	Liao et al. (2015)
Zostera marina L.	PAHs	73% after 60 weeks of treatment	Can degrade PCBs (polychlorinated biphenyls)	Huesemann et al. (2009)

Table 2 Microorganisms, identified in phytoremediation strategies, their rates of PAHs degradation, and the additional benefits

Microorganism	Degraded pollutant	Rate of PAHs degradation/ dissipation	Additional benefit	Reference(s)
Acinetobacter sp.	Phenanthrene and pyrene	90% of phenanthrene and 50% of pyrene in 6 days	It can degrade to 90% of fluorine	Shao et al. (2015)
Bacillus sp.	Diesel	74% in 60 days	Biohydrogen production	Cisneros- de La Cueva et al. (2014), Liu et al. (2015)
Cycloclasticus sp.	Phenanthrene, pyrene, and fluoranthene	98%–99% in 10 days and 52–63% and 49–65% in 21 days, respectively	Unreported	Cui et al. (2014)
Cycloclasticus sp. in association with *Marinobacter* sp.	Pyrene and fluoranthene	63–76% and 65–83% in 21 days, respectively	*Marinobacter* sp. is used for the production of α-amylases	Cui et al. (2014)
Halomonas sp. +Marinobacter sp.	Phenanthrene	90% in 12 days	*Halomonas* sp. has a high capability of bioremediation, due to they can be used as catalysts in different processes	Dastgheib et al. (2012)
Martelella sp.	Phenanthrene	100% in 6 days	Considered as PGPR	Feng et al. (2012)
Micrococcus sp.	Phenanthrene	99% in 21 days	They can degrade industrial substrates such as pyridine, herbicides, and polychlorinated biphenyls	Dellagnezze et al. (2014)
Ochrobactrum sp.	Anthracene, phenanthrene, naphthalene, fluorene, pyrene, benzo(k) fluoranthene, and benzo(e) pyrene	88, 98, 90, 97, 84, 57, and 50%, respectively, within 4–5 days	Considered as PGPR	Arulazhagan and Vasudevan (2011)
Ochrobactrum sp.	Phenanthrene	90% in 7 days	Considered as PGPR	Chang et al. (2011)
Penicillium sp.	Benzo(a)pyrene	83.84% in 5 days	Production of penicillin	Machin-Ramirez et al. (2010)
Pseudomonas sp.	PAHs and benzo(a)pyrene	3–5% of PAHs in 1 day and 12.73% of benzo(a)pyrene in 5 days	Considered as PGPR	Brito et al. (2015), Parray et al. (2015), Machin-Ramirez et al. (2010)

Pseudoxanthomonas sp.	Phenanthrene	100% within 120 h under optimized conditions	Unreported	Patel et al. (2012)
Rhodococcus sp.	Naphthalene and phenanthrene	100% and phenanthrene, respectively, within 9–11 days	Can degrade linear alkanes and branched alkanes	Yang et al. (2014)
Staphylococcus sp.	Phenanthrene	90% in 3 days	Unreported	Chang et al. (2011)
Streptomyces sp.	Naphthalene	81.03–85.23% in 12 days	Production of antibiotics	Ferrafji et al. (2014)
Achromobacter xylosoxidans	Fluoranthene	90% after 14 days	Unreported	Ma et al. (2015)
Acinetobacter venetianus	Diesel	>95% in 60 h	Has industrial applications for the production of the bioemulsifier emulsan	Lin et al. (2015)
Aspergillus niger	Benzo(a)pyrene	45% in 5 days	Used for the production of enzymes and chemicals	Machin-Ramirez et al. (2010)
Aspergillus sclerotiorum	Pyrene and benzo(a) pyrene	99.7% and 76.6% after 8 and 16 days, respectively	Unreported	Passarini et al. (2011)
Bacillus mycoides	Benzo(a)pyrene	27.06% after 5 days	They are in common pesticides and used to inhibit the growth of harmful bacteria and fungi	Machin-Ramirez et al. (2010)
Cladosporium cladosporioides	Pyrene and benzo(a) pyrene	42.1% and 45.3% after 8 and 16 days, respectively	Unreported	Passarini et al. (2011)
Dietzia maris	Phenanthrene	63% in 21 days	Production of canthaxanthin	Dellagnezze et al. (2014)
Ensifer meliloti (before *Sinorhizobium meliloti)*	Phenanthrene	46.3% in 5 days	Considered as PGPR	Muratova et al. (2014)
Fusarium solani	Pyrene	64.1–70.7% after 2 weeks	Acts as degrading plant material in soil	Hong et al. (2010)
Ganoderma lucidum	Phenanthrene and pyrene	>95% in 6 days	Has been investigated extensively for its pharmaceutical applications	Ting et al. (2011)
Hypocrea lixii	Pyrene	69.4% after 2 weeks	Improves plant growth	Hong et al. (2010)
Kocuria flava	Naphthalene	53% in 10 days	Considered as PGPR	Ahmed et al. (2010)
Kocuria rosea	Naphthalene and phenanthrene	36% and 9%, respectively, in 10 days	They can degrade bird feathers	Ahmed et al. (2010)

(continued)

Table 2 (continued)

Microorganism	Degraded pollutant	Rate of PAHs degradation/ dissipation	Additional benefit	Reference(s)
Methylobacterium populi	Phenanthrene	27% in 20 days	Considered as PGPR	Ventorino et al. (2014)
Mucor racemosus	Pyrene and benzo(a) pyrene	33.8$ and 51.7% after 8 and 16 days, respectively	It can be used in the fermentation for the production of bioethanol	Passarini et al. (2011)
Mycobacterium goodii	PAHs	3–4% day^{-1}	Unreported	Brito et al. (2015)
Novosphingobium pentaromativorans	Phenanthrene, pyrene, and benzo(a)pyrene	86.62% of the phenanthrene in 24 h, 31.81% of pyrene in 36 h, and 22.18 of benzo(a)pyrene in 48 h	Unreported	Lyu et al. (2014)
Penicillium commune	Industrial oil (PAHs)	95.4 in 5 days	Production of α-amylases	Esmaeili and Sadeghi (2014)
Pseudomonas aeruginosa	PAHs	100% degraded in 4 weeks	Considered as PGPR and biocontrol agent	Patowary et al. (2015), Goswami et al. (2015)
Rhizobium tropici	Phenanthrene and benzo(a)pyrene	50% and 45%, respectively, after 120 h	Contribute to biological nitrogen fixation	Gonzales-Paredes et al. (2013)
Saccharomyces cerevisiae	Benzo(a)pyrene	46% in 5 days	Industrial use	Machin-Ramirez et al. (2010)
Serratia marcescens	Benzo(a)pyrene	32.41% after 5 days	Unreported	Machin-Ramirez et al. (2010)
Sphingomonas koreensis	Naphthalene, phenanthrene, anthracene, and pyrene	100, 99, 98, and 92.7% within 15 days, respectively	Unreported	Hesham et al. (2014)
Trichoderma asperellum	Phenanthrene, pyrene and benzo(a)pyrene	74.4, 62.63 and 80.94% in 18 days, respectively	Biocontrol agent	Zafra et al. (2015)
Trichoderma harzianum	Benzo(a)pyrene	77% in 5 days	Biocontrol agent	Machin-Ramirez et al. (2010)
Trichoderma longibrachiatum	Phenanthrene	90% in 14 days	Used in biological control	Cobas et al. (2013), Zhang et al. (2014)
Trichoderma viride	PAHs	47% after 12 months of treatment	Biocontrol agent	Szczepaniak et al. (2015)
Yarrowia lipolytica	Crude oil (TPHs)	58–68% in 7 days	Production of lipases with application in biotechnology	Hassanshahian et al. (2012)

Biological Interrelationships Between Plant and Microorganisms in a Polluted Site: Insights into Prior, During, and After of the Bioremediation Strategies

For more than 120 years, the biological interrelationship among plants and microorganisms has been studied. However, the remediation techniques are not older than 30 years. Nevertheless, more and more studies have demonstrated the remediation's potential to recover polluted systems, which are becoming major environmental and human health concerns worldwide.

Lynch and Moffat (2005) were the first to use the term "phytobialremediation" in order to redefine phytoremediation assisted by microorganisms. Recently, it has been reported that plants and microorganisms help each other in the whole process of phytoremediation throughout phytobialremediation, which may be improved with transgenic technologies. Phytobialremediation is a technique, which can be carried out by free-living microorganisms or by symbiotic microbes, which live in the rhizosphere. In addition, it has to be remembered that plant microbial symbionts may constitute the "unseen majority" in phytoremediation of organic compounds (Fester et al. 2014). The rhizosphere is the microecological zone surrounding plant roots, i.e., it is a narrow region of soil that is directly influenced by root secretions and associated soil microbes. In the rhizosphere, the roots release a number of compounds establishing a highly dynamic and active microbial community distinctly different from the bulk soil microbial community. The exudates compounds increase contact among plant roots and the surrounding soil and prevent dehydration during dry spells. The functions of the plant root system include anchorage, the absorption of water and mineral nutrients, synthesis of various essential compounds, and the storage of food. Furthermore, the plant root system aerates the soil and provides a steady-state redox environment and a starting material for colonization of plant growth-promoting rhizobacteria (PGPR). PGPR are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms such as degradation of pollutants, phosphate solubilization, siderophore production, biological nitrogen fixation, antifungal activity, and induction of systemic resistance, among others (López-Valdez et al. 2015).

Chen et al. (2016) studied the potential of interplanting a Zn/Cd hyperaccumulator plant (*Sedum alfredii* L.) with a rhizospheric mediator (perennial ryegrass, *Lolium perenne* L.) for remediation of an actual wastewater-irrigated soil co-contaminated with PAHs and heavy metals in a 2-year greenhouse experiment, using *Microbacterium* sp. strain KL5 and *Candida tropicalis* strain C10. They found that the highest efficiency of PAHs removal, PAHs mineralization, and metal phytoextraction was obtained by interplanting ryegrass with *S. alfredii* associated with regular reinoculation with strain KL5 and C10 in the contaminated soil. Additionally, they reported that microbial inoculation promoted soil enzyme activity, PAHs removal, plant growth, and metal phytoextraction. Their date from qPCR and high-throughput sequencing suggest that reinoculation was necessary for the long-term remediation practice, and plants especially ryegrass were beneficial for PAHs

degraders (Chen et al. 2016). As already explained, it has to be remembered that PAHs degradation in soils is dominated by bacterial and fungal strains belonging to a wide number of taxonomic groups (Fernández-Luqueño et al. 2011), i.e., it is well known that degradation/dissipation rates are strongly influenced by a wide number of soil microbial communities. Fernández-Luqueño et al. (2013b) studied the dynamics of the bacterial community composition, i.e., the diversity and abundance of microbial soil communities through PCR-DGGE of 16S rDNA gene fragments from a saline-alkaline soil polluted with PAHs. They found in a 56-days experiment that some microbial communities were harbored in the nine studied treatments. In addition, they found that the number of ribotypes increased in an alkaline-saline soil amended with wastewater sludge and spiked with phenanthrene and anthracene. Aertsen and Michiels (2005) noted similar results in a soil polluted with PAHs. They showed that both microorganism prokaryotes and eukaryotes possess mechanism that generates genetic and phenotypic diversity upon encountering stress such as PAHs spill.

Fernández-Luqueño et al. (2011) stated that the cutting-edge knowledge in the molecular genetics of plant and microorganisms and the knowledge-based methods of rational genetic modification suggest the possibility to develop plants and/or microorganisms that could decontaminate environments. The genomics and genetic engineering are the main biotechnological techniques to achieve this. Plants and microorganisms naturally respond differently to various kinds of stresses and gain fitness in the polluted environment. However, applying genetic engineering techniques can accelerate this natural process, but it has to be taken into account that ethical and social concerns are important. In addition, it has to be remembered that during the last several decades, plants and microorganisms have been widely investigated as unconventional systems for getting faster production of consumer goods and additional benefits. In genetic transformation processes, the gene of interest of donor plants, microorganisms, or viruses is transferred to host plants using methods such as *Agrobacterium* mediation, bombardment/biolistics, electroporation, a silicon-carbide fiber-based technique, polyethylene glycol-mediated protoplast fusion, and liposome-mediated gene transfer, among others. To date, transgenic plants have been engineered for the following purposes: to increase their tolerance to abiotic and biotic stresses, to improve the nutrient uptake, to reduce the effect of harmful agrochemicals, to increase their yield (grain production, growth rate, and biomass production), to increase the symbiotic interaction ships with soil microorganism, to increase the tolerance to pollutants, and to be used during phytoremediation processes (Abiri et al. 2016). Kotrba et al. (2009) published a review in which they summarize the state of the art on phytoremediation with genetically modified plants. Hannula et al. (2014) stated that the impact of genetically modified plants on natural or agricultural ecosystems showed that specific effects of single transformation events should be tested on a case-by-case basis in a natural setting where the baseline factors are all taken into consideration. In addition, Fernández-Luqueño et al. (2011) suggested that care should then be taken that the genetically modified microorganisms and plants do not outcompete the native ones or that negative traits spread through the soil microbial population.

Therefore, the environmental risk is latent when genetically modified microorganisms and plants are released in the environment in order to phytoremediate natural systems polluted with PAHs. New techniques such as stable isotope probing experiments, high-throughput sequencing, and meta-transcriptomics should be used in parallel with carefully designed field experiments considering a holistic review of the different individual reactions that are simultaneously taking place during the phytoremediation and that should be source of additional effects on the subsequent plant and microorganism species.

Increasing Social Welfare Throughout Remediation

Phytoremediation will become more economically feasible if the harvestable plant biomass results in financial returns (Mench et al. 2010). However, agronomic constraints, such as problems with crop rotation, climate, soil quality, and culture, must be considered. According to Mench et al. (2010), the commercial viability of a phytoremediating crop, which depends on total revenue (minus nonlabor variable costs) earned on the area to be cleaned up and calculated over an appropriate time period, is not decreased from what would be earned by conventional agricultural production. Decision making by the stakeholder must be assisted by a "cost-benefit analysis" accounting for the timely evolution of costs and benefits of phytoremediation. In addition, Mench et al. (2010) stated that assuming a predefined time period for the remediation, a cost-benefit approach could distinguish the cost of the phytoremediation action, capital, and operational costs connected with the contaminant removal, performance of the remediation crop, the soil or water conditions, and the difference between initial and target levels of contamination. Taken as a whole, these determine: (i) the remediation timescale, (ii) the income loss generated by the contaminated matrix, (iii) the potential income through biomass valorization, and (iv) the projected income from the remediated matrix, determined by its functional use (Mench et al. 2010; Ciesielczuk et al. 2014). However, the economics of phytoremediation is frequently favorable, but financial returns from produced biomass and element recycling have yet to be optimized. In addition, strategies for phytoremediation have to be relied on sustainable development, because environment protection does not preclude economic development, and economic development is ecologically viable today and in the long run.

Phytoremediation appears to be a feasible approach for cleaning contaminated matrix with PAHs, but technical hindrances have to be overtaken to shape a sustainable future throughout remediation techniques. In addition, a widespread lack of awareness among governments and societies about the current scale, pervasiveness, and risk to billions of people from environmental contamination hinders the establishment of strategies to stop/reduce the PAHs pollution. However it has to be remembered that phytoremediation is an efficient and cheap technique, but it is not free. Finally, site decontamination should be regarded as integral to bioeconomy and sustainability goals.

Conclusions and Perspectives

A substantially large body of information on the potential of phytoremediation for cleaning up the environment has been gathered together. Here we summarize the gained experience, which has helped to prove the suitability of plants and microorganism to remediate polluted environments. However, it has to be remembered that many technical hindrances currently limit the efficiency of phytoremediation. In addition, it has to be taken into account that to protect human health and the environment is necessary to develop and to promote innovative cleanup strategies that restore polluted sites/matrix to incorporate them to a productive use and promote the environmental stewardship and the sustainable development. Sharing scientific knowledge and technologies for assessing, cleaning, and preventing contamination is necessary, but the lack of environmental education in our society is evident, while the universities and research centers have the commitment of preparing young engineers with strong academic skills to address and decontaminate the increasingly polluted environment. We must not forget that the multidisciplinary nature of assessment and cleanup of polluted sites requires a complex and costly team of experts.

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Organic Micropollutants in the Environment: Ecotoxicity Potential and Methods for Remediation

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Abstract The fate, occurrence, and ecotoxicological significance of organic micropollutants (OMPs) in the environment are a relatively recent challenge faced by societies. The continuous discharge of these pollutants, without any regulatory measures, may cause environmental concerns even at their low concentrations. Recent studies have reported the fate of many OMPs in the environment along with their ecotoxicological potential. While acute toxicity due to OMPs is considered unlikely at environmental concentrations, the chronic exposures may cause damages to biotic elements of ecosystem at large. In this review, we are discussing exposure pathways with particular emphasis on the role of aquatic and terrestrial plants in bioaccumulation, associated potential risks, and remedies for the abatement of OMPs and their metabolites. Further negligence on behalf of concerned authorities and scientific community may lead to unwanted consequences if proper measures are not taken. These measures start with the further development and adoption of sensitive, robust methods to detect and analyze OMPs in the environment. To assess potential risks and hazards of OMPs and their metabolites, methods must be devised to generate data on their usage, environmental persistence, and mobility. Next, strategies must be devised for risk assessment of biologically active toxins within the

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class of OMPs. A consensus ought to be reached to adopt a multi-trophic environmental risk assessment (ERA) regime, as is recommended for ERA of synthetic pesticides and insecticidal proteins produced in transgenic crops.

Keywords Environmental pollution • Organic micropollutants • Ecotoxicity • Remediation

Introduction

Since World War II, global production of anthropogenic chemicals has increased from 1 to 400 million tons. Harmful effects of several of these chemicals, especially persistent organic pollutants (POPs), have been observed and investigated in some and even considerable detail since the publication of *Silent Spring* by Rachel Carson in 1962. Currently, increasing attention is being paid toward understanding the fates and effects of organic micropollutants (OMPs) in the ecosystem (Gavrilescu et al. 2015). OMPs is an operational definition for a group of compounds, which are not covered by existing water quality regulations due to their lower concentrations (i.e., ng/L up to μg/L) but are thought to be potential threats to environmental ecosystems (Farré et al. 2008a, b). The group includes more than 20 classes, which are found in the European aquatic environment (http://www.norman-network.net) (Geissen et al. 2015). The prominent classes are pharmaceutically active compounds, personal care products (PCPs), endocrine-disrupting chemicals (EDCs), pesticides, industrial chemicals, disinfection by products (DBPs), perfluorinated compounds (PFCs), additives, preservatives, detergents, surfactants, flame retardants, plasticizers, and their transformation products (Ojajuni et al. 2015) (Table 1).

As of today, many of the First World countries have been able to reduce the overall concentration of POPs in the environment by adopting appropriate legal measures (Jones et al. 2005); hence, the focus has been subsequently shifted to this new class of contaminants that may harm biotic elements (Larsen et al. 2004). It is not that the pollutants have been only most recently introduced into the environment but the modern developments in analytical procedures have made it possible to detect them despite their low concentrations in the environment (Brack et al. 2015; Gavrilescu et al. 2015; Guibal et al. 2015). Their significance in the environment is not necessarily due to persistency but rather to their active biological nature together with their continuous emission, which renders them "emerging contaminants" or "prospective pollutants" (Bueno et al. 2012; Murray et al. 2010).

Thousands of OMPs and their metabolites have been detected in water bodies around the world (Escher et al. 2014). While their presence at low concentrations is unlikely to cause acute toxicity, it is suggested that their long-term presence may lead to chronic health concerns (Schriks et al. 2010). According to a study conducted in Germany, 55 active pharmaceuticals and 9 metabolites were found in the wastewater of 49 sewage treatment plants at concentrations up to several μg/L (Ternes et al. 2002).

Table 1 Summary of major organic micropollutants (OMPs), their class, mode of entry, fate, and major examples

OMPs	Class	Mode of entry	Fate	Examples
Human pharmaceuticals and veterinary drugs	Antibiotics	Excretion, hospital discharges/disposal, accidental spills, and farmland waste	Wastewater treatment plants, rivers, streams, groundwater (after leaching)	Amoxicillin, cefazolin, chlortetracycline, ciprofloxacin, coprofloxacin, erythromycin, doxycycline, lincomycin, sulfamethoxazole, ofloxacin, norfloxacin, oxytetracycline, penicillin, tetracycline, trimethoprim
	Analgesics			Acetaminophen, acetylsalicylic acid, codeine, dipyrone, diclofenac, indomethacin, ibuprofen, fenoprofen, fluoxetine, ketoprofen, mefenamic acid, paracetamol, naproxen, propyphenazone, salycilic acid
	Antidiabetics			Glibenclamide
	Psychiatric drugs			Carbamazepine, diazepam, gabapentin, primidone, phenytoin, salbutamol
	Blood lipid regulators			Atorvastatin, bezafibrate, clofibric acid, etofibrate, fenofibric acid, gemfibrozil, pravastatin
	X-ray contrast agents			Iopromide, iopamidol, diatrizoate
	Cardiovascular drugs (β-blockers)			Atenolol, metoprolol, propranolol, solatolol, timolol, sotalol, atenolol
	Drugs of abuse			Amphetamine, cocaine, tetrahydrocannabinol
	Veterinary drug			Flunixin
Endocrine disruptive chemicals (EDCs)	Steroids and hormones		Soil and groundwater	Androstenedione, estradiol, estriol, estrone, diethylstilbestrol, ethinylestradiol, progesterone, testosterone

(continued)

Table 1 (continued)

OMPs	Class	Mode of entry	Fate	Examples
Personal care products (PPCPs)	Antiseptics	Shower waste and direct disposal of industrial waste	Wastewater treatment plants, terrestrial runoff freshwater, marine, estuarine environments and sediment	Triclosan, chlorophene
	Fragrances and synthetic musks			Galaxolide, musk ketone, nitro, polycyclic, and macrocyclic musks; phthalates, tonalide
	Stimulants			Caffeine
	Antihypertensive			Diltiazem
	UV Filters			Benzophenone, methylbenzylidene camphor
	Insect repellents			N,N-diethyltoluamide
Agriculture	Herbicide	Agriculture waste	Soil and water	Diuron, mecoprop, MCPA, terbuthylazine
	Pesticides			
Detergents, surfactants, and perfluorinated compounds (PFCs)	Perfluorooctane sulfonate	Industries, laundries, households, and agricultural applications in pesticides, dilutants, and dispersants	Sewage treatment works	Alkylphenol ethoxylates, alkylphenols (nonylphenol and octylphenol), alkylphenol carboxylates
	Perfluorooctanoic acid			
Swimming pool disinfection by-products	Trihalomethanes and haloacetic acids	Chlorination of the materials of human origin such as hair, lotion, saliva, skin, and urine	Freshwater, wastewater treatment plants, and groundwater	Chloroform, bromodichloromethane, chloral hydrate, dichloroacetonitrile, and 1,1,1-trichloropropanone
Additives	Industrial	Food and municipal waste	Soil, water, and air	Chelating agents (EDTA), aromatic sulfonates
	Gasoline	Mobile exhaust and disposal of exhausted engine oil		Dialkyl ethers, methyl t-butyl ether
Flame retardants		Household items (furniture, electronics, appliances, baby products), and industries	Wet and dry deposition on soil lead to bioaccumulation in the food web	C10–C13 chloroalkanes, hexabromocyclododecane, polybrominated diphenyl ethers, tetrabromobisphenol A, tris (2- chloroethyl) phosphate
New classes	Nanomaterials	Research institutes	Water	1,4-dioxane
	Antibiotic resistance genes	Mutations and genetic adaptation	Horizontal gene transfer in microorganisms	sul (I), sul (II), tet (W), tet (O)

Source: Ellis (2006), La Farre et al. (2008), Verlicchi et al. (2010)

Similarly, in the effluents of several European wastewater treatment plants, 27 out of 32 pharmaceuticals and 4 out of 5 metabolites were detected with maximum concentrations exceeding 1.0 μg/L (Larsen et al. 2004). Many of them are active pharmaceutical ingredients and EDCs, excipients, and additives. The situation is worse in developing countries where the number and concentration of many of the OMPs have been observed to be exceedingly high. This could be due to the fact that in the developing world, many of these pollutants are being sold as over-the-counter compounds causing increased levels in the environment (García-Galán et al. 2010; Stackelberg et al. 2004). The widespread scientific viewpoint is that improved management strategies should be developed and implemented (Brack et al. 2015). However, precise management and even legislation regulation of their permissible levels in the environment need understanding of their fates in the environment along with their harmful effects. This review compiles information on fates of OMPs including their transformation mechanisms in the environment, toxicity to organisms and associated effect on ecosystem, and remediation approaches.

Exposure Pathways of OMPs in the Environment

The mode of entry for OMPs into the environment depends on their application and usage, with wastewater and intensive agriculture as significant sources. Once they are in the environment, OMPs follow various paths such as (bio)chemical transformation, dissolution in water, and/or binding to solids (Farré et al. 2008b; Metcalfe et al. 2013). Only a fraction of OMPs are degraded rapidly; the remainder are dispersed in the environment where their fate depends on physicochemical properties of the compound such as stability, water solubility, vapor pressure, water partitioning, the environmental compartment in which the pollutant is being released, as well as a respective metabolic activity of microbes (Corcoran et al. 2010; Daughton and Jones-Lepp 2001). It has been found that compounds with less water solubility are generally more persistent, toxic, and bioaccumulative compared to compounds with higher solubility and may thus be detected at places far removed from their source (Farré et al. 2008b). In contrast to this, compounds with higher solubility and higher transformation rates are widely distributed in relatively short periods of time. Subsequently, metabolic activities can transform them from less available forms to more available compounds through different pathways (Fig. 1; phase I and phase II) (Boxall et al. 2012). Animals cannot completely metabolize most of these compounds within their bodies, and therefore the parent molecules are excreted with urine and feces into the environment along with their metabolites and/or conjugates (Carballa et al. 2004; Zhang et al. 2008). Similarly, the majority of antibiotics are excreted without any change in their molecular structure (Sarmah et al. 2006; Kümmerer 2009). Thus, many OMPs enter into either wastewater treatment plants where they are usually ignored during secondary and tertiary treatments or join freshwater resources and consequently may end up into food chains (Castiglioni et al. 2006; Lishman et al. 2006; Santos et al. 2007). Direct discharge from households, animal feedlots and

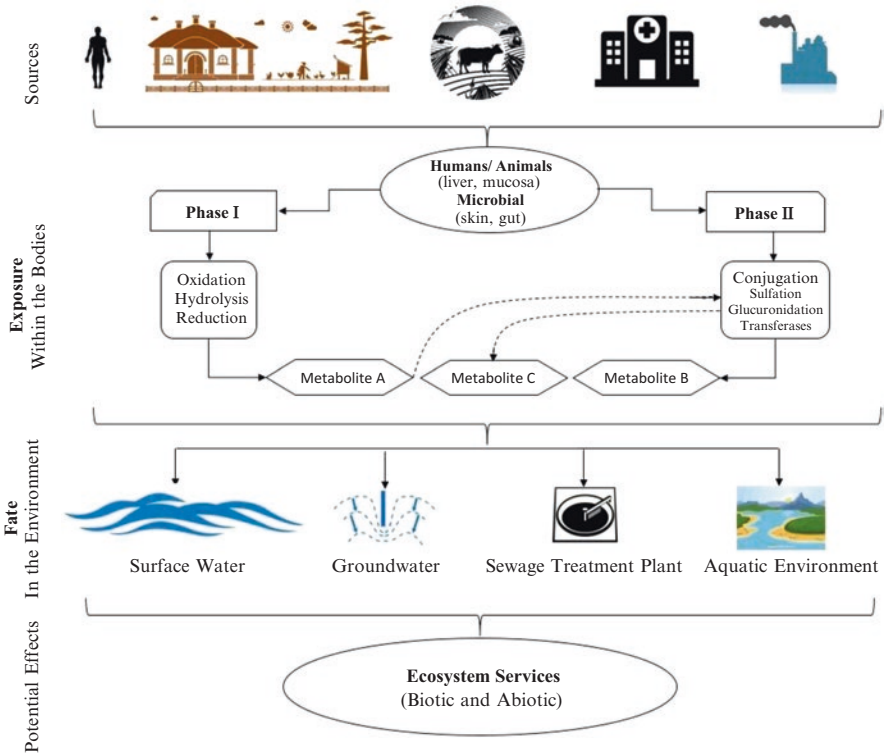

Fig. 1 Schematic representation of the biological transformation of organic micropollutants (OMPs) along with their fate and effects in the environment

agricultural fields, hospitals, and industries also contributes to the OMPs' load in surface waters (Eggen et al. 2010; Verlicchi et al. 2010). The detailed description on fate of OMPs along with their metabolites is presented in the following sections.

Human- and Animal-Associated OMPs

Many of the OMPs are pharmaceuticals and derivatives thereof such as human and animal drugs, antibiotics, EDCs, and personal care products (PCPs). These organic chemicals are generally designed to regulate metabolic reactions within humans and other animals. Pharmaceutical OMPs have been consistently detected in urban wastewater, deposited primarily from human and animal excretions. Active pharmaceutical compounds from unused drugs and hospital waste also find their way into waterways. Sewage treatment plants can remove only some of these chemicals from water by basic filtration practices and metabolism, while others pass through unabated. Most of the over 4000 prescription drugs used for human and animal treatment have been detected in the environment (Scudellari 2015).

Generally, their environmental fate is affected by the adsorption potential of the chemicals as a function of hydrophobic and/or electrostatic properties (Fent et al. 2006). These chemicals are typically designed to be kinetically rather inert; hence, a majority of these OMPs pass through the organism body unmodified or in the form of conjugates. Resultantly, the compounds become constituents of wastewater and may end up in irrigation water, rivers, canals, and treatment plants where their fate depends upon their chemical composition (Daughton and Ternes 1999). For example, acidic pharmaceuticals (being ionic at neutral pH) possess little adsorption potential to wastewater sludge compared to basic pharmaceuticals (Ternes et al. 2004; Urase et al. 2005; Urase and Kikuta 2005). Therefore, acidic pollutants are more likely to remain in the water and thus move into the receiving surface waters. In contrast to this, basic pharmaceuticals can easily adsorb on to the sludge sediments and therefore may be removed from the water stream as adhered compounds (Golet et al. 2002; Ternes et al. 2002; Ternes et al. 2004). Similar results have been obtained for PCPs and EDCs. The high-adsorption efficiency (70–80%) of estrogens is a good example of fates of EDC in the wastewater treatment facilities where they exist in μg/L (Auriol et al. 2006; Johnson et al. 2007), as well as antidepressants which have been found to be partitioned into sludge or sediment at ng/g concentrations (Furlong et al. 2003; Kwon and Armbrust 2003).

Besides an adsorption-dependent fate, variety of OMPs may also undergo microbial and photodegradation processes (Klöpffer and Wagner 2007; Saleem 2016). Such transformations often result in chemical entities with new physicochemical properties, hence having fates distinct of the parent compounds. For example, in treated wastewater, metabolites are sometimes more concentrated than the parent compounds. This could be due to the fact that the resulting compound is metabolically more persistent (or pseudo-persistent) compared to the parent compound after structural modifications (Boxall et al. 2004; Daughton 2004; Escher and Fenner 2011). A good example is the generation of higher concentrations of carboxy-ibuprofen, a metabolite of ibuprofen, in the effluent than in the influent of a domestic WWTP in Norway (Weigel et al. 2004). Likewise, another study revealed higher levels of desmethylcitalopram compared to its parent compound, citalopram, in effluent wastewater (Vasskog et al. 2008). However, this is not always the case as some OMPs are back-transformed into parent compounds. For instance, in the case of antimicrobials, the concentration of a metabolite of sulfamethoxazole (N^4-Acetylsulfamethoxazole) was significantly reduced in the effluent of the municipal wastewater, which is presumed to be retransformed to the antibiotic itself (Göbel et al. 2007), although no change is reported in the metabolite concentration before and after the treatment (Farré et al. 2008b). It is apparent that the fate of a pollutant depends on more than one factor.

Another class of OMPs encompasses the chemicals used for sanitation purposes. For example, disinfection in swimming pools is performed to control the waterborne pathogens and diseases. During the process, different disinfection agents such as ozone, chlorine, chlorine dioxide, and chloramines react with natural organic matter introduced by pool users, resulting into the production of a wide range of disinfection by-products (DBPs). Trihalomethanes (THMs) and haloacetic acids have been observed to predominate the DBP load (Pavelic et al. 2006; Pavelic et al. 2005); however, their ultimate fate depends upon the nature of pollutant and the treatment

process they go through before entering water bodies. For instance, during wetlands treatment, some less stable DBPs can be easily removed by volatilization, sorption, and/or degradation (Barber et al. 1997; Rostad et al. 2000; Keefe et al. 2004). Moreover, chlorination at water treatment facilities also leads to the formation of DBPs, especially trihalomethanes, during rainstorm events, land surface runoff, and resuspension of bottom sediments (Ates et al. 2007; Alkhatib and Peters 2008).

Agriculture-Associated OMPs

Many pesticides are persistent in the environment; only a fraction of them is degraded by physicochemical and biological processes while the rest may attach to thin soil particles and organic matter, evaporates into air, and/or leaches into the groundwater. Runoff water from agricultural lands carries these residues and their metabolites to surface drinking water resources such as lakes, rivers, and canals (Stuart et al. 2012). Residues of pesticides, including some of discontinued use, have been detected at trace concentrations in surface and groundwater throughout the developing and developed world. For example, Sinclair et al. reported metabolites of DDT, heptachlor, and atrazine in groundwater of the United Kingdom, although these pesticides had been banned in the United Kingdom since decades (Sinclair et al. 2010). The same authors also described the presence of 53 pesticide metabolites in surface drinking water on the basis of parent compound usage, formation rates in soil, persistence and mobility, and pesticide activity along with other parameters. Quantifying persistent pesticides and other OMPs present at very low concentrations in drinking water is analytically demanding. The assessment of transformation products is often a great challenge, yet some pesticide metabolites are more toxic than their parent compounds (Sinclair and Boxall 2003) and thus pose considerable risks to biological elements of the ecosystem. In some cases, the pesticide metabolites have been detected at even higher concentrations compared to the parent compounds in groundwater (Kolpin et al. 2004; Lapworth and Gooddy 2006). A recent example is of one of the most widely used herbicide glyphosate. Microbial degradation of glyphosate generates aminomethylphosphonic acid (AMPA) whose chronic accumulation may cause minor to moderate toxic effects in aquatic organisms (Levine et al. 2015). Both glyphosate and AMPA are highly water soluble, and Kolpin et al. (2006) reported the detection of AMPA four times more frequently than that of glyphosate in affected surface water.

Industry-Associated OMPs

Another group of OMPs includes chemicals used in or derived from industrial applications such as carpeting, upholstery, apparels, food paper wrappings, firefighting foams, and metal plating. Many of the chemicals from these sources are already declared as priority pollutants such as adipates and phthalates, chlorinated solvents,

fuel oxygenates, methyl *tert*-butyl ether, plasticizers/resins, and bisphenols (Moran et al. 2005, 2007; Verliefde et al. 2007); however, upon transformation some of the pollutants are turned into metabolites with more toxicity such as benzotriazole intermediates, 1, 4-dioxane, dioxins, and perfluorinated compounds (PFCs). Although reports on toxicity for these chemicals are limited, they are of major concern due to their hydrophilic properties as well as resistance to natural degradation. For instance, 1,4-dioxane, a stabilizer used with 1,1,1-trichloroethane, has been found to readily leach to groundwater without being bound to soil particles (Abe 1999). Similarly, perfluorinated compounds (PFCs) such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid are of significant concern since they are weakly carcinogenic (Jahan et al. 2008; Ju et al. 2008; Strynar and Lindstrom, 2008). Moreover, presence of certain PFCs (e.g., PFOS) at levels above the limit of detection may cause endocrine disruptions. According to a global survey, PFOS is detected in both nonexposed and exposed populations, which increase its importance to be considered as priority pollutant. In this regard, a noncompetitive enzyme-linked immunosorbent assay (ELISA) has been developed to investigate estrogenic activities of selected PFCs (Liu et al. 2007). However, while many of the PFCs have been produced since five decades, their presence in the environment has only recently been reported along with their toxicity, persistence, and bioaccumulative nature. Similar to the other pollutants of same group, PFCs also remain in water bodies without or hardly any degradation and are thus found at remote places like the deep sea. Some PFCs with relatively volatile nature are subject to transformation, leading to the formation of persistent sulfonate and carboxylic acid forms (Farré et al. 2008b). Direct discharges from pharmaceutical and pesticide industries also contribute to major amount of OMPs in the environment.

Potential Risks to the Environment

As many of the OMPs are specifically designed to be bioactive at low concentrations, their environmental presence at such low concentrations may cause harmful effects to living organisms (Baguer et al. 2000; Migliore et al. 2003; Ferrari et al. 2004; Kumar et al. 2005; Ankley et al. 2007; Corcoran et al. 2010). To date, different studies have been conducted to evaluate the acute (short-term) and chronic (long-term) effects of different OMPs (Cleuvers 2003; Ferrari et al. 2004; Hernando et al. 2004). In most cases, acute toxicity is less likely to occur at present environmental concentrations (ng/L up to several μg/L); however, chronic effects of OMPs' toxicity are widely acknowledged and being investigated at large in developed countries (Farré et al. 2008b). For example, Webb observed chronic effects on more than 90% of the studied organisms, including algae, invertebrates, and fish at concentrations close to 1 μg/L (Webb 2001). On the other hand, 10% of the organisms showed acute toxicity symptoms only at concentrations orders of magnitude higher, i.e., approaching 1.0 mg/L. Similarly, Ferrari et al. reported higher toxicity in chronic tests than acute tests for six pharmaceuticals (Ferrari et al. 2004). The toxicity due to chronic exposure is getting more

attention particularly when present as components of complex mixtures (Schwarzenbach et al. 2006). Environmental concentrations may be of concern, therefore, and sublethal effects on non-target organism may arise in different levels of ecosystem services (Franzellitti et al. 2014).

Long-term presence of OMPs in an ecosystem may affect its biotic characteristics and thus eventually impair the ecosystem's function and service. For instance, OMPs with antimicrobial properties may have pronounced effects. Westergaard et al. showed that veterinary antibacterial compounds affected sulfate reduction in soil as well as inhibition of dung decomposition resulting in disturbance of organic matter balance (Westergaard et al. 2001). Furthermore, some of the OMPs have a distinct activity spectrum, i.e., they target either gram-positive or gram-negative species resulting into replacement of sensitive species with more tolerant ones. This may result into succession of a tolerant community known as pollution-induced community tolerance (PICT) (Blanck 2002; McClellan et al. 2008). The PICT may be of structural changes (changes in species composition) in the community or even biochemical changes in the species present, rendering them more tolerant, i.e., genetic changes or physiological adaptations (Blanck and Wängberg 1988; Blanck 2002). The genetic changes may be of high significance during chronic exposure of OMPs leading to the development of antibiotic resistance as a principle of selection pressure. Heuer and Smalla investigated the effects of pig manure and sulfadiazine on bacterial communities in soil microcosms using two soil types (Heuer and Smalla 2007). In both soils, manure and sulfadiazine positively affected the quotients of total and sulfadiazine-resistant culturable bacteria. The results suggest that manure from treated pigs enhanced horizontal transformation of antibiotic resistance genes in the soil bacterial communities. The presence of antibiotics may also induce the SOS response systems in bacteria, resulting into increased mutation rates, activation, and mobilization of many mobile elements ultimately increasing rate of gene transfer (Fajardo et al. 2008; Aminov 2009; Martinez et al. 2012).

Likewise, a few studies have also explored effects of veterinary antibiotics on aquatic microbes. Schallenberg and Armstrong (2004) explored the impacts of filtered water from an agricultural drain on lake bacteria. They showed that drainage water reduced the abundance of aquatic bacteria in a shallow coastal lake, and the data indicated that these effects might be due to antibiotics. In addition to this, veterinary antibiotics can reduce the rate of degradation of human drugs as an indirect effect to the ecosystem (Boxall et al. 2012). This observation may have serious implications for the risks of other compounds that are applied to the soil environment such as pesticides.

Risks in the Food Web

The potential risks associated with OMPs are particularly critical when involving photosynthetic organisms due to their primary position (i.e., trophic level) in the food web. It is well established that many of the OMPs have octanol-water partition coefficient (log K_{ow}) values lower than 3.5 and, therefore, may be taken up readily

Table 2 Major organic micropollutants (OMPs) with log K_{ow} values <3.0 and their successful uptake by plants

Generic name	Log K_{ow}	Plant used	Reference
Ibuprofen	0.2	*Populus nigra* L.	Iori et al. (2012)
Carbamazepine	2.45	*Zea mays* and *Helianthus annuus*	Spoustová et al. (2015)
Ciprofloxacin	0.3	*Acrostichum aureum* L. and *Rhizophora apiculata Blume Fl. Javae*	Hoang et al. (2013)
Erythromycin	3.0	*Lemna minor*	Pomati et al. (2004)
Sulfamethoxazole	0.9	*Phalaris arundinacea* L. var. *picta* L.	Nowrotek et al. (2016)
Diclofenac	0.70	*Scenedesmus vacuolatus*	Schmitt-Jansen et al. (2007)
MCPA	2.94	*Typha* and *Phragmites*	Matamoros et al. (2008)
Atenolol	0.16	*Typha* spp. and *Phragmites australis*	Dordio et al. (2009)
Levofloxacin	0.28	*Lactuca sativa*, *Medicago sativa*, and *Daucus carota*	Hillis et al. (2011)
Dilantin	2.47	*Lactuca sativa*, *Spinacia oleracea*	Wu et al. (2012)

by aquatic plants and thus may enter the food web (see examples in Table 2) (Yebra-Pimentel et al. 2015). Even a slight toxic effect of OMPs to plants may result in deteriorated ecosystem quality and can thus have drastic consequences (Liener 2012). When plants are influenced by any change in their host environment, the environment is also influenced by any change in the physical, physiological, or biological properties of plants they are hosting (Orcutt 2000; Wardle et al. 2004). This may result into reverse effects on soil, water, air, and organism living on the plants. Therefore, phytotoxicity of OMPs must be taken very seriously, and strategies must be devised in advance to avoid or minimize potential risks (Kabata-Pendias 2010). The following sections describe potential aspects of OMP-associated toxicity in the food web at different trophic levels.

Many studies have been carried out to investigate the harmful effects of OMPs to the aquatic, free-dwelling, photosynthetic organisms, e.g., microalgae, phytoplanktons, zooplanktons, and small plants (weeds) (Day and Saunders 2004; Reinhold 2007; Reinhold and Aryal 2011). Pomati et al. studied toxic effects of erythromycin and tetracycline on the growth of a cyanobacterium, *Synechocystis*, and *L. minor* (Pomati et al. 2004). The study reported 15% reduction in the cell density of *Synechocystis* at 1.0 μg/L, 66% at 100 μg/L, and 70% at 1000 μg/L of erythromycin, whereas no negative effects were observed for *L. minor* at 1.0 μg/L, but 15% reduction in biomass was observed at 100 μg/L. Similarly, tetracycline inhibited the growth of *Synechocystis* up to 20% at 10 μg/L and 22% at 100 μg/L, while the growth of *L. minor* was inhibited to 18% at 1.0 μg/L and 26% at 10 μg/L. Similar findings have been reported for toxicity of sulfamethoxazole, levofloxacin, and atorvastatin to *Lemna gibba* and *Myriophyllum sibiricum* (Brain et al. 2004);

however, it is not always the case that all of the OMPs are toxic to the aquatic plants as some of the OMPs express phytotoxicity only at very high concentrations, e.g., diclofenac (Cleuvers 2003; Fent et al. 2006). The majority of the toxicity studies on aquatic plants have been conducted for pharmaceuticals, and less information is available on other classes of OMPs.

In case of higher plants, toxicity begins at germination stage as many of the pollutants directly affect the plant embryo by altering the growth conditions. Later on, if the embryo is able to survive, the presence of pollutants inhibits plant growth by affecting several biochemical pathways such as chlorophyll synthesis, enzymatic reactions, or plant-microbe interactions. One reason behind these inhibitions is the disturbance of cell redox status due to production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Hence, oxidative stress results in major changes in the electron transport chain particularly in the chloroplast and mitochondrial membranes leading to membrane dismantling, ion leakage, chlorophyll synthesis inhibition, biological molecule degradation, lipid peroxidation, and DNA strand cleavage (Carrasco-Gil et al. 2011). Earlier studies presented toxic effects at higher concentration only; however, some of the recent studies have also confirmed the harmful effects of OMPs at slight concentration with shorter period. Kummerová et al. (2016) report significant reduction of *Lemna minor* biomass production along with the increased amount of ROS and RNS in roots during 10-day exposure to diclofenac and paracetamol at 10 and 100 μg/L. The diclofenac assay increased lipid peroxidation along with the disturbing of membrane integrity, ultimately lowering the dehydrogenase and oxidoreductase activities, while paracetamol increased the content of soluble proteins and phenolics. This could have happened due to the changes in biochemical processes associated with activation of defense mechanisms against oxidative stress. Similarly, another study reports the differences in biomass production for periphyton communities in short-term field tests, at reference and polluted localities. The community tolerance of polluted communities was observed to be significantly high for prometryn confirming the site-specific effects on local periphyton (Rotter et al. 2015). However, less toxicity to higher plants have been reported which could be due to the fact that those plants have several degradation mechanisms such as oxidation reactions, hydrolysis, and can harbor a pollutant-degrading endophytic community. Therefore, the mutual degradation potential of plant and associated microorganisms manages to eliminate the OMPs without getting affected by their toxic nature (Chaudhry et al. 2004). However, this is not the case always as the chronic exposure of pollutant may deteriorate plant health (Farré et al. 2008b). It has been observed that some of the OMPs, due to their persistent and reactive nature, strengthen the toxicity index for certain plant species (Forni et al. 2002). It is reported that the chronic exposure of sulfadimethoxine significantly reduced the average biomass along with the chlorophyll content in *Azolla filiculoides* even at low concentrations. In contrast to this, acute toxicity (short-term exposure) of OMPs has never revealed any significant effects to plant species. For instance, *L. minor* was only weakly affected in both plant growth and photosynthetic pigments during acute exposure of sulfadimethoxine (Białk-Bielińska et al. 2012).

Harmful effects of OMPs on plants may influence other organisms present at higher trophic level within the food web (Boxall et al. 2004; Quinn et al. 2008). The OMPs can impart negative effects on the biotic factors due to a variety of reasons. Firstly, they may be taken up as such by plants and can accumulate within the plant's tissues and organs. For example, Hu and colleagues analyzed the migration of veterinary antibiotics in organic vegetable bases through water transport and passive absorption (Hu et al. 2010). The distribution of antibiotics in a plant was in the sequence leaf > stem > root and bioaccumulated accordingly. The organisms hosted by or feeding on such plants may take up high amounts of the OMPs similar to the accumulation of persistent organic pollutants. For example, polybrominated diphenyl ether (PBDE) flame retardants have been reported to be biomagnified along with the other endocrine-disrupting chemicals (EDCs) in three trophic levels of food web in the lower Columbia River (Rayne et al. 2003). Secondly, the OMPs may be transformed into more toxic chemicals and be taken up by the organisms eating plants. For example, Isidori et al. reported that the metabolites of naproxen possess significantly higher toxicities than the parent compound (Isidori et al. 2005). Moreover, the toxicity index was found higher in chronic exposure experiments. Sometimes the respective metabolites are more concentrated than the parent compound. For instance, it was reported that the use of iodinated X-ray contrast media (ICM) as a source of iodine leads to the formation of iodo-trihalomethane and iodo-acid disinfection by-products (DBPs), both of which are highly genotoxic and/or cytotoxic in mammalian cells (Steger-Hartmann et al. 1999; Kormos et al. 2011). Similarly, higher levels of desmethylcitalopram, a metabolite of citalopram, have been observed in the effluent wastewater compared to the influent concentration (Vasskog et al. 2008). Thirdly, OMPs may affect plants in a way that they alter their physiological processes or produce harmful products. In such cases, organisms depending solely on plants will inevitably be affected. For example, the presence of pollutants induces the plant to produce reactive oxygen species (ROS) by reaction with singlet oxygen ($^1O_2$), photoexcited organic matter (Brame et al. 2014). The high production of ROS damages biological molecules (nucleotides, proteins, carbohydrates, and fatty acids) and membranes (Ünyayar et al. 2006). Resultantly, plant endophytes and rhizobacteria living on plant proteins and root exudates may be affected by metabolizing ROS-damaged plant matter and/or directly interacting with the reactive species, resulting into the disturbance in plant microbial communities. Moreover, as many of the plant-associated bacteria possess plant growth promoting (PGP) activities, the inhibition of microflora may contribute to weaken the plant defense against the pollutants. Last but not least, the production of ROS in the food may affect the health of higher animals including human beings directly feeding on the plant (Wettasinghe and Shahidi 2000; Choe and Min 2005).

The harmful effects of OMPs are not limited to the microorganisms and plants but may also cause indirect effects to animals especially by feeding of macroinvertebrates in the food web. For example, polybrominated diphenyl ethers (PBDE) flame retardants were found to be biomagnified in large-scale suckers and osprey eggs whose concentration was not prominent in invertebrate biomass (Nilsen et al.

2014). This may be due to the chemical nature of OMPs rendering them inevitable in the ecosystem whose risk can be conjectured on all possible risk pathways. As mentioned above, these chemicals tend to accumulate, degrade, reassemble, and undergo novel transformations under the influence of certain environmental conditions; hence, their presence may lead to bioaccumulation and potential negative effects in multiple levels of the food web.

Genotoxicity

The chronic exposure of OMPs is not limited to the ecotoxicity in the food web but may also lead to genotoxicity including point mutations, chromosomal rearrangements, inversions, deletions, insertions, and/or persistent epigenetic modifications (DNA methylation, phosphorylation, histone modifications) (Hoffmann and Willi 2008). These genetic changes primarily depend on the toxicity potential and exposure period of the contaminant. As many OMPs are designed to regulate metabolic processes, their chronic exposure may cause disruptions in the genome of the exposed organism. Many studies have reported chemical-induced genetic alterations in both laboratory- and field-exposed plants and animals (Dixon et al. 2002). In addition, genotoxicity could happen to both somatic and germinal cells; hence, harmful effects may appear in the same generation or in trans-generations, respectively. In case of somatic cells' genotoxicity, physiopathological changes such as impairment of vital metabolic functions may result into reduction of average fitness of the affected group whereas germinal toxicity can pass detrimental damage to the next generation (Kurelec 1993). Likewise, the long-term presence of active metabolites may also cause epigenetic changes. For example, minute concentrations of hydralazine, an antihypertensive, have been reported to inhibit DNA methylation resulting into uncontrolled gene expression. In a recent report, a two-tier effect of a drug was evaluated in which acute exposure had profound effects on signaling pathways resulting into alteration of transcription factor activity at gene's promoter sequence, whereas chronic exposure resulted into more permanent modifications to DNA methylation and chromatin structure. Moreover, the epigenetic changes became persistent even after the drug was discontinued (Csoka and Szyf 2009).

Detection Methods of OMPs

The potential (eco)toxicological risks of OMPs necessitate the need to deal with the pollutants before the detrimental effects may appear. However, many of the existing methods fail to detect the range of OMPs at their environmental concentrations; hence, the presence and fate of such trace chemicals can be underestimated.

Sampling Methods

The detection of OMPs starts with appropriate sampling procedure. The conventional sampling methods such as grab and spot sampling have not been very efficient/effective (Ibrahim et al. 2013). Moreover, environmental factors can alter the concentration of pollutants in aqueous environment. The bias can be resolved by frequent sampling; however, the procedure is expensive and time consuming. To deal with these challenges, online monitoring and passive sampling procedures have recently been developed which assure precise detection of OMPs at environmental concentrations. Online monitoring has improved the reliability of monitoring data but involves expensive equipment and high maintenance cost. On the other hand, passive sampling procedures, such as semipermeable membrane devices (SPMDs), polar organic chemical integrative samplers (POCIS), Chemcatcher, and ceramic dosimeter, can sample water over a long period of time. They provide time-weighted average (TWA) concentrations, are cost effective, and have great potential to be employed with little modifications (MacLeod et al. 2007).

In passive sampling, analyte molecules move from the sampled medium to the reference phase in a sampler, as a result of chemical potential differences between water and the sampler. Reference or receiving phase can be a chemical reagent, solvent, or a porous medium, which further traps the analytes. When the equilibrium is reached, the net movement of molecules stops (Górecki and Namieśnik 2002). The whole process does not require any kind of additional energy and generally depends upon the difference between chemicals' potential. Nevertheless, passive sampling simplifies the process of sample collection as well as preparation by excluding power requirements, reducing the analysis costs, and protecting decomposition of analytes before analysis (Kot-Wasik et al. 2007). In contrast to grab sampling, analyte extraction from deionized water in passive samplers provides concentrations of freely available analytes rather than the total concentrations in the samples. Furthermore, the efficiency of passive sampling allows detection of more micropollutants than active sampling (Terzopoulou and Voutsa 2015).

Semipermeable Membrane Devices

Semipermeable membrane devices (SPMDs) were introduced by Huckins et al. (1990) and are being used for aquatic hydrophobic contaminant measurements. SPMD consists of a lay-flat low-density polyethylene (LDPE) tube, filled with high-purity triolein and sealed at both ends. Its hydrophobicity confines its usage to hydrophobic contaminants typically characterized by log $K_{ow} > 3.0$ (Huckins et al. 2006). A process of dialysis or solvent extraction of triolein and LDPE membrane is carried out, followed by analytical measurements. These samplers provide time-dependent pollutant concentration. A wide range of contaminants, including organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dioxins, furans, and other hydrophobic compounds, can be analyzed using SPMDs (Seethapathy et al. 2008).

Polar Organic Chemical Integrative Sampler

In contrast to SPMDs, the polar organic chemical integrative sampler (POCIS) was developed to sample polar organic compounds (with log $K_{ow} < 3.0$) from aqueous environments. A typical POCIS used for sampling of hydrophilic organic compounds consists of a solid sorbent medium enclosed between microporous polyethersulfone membranes (Alvarez et al. 2004; Seethapathy et al. 2008). Currently, there are two phase configurations of POCIS available. One is pesticide specific and contains a mixture of three sorbents whereas the other is pharmaceutical specific and contains a single sorbent. In POCIS, the microporous membranes act as filter, while the enclosed sorbent performs the role of accumulator (Miège et al. 2012). Generally, a wide range of pollutants including personal care products, pharmaceuticals and pesticides, and other hydrophilic compounds are analyzed using POCIS.

Chemcatcher

The passive sampler Chemcatcher was designed by Kingston et al. (2000) to sample organic compounds from aqueous environments. In this kind of sampler, a PTFE body containing a C18 Empore disk is used as sorbent phase. The Empore disk consists of 90% (w/w) silica particles and 10% (w/w) PTFE fibers, chemically bonded to octadecyl (C18) groups. Polyethersulfone is used as limiting barrier and is suitable for polar organic compounds with log K_{ow} range 2.0–4.0. However, polyethylene can be used as limiting membrane to sample nonpolar organic compounds with log $K_{ow} > 4$ (Kingston et al. 2000).

Ceramic Dosimeter

A ceramic dosimeter consists of a ceramic tube of 5.0 cm length and 1.0 cm OD and suitable sorbent and was developed by Martin et al. (2003). It is used for time-dependent, long-term monitoring of organic compounds in aqueous environments. This method of sampling has been tested for volatile aromatic compounds (BTEX), volatile chlorinated hydrocarbons, and polycyclic aromatic hydrocarbons (PAH) and can be used for many other organic and inorganic contaminants.

Analytical Methods

In order to assess the concentration and fate of micropollutants, sensitive analytical methods are necessary. Conventional analytical methods for detection of organic micropollutants include simple gas chromatography-mass spectrometry or liquid

chromatography with UV/fluorescence; however, in these methods, the sample preparation is very complicated, requiring many steps (Chusaksri et al. 2006). Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become a robust analytical tool for analyzing environmental contaminants (Chusaksri et al. 2006; Díaz-Cruz and Barceló 2005; Li et al. 2006). LC-MS/MS is a more efficient and reliable method with less complicated sample preparation and detects a wide range of compounds in water (Hernández et al. 2005). Typically, the ionization method for analysis by LC-MS/MS is electrospray ionization (ESI) (Alder et al. 2006). While time-of-flight (TOF) mass analyzers are less used for detection of micropollutants, they are considered as more precise and sensitive tools (Petrovic et al. 2006; Sancho et al. 2006). In contrast to other MS analyzers, high sensitivity and resolution of TOF-MS makes it suitable for screening purposes. Furthermore, use of hybrid quadrupole-time-of-flight (QqTOF) enhances the TOF performance by facilitating the simultaneous detection of pollutants in samples (Bobeldijk et al. 2001). Therefore, QqTOF is one of the most reliable and advanced tool to monitor unknown micropollutants in water (Lacorte and Fernandez-Alba 2006).

The triple quadrupole mass instrument (QqQ) works under the same principle as the single quadrupole; however, the sensitivity, precision quantification is increased during operation at multiple reaction monitoring mode (MRM). Moreover, the QqQ is easy to operate, cheaper, efficient, highly sensitive, and can detect very low concentration of pollutants (Peng et al. 2014), hence, is a common analytical tool in environmental monitoring by LC-MS/MS. Therefore, both these setups, i.e., LC-QqQ and LC-QqTOF, have been considered as more useful, reliable, and ideal instruments for detection of pollutants in minute concentrations. Transition from GC-MS to LC-MS/MS is significantly effective by reducing the cost and time and increasing the analytical sensitivity.

In addition, the combination of a linear ion trap in the third quadrupole (QTrap) can be introduced where Q3 is operated either in the normal RF/DC mode or in LIT mode (Hager 2002), forming quadrupole ion trap mass spectrometer. The QTrap mass spectrometry is also considered as a versatile technique with high specificity and sensitivity. This technique is relatively less costly and therefore gained substantial growth in the field of mass spectrometry. Once the pollutant concentration is detected precisely, their removal potential can be studied more accurately in different systems.

Methods of Remediation and Their Removal Potential

Nonbiological Methods of Remediation

There are several conventional and modern nonbiological technologies for remediation of OMPs, including advanced oxidation, activated carbon adsorption, membrane ultrafiltration, hydrostatic exclusions, and electrostatic exclusions (Ojajuni et al. 2015). *Advanced oxidation* is an aqueous phase oxidation procedure that can eliminate a wide range of OMPs primarily by mineralization

through production of highly reactive species or converting them to less harmful products (Tawabini 2014). Key methodologies include photocatalysis based on solar visible or near-ultraviolet irradiation, ozonation, Fenton's reaction, ultrasound, and wet air oxidation (Ikehata et al. 2008). Recently, ionizing radiation, pulsed plasma, microwaves, and Fenton's reagent have also been considered as evolving procedures in oxidation. The technique has gained tremendous popularity in the removal of OMPs but has limitations when the effluents contain higher loads of organic and/or inorganic matter. To overcome the problem of dealing with an effluent containing high organic load, *activated carbon adsorption* is a suitable option as the hydrophobic nature of the pollutants (compounds with $K_{ow} > 2.0$) favor their adsorbance on the activated surface (Jones et al. 2005). However, the adsorption success depends on the type of activated carbon, i.e., granular and powdered. *Membrane-assisted processes* also have abundant potential in removing wide range of OMPs (Snyder et al. 2007; Schäfer et al. 2011; Ojajuni et al. 2015). Membranes work as a physical barrier that rejects pollutants of dimensions greater than the membrane's porosity while allowing water to permeate through it (Kimura et al. 2003; Kim et al. 2005). Recently, several membrane processes have been exploited to remove the OMPs including microfiltration, ultrafiltration, nanofiltration, membrane adsorption, size exclusion, reverse osmosis, electrostatic exclusion, electrodialysis reversal, membrane bioreactors, and combinations of membranes in series (Kim 2011; Boonyaroj et al. 2012; Nguyen et al. 2012; Ojajuni et al. 2015). However, research is ongoing to improve the selectivity of the removal systems, lessening of secondary effluent, lowering of operating cost, and possibility of waste recovery, hence, making the existing methods more promising and potential alternative compared to the currently employed methods.

Biological Methods of Remediation

Since many of the nonbiological processes are expensive and may result in the generation of new waste products, biological removal of OMPs appears as a promising alternative due to reduced capital investment and eco-friendly nature. In principle, many of the biological treatment procedures primarily depend on microorganisms containing appropriate metabolic capabilities. Although biological degradation pathways have been reported to have great influence on wide range of contaminants, their efficiency has always remained a challenge to environmental biologists. Many of the conventional bioremediation systems remove only a part of OMPs from the environment, and hence many of the polar compounds are discharged without being reduced in concentration (Koh et al. 2008). To overcome this issue, different improvements in the design and principle of biological system have been adopted to reduce OMPs load such as phytoremediation, plant-bacteria partnership system, constructed wetlands, ligninolytic enzymes application (e.g., laccase), and biofiltration (Majeau et al. 2010; Rauch-Williams et al. 2010; Verlicchi et al. 2010; Reinhold and Aryal, 2011; Sadef et al. 2014).

Phytoremediation of OMPs

Phytoremediation is an eco-friendly technology that utilizes plants to detoxify and remove the pollutants present in sediments, soil, water, and in the atmosphere (Chigbo and Batty 2014; Samardjieva et al. 2015; Weyens et al. 2015). In principle, success of phytoremediation appears to be the function of pollutant uptake, translocation, transformation, extraction, sequestration, and/or detoxification depending upon the physicochemical characteristics of the pollutants. These physicochemical characteristics are log K_{ow}, aqueous solubility (S_w), octanol solubility (S_o), acidity constant (pK_a), and the pollutant concentration (Admire et al. 2014; Arslan et al. 2015; Zeng et al. 2012). Among them, log K_{ow} is of prime importance as it determines the hydrophobicity and lipophilicity of the compound, which can have a strong impact on the fate of a pollutant in a phytoremediation system.

As microorganisms, plants were not under selection pressure during the course of evolution for OMP transformation and hence have not adopted robust pathways for their mineralization (Burken 2003; Gerhardt et al. 2009). However, partial degradation of pollutants has been observed within plants through *in planta* detoxification mechanisms, i.e., transformation (phase I), conjugation (phase II), and compartmentalization (phase III) (Fig. 2). The mechanism has been named as green-liver model, and its significance has been expressed in terms of oxidation, hydrolysis, and epoxide formation reactions (Chaudhry et al. 2002; Arslan et al. 2015). Oxidation is the most prevalent mechanism in plant-derived remediation and is catalyzed by some microsomal enzymes, e.g., peroxidases, cytochromes P450, and flavin-dependent monooxygenases (Naumann et al. 2002; Khandare et al. 2012). These enzymes can

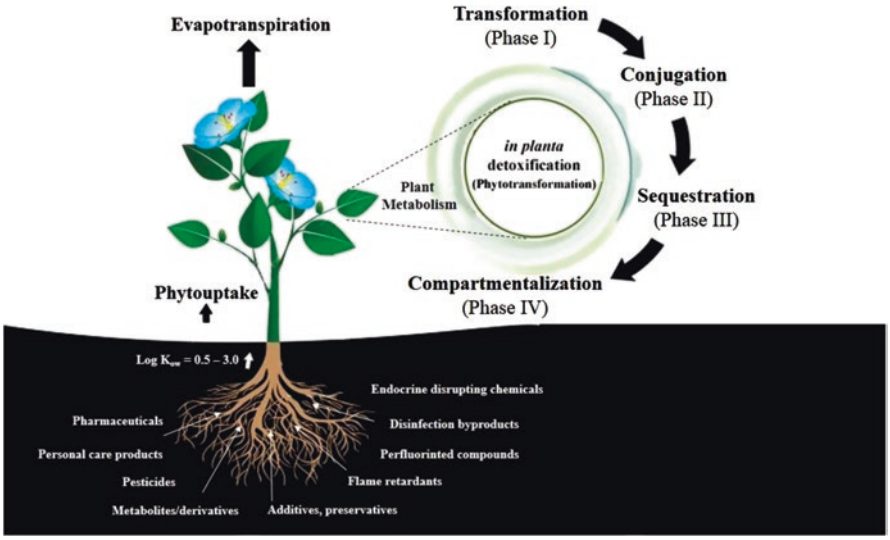

Fig. 2 Schematic representation of *in planta* degradation of organic micropollutants (OMPs) and "green-liver model"

degrade a variety of organic compounds including OMPs; for example, cytochrome P450 has been reported to degrade organophosphates (P = S → P = O) with the release of sulfur atom (Neal 1980). In addition to this, different PCB congeners have also been degraded using cytochrome P450 system (Chaudhry et al. 2002).

Plant-Bacteria Interactions in Phytoremediation of OMPs

The direct role of plants in OMP degradation is limited and is generally aided by plant-associated microbial communities, i.e., rhizo- and/or endophytic bacteria (Mackova et al. 2009; Weyens et al. 2009; Glick 2010; Afzal et al. 2014a). Plant-rhizobacteria partnership is a synergistic relationship between plants and associated bacteria (Khan et al. 2013; Arslan et al. 2014), whereas plant-endophyte associations are the partnerships of plants with the bacterial communities residing within plant tissues. Plants provide nutrients and residency to endophytes, whereas endophytes protect plants from the toxic effects of pollutants through plant growth promoting (PGP) activities (Rylott 2014). Furthermore, microbes are found to be chemoattracted to root exudates, which allow them to colonize and proliferate successfully (Lugtenberg and Kamilova 2009).

In plant-bacteria partnership, pollutants are degraded by the action of bacterial catabolic enzymes, which can be expressed under stress conditions. Among these enzymes, dioxygenases have been found to be key enzyme in the degradation of a number of OMPs such as basic ring compounds, pharmaceuticals, and pesticides. Nevertheless, the transformation pathways for OMPs by bacteria are mostly unknown and therefore need investigations in particular to each class of the pollutants.

The plant microflora not only comprises catabolic machinery but also harbors necessary genes for PGP activities. Expression of these genes helps strengthen innate defense of plants, reduce accumulation of stress hormones, and enhance accumulation of PGP enzymes (Bhattacharyya and Jha 2012). Recent investigations have categorized PGP activities into the following five processes: (1) ACC deaminase production which reduces the ethylene production and hence alleviates plant stress (Dey et al. 2004); (2) plant phytohormones production such as indole acetic acid (IAA), gibberellic acid, and cytokinins (Narula et al. 2006; Saleem et al. 2007; Mishra et al. 2010); (3) asymbiotic nitrogen fixation by rhizobacteria which enhances nutrient assimilation (Ardakani et al. 2010); (4) production of siderophores, β-1,3-glucanase, antibiotics, chitinases, cyanide, etc. for antagonistic activities against phytopathogenic microorganisms (Pathma et al. 2011); and (5) solubilization of mineral phosphates and other nutrients (Hayat et al. 2010). The significance of such activities during OMPs' toxicity appears to be pivotal as many of microorganisms have been found to employ these mechanisms altogether (Martínez-Viveros et al. 2010). Nevertheless, harmful effects of OMPs stress on plants have been reported up to a certain level (explained in ecotoxicology section), while little information is available on pollutant toxicity to PGP bacterial communities.

In addition to all the foregoing facts, when the plant environment is deprived of beneficial microorganism, the isolation of microbes from a different host environment and inoculation in the polluted environment may bring positive results toward improvement of plant health and pollutant degradation (Afzal et al. 2012; 2013). Once the bacteria are inoculated, production of root exudates allows successful colonization of plant growth promoting bacteria, which not only enhance the plant biomass but also help in pollutant degradation as a feedback effect. Recently, researchers have reported the successful removal of a wide range of xenobiotics using the plant-bacteria partnership (Afzal et al. 2014b); however, there is a paucity of reports on removal of OMPs in natural environment. According to one study, introduction of ciprofloxacin in constructed wetlands had an initial adverse effect on the bacterial communities, which were recovered after 2–5-weeks acclimation period, resulting into successful colonization of bacterial communities leading to removal of pollutant and regaining of plant health (Weber et al. 2011). Yet the available information is limited to laboratory scale experiments. Based on the facts, further improvements and optimization in design of the remediation system may bring convincing results at pilot-scale experiments.

Constructed Wetlands in Phytoremediation of OMPs

Till today, different variations of constructed wetlands (CWs) have been developed for the removal of OMPs. CWs are land-based engineered wastewater treatment facilities that comprise shallow ponds, beds, or trenches in which floating or emergent rooted plants are involved in transforming pollutants (Stottmeister et al. 2003). Applying such systems aims at harnessing the interactions among plants, microorganisms, water, soil, and atmosphere to remove contaminants from large water resources (White and Cousins 2013; Ijaz et al. 2015). Hijosa-Valsero et al. studied removal of PPCP (i.e., ketoprofen, naproxen, diclofenac, ibuprofen, carbamazepine, salicylic acid, caffeine, tonalide, galaxolide, and methyl dihydrojasmonate) in seven mesocosm-scale CWs over a period of 9 months (Hijosa-Valsero et al. 2010). Their results illustrated the successful removal in all of the systems; however, soilless CWs showed highest removal efficiency for ibuprofen, ketoprofen, and carbamazepine, whereas free-water CWs performed well for the removal of salicylic acid, ketoprofen, galaxolide, and tonalide. Similarly, Zhang et al. reported successful removal of pharmaceuticals in planted bed reactors compared to the unplanted bed reactors (Zhang et al. 2012). In contrast, weak correlations between removal efficiencies and log K_{ow} suggested that the removal is not well related to the compound's hydrophobicity in subsurface flow CWs. This suggests that the optimization of design parameter can enhance the removal potential even for those pollutants, which have high log K_{ow} values. Another study illustrates that the type of organic matter added to CWs had no apparent effect on removal of pharmaceuticals; nevertheless, the system fed with glucose resulted earlier onset of pollutant removal compared to the system fed with starch, which could be due to higher biofilm development in the system fed with glucose (Matamoros et al. 2008). Further research in this area

especially the role of three main components (substrate, plants, and microbes) in various CW systems may unravel the potential of CWs in OMPs removal. The successful examples of OMPs removal in CW are presented in Table 2.

Enzymatic Degradation of OMPs

OMPs degradation can also be enhanced by direct application of ligninolytic enzymes (e.g., laccases) instead of facilitating microbial population in different environmental compartments as mentioned earlier (Majeau et al. 2010). The reasons behind the direct application are (1) the expression in the microorganisms is controlled by a number of factors and the presence of inhibitors may affect the catabolic genes expression, and (2) there may not be an effective enzyme for a particular OMP for the particular system. Hence, in such situations, direct application of microbial extracts could bring positive results compared to bioaugmentation. As a result, application of such enzymes can polymerize, oxidize, or transform variety of phenolic compounds to less toxic compounds. The substrate ranges for such enzymes are quite diverse and include phenols, EDCs, pesticides, dyes, and polycyclic aromatic hydrocarbons. Laccase production has been widely achieved using different types of fungi and microorganisms. The production of laccase by fungi depends on a number of parameters such as type of species, cultivation procedure, agitation, and aeration. Nevertheless, the most critical factors are the glucose and nitrogen sources, their concentration and the ratio between them, and the nature and concentration of the inducer. The culture growth medium can be synthetic, natural, or semisynthetic, for example, solid lignocellulosic waste in artificial liquid medium (Majeau et al. 2010).

Among microorganisms, basidiomycetes species are extensively studied organisms for laccase production because of their outstanding lignin-degrading capacity. Some species have been reported to produce laccase predominantly, or as the sole ligninolytic enzyme – a characteristic often considered valuable as it can simplify purification procedures for industrial purposes. Some other laccase-producing organisms include *Pleurotus ostreatus* (Hou et al. 2004), *Marasmius quercophilus* (Farnet et al. 2000), *Pleurotus pulmonarius* (Marques De Souza et al. 2002), *Ganoderma adspersum* (Songulashvili et al. 2006), *Pycnoporus cinnabarinus*, and *Pycnoporus sanguineus* (Eggert et al. 1996; Pointing and Vrijmoed, 2000). However, removal of one or many OMPs in wastewater may necessitate the presence of other ligninolytic enzymes to improve decontamination efficiency.

The success of laccase toward OMPs removal can be achieved by the application of (1) purified free enzyme, (2) purified immobilized enzyme, (3) enzymes derived from culture broths, and (4) reactor-based bioremediation with immobilized or free cells. Previous studies have generally focused on oxidative capacity of purified laccase on a variety of contaminants; nevertheless, further efforts have to be made on optimization of real wastewater treatment. Moreover, it has been reported that some phenolic compounds can inhibit the growth of white-rot fungi thereby affecting overall yield (Buswell and Eriksson 1994). However, appropriate dilutions and

selection of appropriate fungal growth stages may reduce the associated adverse effects. Ryan et al. (2005) added small volumes of stripped gas liquor from a coal gasification plant to *Trametes pubescens* culture to induce laccase activity for removal of phenol in the effluent. Furthermore, exploiting appropriate flow rate of wastewater for remediation was shown to be particularly important in experiments with dye-laden effluent (Romero et al. 2006), where higher or lower rates led to laccase deactivation or inefficient system operation, respectively.

Despite their enormous potential, the state of knowledge of parameters regulating production of such enzymes in microorganisms is still in its infancy (Majeau et al. 2010). Moreover, enzymatic degradation of several phenolic compounds has been found to generate unwanted by-products, which are more toxic than the parent molecules. Therefore, enzymatic degradation shall be handled with great care. Moreover, risk assessment of OMPs and their by-products is necessary before implying it at wider scales. The available information is limited in terms of high enzyme production costs. In this regard, lignocellulosic waste has been recognized as a potential source of growing laccase-producing microbial cultures (Lorenzo et al. 2002; Howard et al. 2004); however, further research is necessary to unravel their potential with regard to the economical production of these enzymes.

Biofiltration of OMPs

During biofiltration, removal of contaminants happens due to the action of mixed cultures of microorganisms. Recently, it has been reported that the efficiency of microorganisms toward degradation can be enhanced by optimizing various physiological processes such as oxygen concentration, nutrients availability, temperature, sludge retention time (SRT), and hydraulic retention time (HRT) of the pollutant (Sadef et al. 2014; Sadef et al. 2015). Moreover, it is revealed that the lack of oxygen and higher concentration of inorganic nitrogen reduces OMPs degradation. By contrast, degradation in a trickling filter may not be efficient for compounds with low solids retention time (SRT) and/or hydraulic retention time (HRT), e.g., estrogens (Koh et al. 2008). Another study reported that the highest removal could be achieved at plants with HRT >27 days and SRT >35 days (Servos et al. 2005).

Conclusions and Recommendations

There is an increasing awareness of OMPs as an important group of emerging pollutants. These chemicals are present everywhere but go almost unnoticed due to low concentrations in the environment. The key reason for this relatively recent viewpoint is the necessary sophistication in analytical techniques for detecting these compounds at such low concentrations (Caliman and Gavrilescu 2009). Hence, the environment of many developed countries, once considered as clean and green, is now recognized as contaminated with OMPs. The list of OMPs and their impact on

the environment can be documented in the form of an inventory of already available information in the literature. Such an inventory may include the information regarding the interactions and fate of metabolites in the ecosystems. The information describing persistence, leachability, and toxicity at individual and at complex levels may help deal with and plan for the potential coercions beforehand. Working groups involving intergovernmental and nongovernmental organizations may be formed to discuss potential risks of OMPs and their likely remedies on scientific, managerial, and ethical basis.

Modern nonbiological methods of remediation could be useful for removal of OMPs from the environment. These methods employ a range of physicochemical processes such as advanced oxidation, activated carbon adsorption, membrane ultrafiltration, hydrostatic exclusions, and electrostatic exclusions. In addition, biological transformation of OMPs to innocuous products can be achieved through modifications in conventional bioremediation methods. These methods include biological trickling filter beds, ligninolytic enzyme application (e.g., laccase), membrane biological reactors, constructed wetlands, and plant-bacteria partnership. Moreover, combinations of both nonbiological and biological methods can be interesting to achieve maximum removal efficiency in certain cases. In a nutshell, we are of the view that the authorities responsible for environmental integrity and ecological safety world over must show concern and become proactive before OMPs may instigate any severe environmental impairment. The needs are to (1) develop and adopt robust and cost-effective methods for detection and analyses of different chemicals within the class of OMPs and their metabolites, (2) an improved understanding of their ecotoxicological relevance, and (3) the development of cost-efficient abatement technologies. It is important to assess the environmental fate of OMPs before we move on to risk assessment and hazards management. Finding environment-friendly alternatives to the most harmful OMPs could be a huge step forward. Meanwhile, workable risk assessment approaches must be considered for evaluation of biologically active OMPs. It is time to move into implementation of a multi-trophic environmental risk assessment (ERA) program, as is practiced for commercial release of synthetic agrochemicals and insecticidal proteins produced in transgenic crops (Ullah et al. 2015).

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The Contributions of Mycorrhizas in the Mineralization of Organic Contaminants

Chris O. Nwoko

Abstract This chapter aimed to (a) overview organic pollutants and their sources, (b) introduce mycorrhizas and highlight their relationship with plants and role in the degradation of organics providing some experimental evidence, (c) discuss interaction of mycorrhizas with other soil microbes, and (d) point resultant effect of degradation on soil health.

Keywords Organic pollutants • Mycorrhizas • Degradation • Soil microbes • Soil health

Introduction

Organic Pollutants and Sources

Various types of organic pollutants can be grouped into three categories, namely, (a) hydrocarbons; (b) oxygen, nitrogen, and phosphorus compounds; and (c) organometallic compounds. Probably, the major category is the hydrocarbons and related compounds, which contains such compounds as DDT, dioxins, and polycyclic aromatic hydrocarbons (PAHs). These compounds contain the elements of carbon and hydrocarbon, with some containing chlorine and oxygen as well. There are limited number of types of chemical bonds present, which are principally C-H, C-C, C-Cl, C=C, and C=C (aromatic). All of these bonds are relatively stable and have limited polarity, and this property is then conferred into the related compounds. Some typical structures are shown below (Fig. 1).

The low polarity of these compounds has made them fat soluble (i.e., lipophilic), poorly soluble in water, and persistent in the environment. They have the tendency to sorb in sediment and can be bioaccumulated by organisms (in fatty tissues) and have low concentration in water and air. The lipophilicity of compound can be measured

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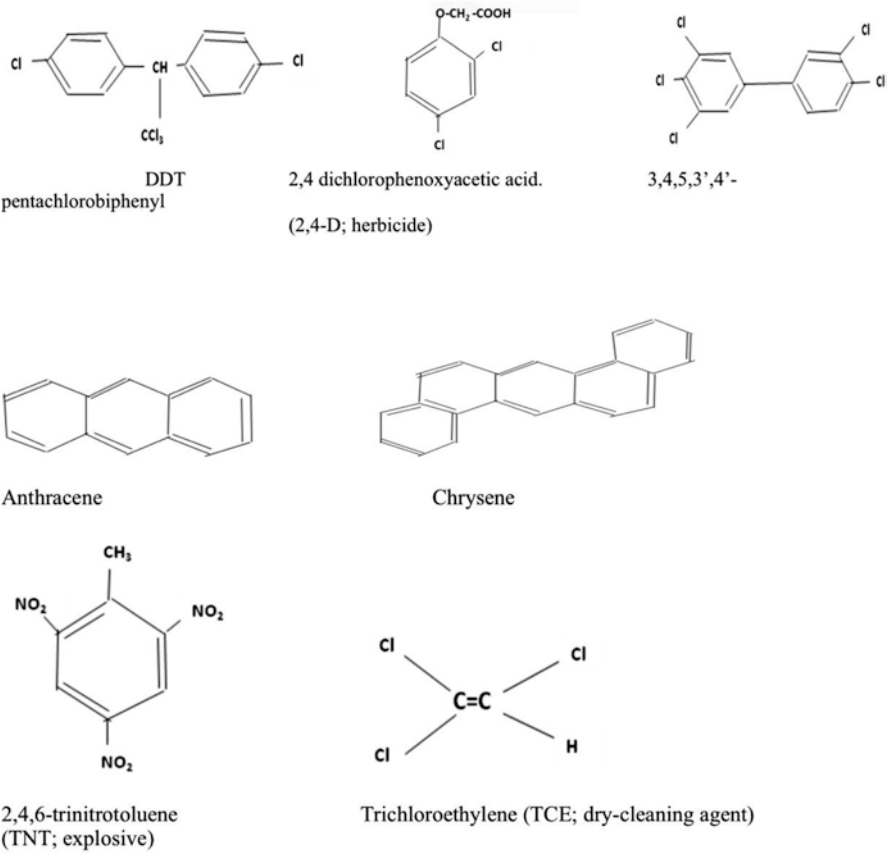

Fig. 1 Representative organic pollutants

in the laboratory as the octanol-water partition coefficient (K_{ow}). This class includes the toxic compounds of abiotic origin 2,3,7,8-tetrachlorodibenzo(1,4)dioxin, also known as 2,3,7,8-TCDD. Notably, the group with oxygen, nitrogen, and phosphorus compounds is very diverse and contains compounds with relatively high solubility in water and low fat solubility and relatively low persistence in the environment. This is due to the presence of bonds with relatively high levels of polarity due to carbon and other atoms being attached to oxygen, nitrogen, or phosphorus conferring a high level of polarity onto the related compounds. Their K_{ow} value is relatively low compared to that of hydrocarbons, and also their bonds are relatively dissolved by environmental processes and are less persistent. The substances within this group only rarely form residues in the environment due to their low persistence, low accumulation in sediments, and low bioaccumulation capacity in organism. The organometallic group is probably the least important from an environmental perspective and includes compounds that are combinations of metal, such as lead (Pb) and tin (Tn), with organic components based on carbon. Organic substances are produced by human society and

are discharged into the environment as sewage, storm water, and industrial waste. Automobile vehicle use and repairs are major sources of petroleum hydrocarbons, polycyclic hydrocarbons, and dioxins, which are often discharged to the atmosphere in particulate form. These particulates are deposited close to busy roadways and urban centers leading to contamination of urban soils and potential human exposure. Agricultural inputs such as pesticides are major sources of pollutants into soil and watercourses. The use of agrochemicals for enhanced agricultural output has gained wide acceptance among farmers across the globe. This practice has doubled the input of hydrocarbon pollutants into the environment. The accidental spillage of petroleum and petroleum products into the environment is the most spectacular source of hydrocarbon pollutants. Over the years, many disasters of this kind have occurred releasing tens of thousands tons of petroleum into the soil and aquatic environment.

The Organism: Mycorrhizas

The name mycorrhizas, which literally means fungus root, are symbiotic associations essential for one or both partners, between a fungus (specialized for life in soils and plants) and a root (or other substrate-contacting organ) of a living plant that is primarily responsible for nutrient transfer. Mycorrhizas occur in a specialized plant organ where intimate contact results from synchronized plant-fungus development. There are five major categories as described in Table 1.

Arbuscular Mycorrhiza

Arbuscular mycorrhizas (vesicular-arbuscular mycorrhizas, VAM or AM) are associations where *Glomeromycete* fungi produce arbuscules, hyphae, and vesicles within root cortex cells. These associations are defined by the presence of arbuscules (Fig. 2a, b).

Ectomycorrhiza

Ectomycorrhizal associations (abbreviated as ECM or EM) are mutualistic associations between higher fungi and gymnosperms or angiosperms. ECM associations consist of a soil mycelium system, linking mycorrhizal roots and storage or reproductive structures. Ectomycorrhizal roots (formerly known as ectotrophic or sheathing mycorrhizas) are characterized by the presence of a mantle and Hartig net. Ectomycorrhizal associations are formed predominantly on the fine root tips of the host, which are unevenly distributed throughout the soil profile, being more abundant in topsoil layers containing humus, than in underlying layers of mineral soil (Fig. 2c above).

Table 1 Major categories of mycorrhizas

Sl. No.	Category	Definition	Host	Fungi
1	Arbuscular mycorrhizal	Associations formed by *Glomeromycotan* fungi in plants that usually have arbuscules and often have vesicles (also known as vesicular-arbuscular mycorrhizas, AM, VAM).	Herbaceous plant	*Glomeromycota*
1.1	Linear AM	Associations that spread predominantly by longitudinal intercellular hyphae in roots	Plant	As above
1.2	Coiling AM	Associations that spread predominantly by intracellular hyphal coils within roots (formerly known as Paris series VAM).		As above
2.0	Ectomycorrhiza (ECM)	Associations with a hyphal mantle enclosing short lateral roots and a Hartig net of labyrinthine hyphae that penetrate between root cells.	Host	Higher fungi (asco-, basidio-, and zygomycetes)
2.1	Cortical ECM	Hartig net hyphae penetrate between multiple cortex cell layers of short roots	Most are gymnosperm trees	As above
2.2	Epidermal ECM	Hartig net fungal hyphae are confined to epidermal cells of short roots	Angiosperms (most are trees)	As above
3.0	Orchid	Associations where coils of hyphae (pelotons) penetrate within cells in the plant family Orchidaceae.		Most are basidiomycetes in *Rhizoctonia* alliance
3.1	Orchid root	Associations within a root cortex.	Orchidaceae	As above
3.2	Exploitative orchid	Associations of myco-heterotrophic orchids.	Orchidaceae (achlorophyllous)	Orchid, ectomycorrhizal, or saprophytic fungi
4	Ericoid	Coils of hyphae within very thin roots (hair roots) of the Ericaceae.	Ericaceae (most genera)	Most are Ascomycetes
5	Subepidermal	Hyphae in cavities under epidermal cells, only known from an Australian monocot genus.	*Thysanotus* spp. (Laxmaniaceae)	Unknown

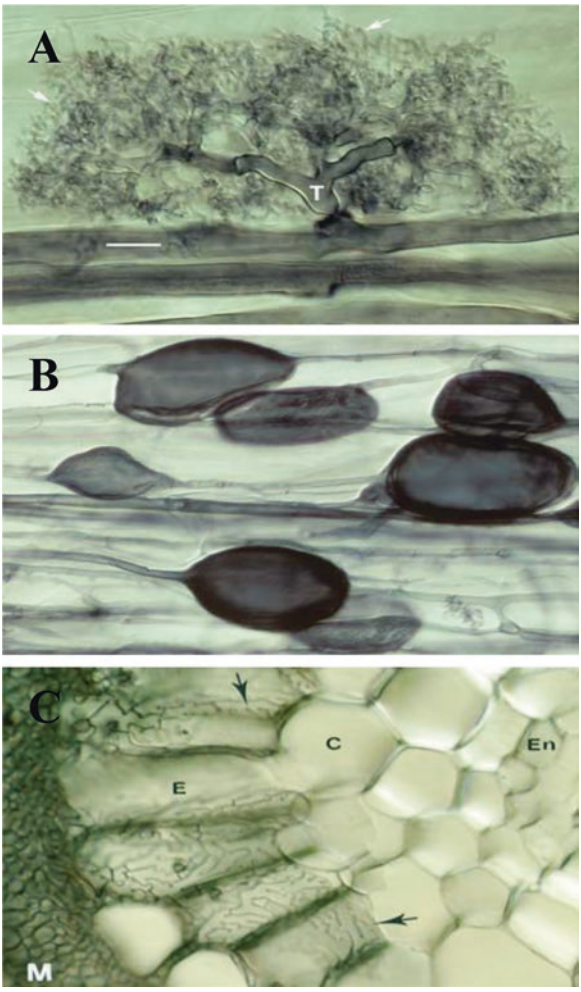

Fig. 2 Arbuscule (**a**) and vesicles (**b**) of a *Glomus* species in a root cortex. Epidermal Hartig net of *Populus* ECM can be seen **c** (Adapted from http://mycorrhizas.info/ecm.html)

The major function of mycorrhizas is nutrient transport. Extra-radical hyphae attached to the root exploit soil outside the root where it absorbs mineral nutrients (mainly N, P, and micronutrients), translocate them back to the root, and transfer them to the host plant in exchange for photosynthetically fixed C in the form of sugars. The fact that these hyphae are fed with C and energy from the host plant gives them an advantage over other microorganisms with respect to growth and active metabolism in nutrient-poor substrates. Mycorrhizas have very different saprophytic capacities. The ericoid mycorrhizal fungi are potent degraders; ECM fungi are moderately capable. However, AM fungi are obligate symbionts with little or no capacity for degradation of organic materials (Michelsen et al. 1996, 1998). All groups of mycorrhiza do, however, interact with and modify the microbial communities that the hyphae encounter in soil, and in this manner, they may all affect microbial degradation processes indirectly.

Mycorrhiza-Plant Relationship

Mycorrhiza-plant relationship has profound beneficial effects on host plant nutrition and physiology. One aspect of these effects is related to stress tolerance of plants, most commonly direct or indirect stress induced by nutrient deficiency. In the case of mycorrhiza, enhanced plant tolerance against other types of stress (toxic metals, salinity, drought, pathogens, etc.) is also a well-established phenomenon (Gianinazzi and Schüepp 1994). The mycorrhiza symbiosis may alleviate plant responses to moderate moisture deficit by several mechanisms, including increased water uptake due to extraction of water in the soil by hyphae (Auge et al. 1992; Davies et al. 1992), altered hormonal levels causing changes in stomatal conductance (Druge and Schonbeck 1992), increased turgor by lowering leaf osmotic potential (Davies et al. 1993), improved nutrition of the host (Johnson and Hummel 1985), and improved plant recovery after drought through improved maintenance of the soil-root continuum (Sweatt and Davies 1984). Mycorrhizal associations seem to help a plant avoid drought effects. When plants are experiencing moderate moisture deficits, they can still photosynthesize and will allocate an increased amount of carbon to roots and mycorrhizas, and hyphal strands of the mycorrhizal fungi will then exploit a greater volume of soil than roots alone, obtaining more water. When plants are experiencing severe moisture deficits, photosynthesis is impaired and less carbon is allocated to roots; therefore, mycorrhizal associations and water uptake decrease.

Soil temperature along with moisture exerts a major influence on mycorrhizal colonization of plants (Braunberger et al. 1997). The influence of temperature on AM fungi is variable; it may be affected by the fungal host species combination, temperature range for germination of the AM fungi, optimal temperature range for photosynthesis of a host plant, and the developmental stage of the plant. Schenck and Smith (1982) noted the efficacy of mycorrhizas at soil temperatures ranging from 18° to 41 °C to have maximum colonization, sporulation, and growth enhancement in soybean. Responses were related to the various combinations of soil temperatures and AM fungal species. Infected root length and number of vesicles increased as temperature increased from 20° to 30 °C in *Eupatorium odoratum* inoculated with *Glomus macrocarpum*. The influence of temperature on AM formation is variable, and optimum appears to be between 18° and 40 °C with most fungal host species combinations exhibiting an optimum near 30 °C. Optimal temperature ranges are dependent on germination temperature of the AM fungi and the optimal temperature of host plant photosynthesis and carbon flow to roots.

The response of AM-inoculated plants to pH has been studied for some very practical reasons including potential negative effects of the hydrogen ion activity on plant productivity via direct effects on the endophyte and host plant physiology and indirect effects via changes in soil processes, e.g., heavy metal and base cation availability (Habte and Soedarjo 1996). AM fungi play an important role in improving plant productivity by enhancing the uptake of nutrients, particularly phosphorus (Smith and Read 1997). The influence of the AM symbiosis in nutrient absorption depends on the uptake capabilities of the host and the endophyte, the extent of colonization of the

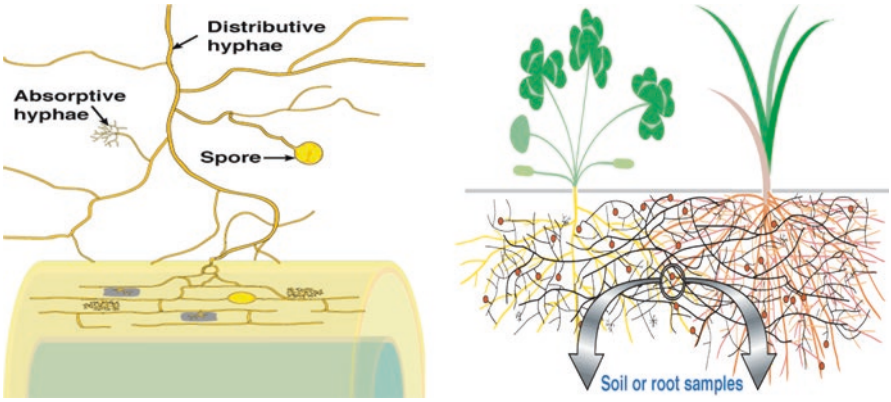

Fig. 3 The structure of mycorrhizas in association with plant roots

root and surrounding soil, and the factors affecting the formation and reproduction of the endophyte symbiosis (Habte 1995). The hydrogen ion activity can affect most, if not all, of these characteristics. For example, availability of *P* in soil is low at all pH values because *P* reacts with soil constituents forming insoluble compounds.

Arbuscular mycorrhizal associations enhance plant acquisition of nutrients by increasing the effective surface area of the root system. Mycorrhizas are especially important for plant survival and growth when the soil has low concentrations of plant available nutrients, especially phosphorus. Mycorrhizal roots are able to obtain more nutrients from deficient soils than roots that are non-mycorrhizal because hyphal strands exploit a greater volume of soil than roots alone. Mycorrhizal roots can use those sources of nutrients that are not available to roots. This could involve an increased rate of nutrient solubilization of insoluble nutrients, production of organic acids that act as chelating agents, and production of enzymes that can degrade soil organic materials, mineralizing nutrients and translocating them to roots. Mycorrhizas in association with plant roots have unique structure (Fig. 3).

Mycorrhizas and Degradation of Organics

Mycorrhiza fungi are known to be indirectly associated with bioremediation processes due to the so-called mycorrhizosphere effect which stimulates soil microbial activity, improves soil structure, and contributes to overall bioremediation of pollutants (Meharg and Cairney 2000; Joner and Leyval 2003a). The activity of AM fungus in the mycorrhizosphere could be a source of different soil enzymes required for biochemical reactions. There are reports indicating that soil enzymatic activities, such as phosphatases and dehydrogenases, are increased by AM fungus inoculation (Kothari et al. 1990). Mycorrhizal fungi do not only increase the absorption surface but also the organic surface through their hyphae and spores, which contributes to

organic pollutant mobilization and binding to the root and consequently to a more complete removal of organics from the medium (Aranda et al. 2013). AM fungi have a large surface area, which enables mycorrhizal fungi to adsorb considerable amounts of pollutants from soil (Rajkumar et al. 2012).

Mycorrhiza modifies root physiology by influencing enzyme activity, exudation, and longevity in a manner that stimulates organics degradation, either by root-derived enzymes or by rhizosphere organisms. This explanation can be supported by several observations reported in the literature: First, mycorrhiza enhances the level of hydrogen peroxide and oxidoreductases in roots (Joner and Leyval 2003a, b; Ma et al. 2003) and the activity of oxidative enzymes in roots and rhizosphere soil (Aranda et al. 2013), which may lead to enhanced levels of peroxidase activity and oxidation of organics around mycorrhizal roots (Criquet et al. 2000). Second, mycorrhizal colonization results in quantitative and qualitative changes in root exudation (Rajkumar et al. 2012), which in turn modify the microbial community colonizing the rhizosphere of mycorrhizal roots. Another mechanism for organic stabilization is the accumulation of organics in spores of mycorrhiza; also the external mycelium may play a significant role in sequestration and accumulation of organics. In addition, AM fungal biomass may indirectly create favorable microhabitat for microbial activity (Rillig and Steinberg 2002), which may also stimulate hydrocarbon degradation.

Experimental Evidence on Organics Degradation

Some studies have shown that the presence of petroleum hydrocarbons in soils is detrimental to the expression of AM fungi associations with the roots of higher plants (Cabello 1997; Leyval and Binet 1998; Davies et al. 2001). Some species of AM fungi such as *Gigaspora margarita* and *Acaulospora delicata* have been detected in soils chronically contaminated by petroleum in Veracruz, México (Varela et al. 2000). Arbuscular mycorrhizal fungal species isolated from petroleum-contaminated soils have been shown to be more efficient in promoting plant growth and nutrient uptake than introduced fungal species (Cabello 1999). In addition, plant survival and growth has been enhanced by the presence of AM fungi in PAH-contaminated soil (Leyval and Binet 1998). The establishment of AM fungi in the root system of some plants may represent an important biological process for the dissipation/degradation of PAH toxicity (Binet et al. 2000; Joner et al. 2002). Research findings have shown that ectomycorrhizal fungi may degrade several recalcitrant compounds such as 2, 4-dichlorophenol (Meharg et al. 1997), 2,4,6-trinitrotoluene (Scheibner et al. 1997), atrazine (Donnelly et al. 1993), polychlorinated biphenyls (Donnelly and Fletcher 1995), and some 3–5 ring PAHs (Braun-Lüllemann et al. 1999). Aranda et al. (2013) in their experiment evaluated the impact of anthracene, phenanthrene, and dibenzothiophene on the AM fungus *Rhizophagus custos*, isolated from soil and contaminated by heavy metals and PAHs, under monoxenic conditions. They found a high level of tolerance in *R.*

custos to the presence of PAHs, especially in the case of anthracene, in which no negative effect on AM-colonized root dry weight (root yield) was observed, and also a decrease in the formation of anthraquinone was detected. Increased PAH dissipation in the mycorrhizal root culture medium was observed; however, the level of concentration and the specific affected dissipation PAH. In another instance, Joner and Leyval (2003a, b) conducted a time course pot experiment to measure dissipation of polycyclic aromatic hydrocarbons (PAHs) in the rhizosphere of clover and ryegrass grown together on two industrially polluted soils (containing 0.4 and 2 g kg^{-1} of 12 PAHs). The two soils behaved differently with respect to the time course of PAH dissipation. The less polluted and more highly organic soil showed low initial PAH dissipation rates, with small positive effects of plants after 13 weeks. At the final harvest (26 weeks), the amounts of PAHs extracted from non-planted pots were higher than the initial concentrations. In parallel planted pots, PAH concentrations decreased as a function of proximity to roots. The most polluted soil showed higher initial PAH dissipation (25% during 13 weeks), but at the final harvest, PAH concentrations had increased to values between the initial concentration and those at 13 weeks (Table 2). Mycorrhiza enhanced PAH dissipation when plant effects were observed. The AM fungi percentage colonization of roots of plants positively affects level of plant physiological development and organics degradation. Nwoko et al. (2013) in their experiment, where they assessed the influence of soil textural class and AM fungi in the performance of *Phaseolus vulgaris* under crude oil contaminated soil, noted that increased AM colonization enhanced physiological characteristics of *P. vulgaris* and total petroleum hydrocarbon (TPH) decomposition.

Interaction of Mycorrhizas with Other Soil Microbes

Mycorrhizosphere bacteria may affect AM fungi and their plant hosts through a variety of mechanisms. Some of these have been more fully studied in ectomycorrhizal fungi (Garbaye 1994), but possibilities include (1) effects on the receptivity of the root, (2) effects on the root-fungus recognition, (3) effects on the fungal growth, (4) modification of the chemistry of the rhizospheric soil, and (5) effects on the germination of the fungal propagules (Fig. 4). From Fig. 4, (1) there is supply of energy-rich C compounds via the hyphae, (2) effects on germination of fungal spores/propagules, (3) modification of soil chemistry and structure, (4) competition for nutrient and other inhibitory and stimulatory compounds, and (5) effects on receptivity of roots to AM/root-fungus recognition.

Colonization of plant roots by AM fungi can affect bacterial communities associated with the roots in both direct and indirect ways. Direct interactions include provision of energy-rich carbon compounds derived from host assimilates, which are transported to the mycorrhizosphere via fungal hyphae, changes in pH of the mycorrhizosphere induced by the fungus, competition for nutrients, and fungal exudation of other inhibitory or stimulatory compounds. Indirect interactions can also take place in the form of mycorrhiza-mediated effects on host plant growth, root exudation, and soil structure.

Table 2 PAH concentrations in two soils as a function of proximity to roots measured after 13 and 26 weeks of cultivation of mycorrhizal and non-mycorrhizal plants

Soil sample	Soil 1 Σ12 PAH (mg kg^{-1})	SEM	Soil 2 Σ12 PAH (mg kg^{-1})	SEM
Start	405	18	2030	64
	13 Weeks			
No plants	348 c	12	1494 cd	50
Non-mycorrhizal Loosely adhering	315 cd	25	1577bcd	99
Strongly adhering	327 cd	13	1801ab	21
mycorrhizal Loosely adhering	311 cd	10	1539bcd	56
Strongly adhering	298d	13	1777b	97
	26 Weeks			
No plants	460 a	19	1763b	47
Non-mycorrhizal Loosely adhering	477a	19	1382de	96
Strongly adhering	413b	20	1689bc	153
Rhizoplane soil	275d	18	1149 ef	164
Mycorrhizal Loosely adhering	435 ab	12	1042 f	62
Strongly adhering	362bc	24	1182ef	92
Rhizoplane soil	222 e	13	655 g	57

Adapted from Joner and Leyval (2003a, b)
Values followed by the same letter within a column are not significantly different ($p > 0.05$, $n = 5$)

The so-called mycorrhiza helper bacteria (MHB) promote the establishment of symbiosis by stimulating mycelial extension, increasing root-fungus contacts and colonization, and reducing the impact of adverse environmental conditions on the mycelium of the mycorrhizal fungi. For example, spore germination and mycelial growth may be enhanced by mycorrhiza helper bacteria through the production of growth factors, through the detoxification of antagonistic substances, or through the inhibition of competitors and antagonists. Xavier and Germida (2003) reported that direct contact between the AM spores and bacteria is necessary for the induction of spore germination in *Glomus clarum*, indicating a ligand-receptor interaction between the two microbes. Spore germination stimulatory bacteria can be accompanied by other bacterial isolates, which produce antagonistic volatiles, suggesting the presence of a complex bacterial consortium on the *G. clarum* spore surface that regulates germination. Also, volatile compounds produced by different species of *Streptomyces* are proved to promote the germination of *G. mosseae* spores (Tylka et al. 1991).

Soil and rhizosphere microbes, including fungi as well as bacteria, are known to contribute to mineral weathering by secreting protons and complexing agents such as low-molecular-weight organic anions or siderophores. In situ evidence exists that ectomycorrhizas and their extramatrical mycelium can locally solubilize minerals (Landeweert et al. 2001), but the roles of the different partners of the ectomycorrhizal

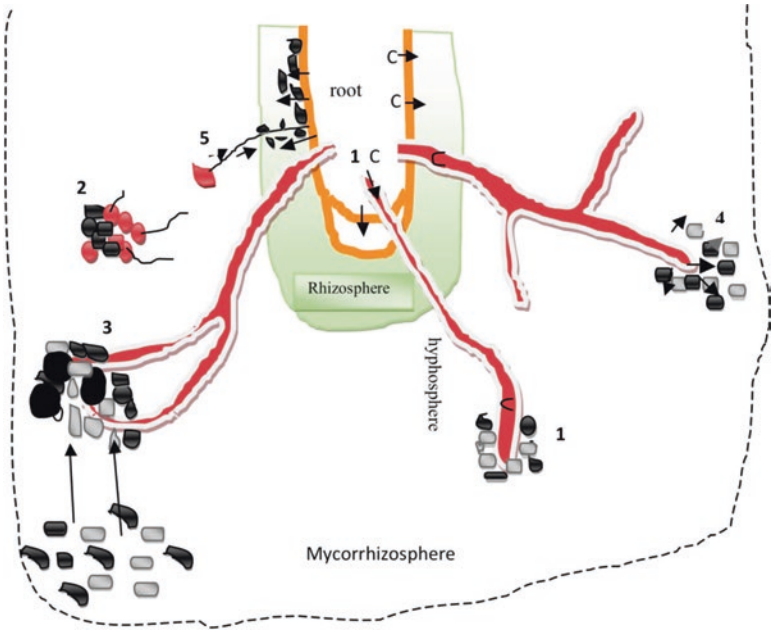

Fig. 4 Schematic representation of interactions among the components of mycorrhizosphere

complex are poorly understood. Nitrogen-fixing bacteria, no doubts, have the potential to influence AM fungi. The presence of genes for N fixation has been shown in endosymbiotic *Burkholderia* sp. (Minerdi, et al. 2001), but expression of this activity at levels significantly influencing the growth of the mycorrhizal association is yet to be demonstrated. *Rhizobium* spp. may act synergistically with AM fungi on their plant hosts. Nodulation and N fixation are commonly increased in legumes following AM colonization, probably because the mycorrhiza supplies the plant and the rhizobacteria with P, which is essential for the enzymes involved in the N fixation process. Nitrogen fixation further promotes mycorrhizal development (Puppi et al. 1994). Some mycorrhizosphere bacteria may be able to promote mycorrhizal establishment through improved spore germination (Tylka et al. 1991), but so far there are no direct demonstrations of this in the field. The colonization enhancement may also be mutual between associated microorganisms, and this has been reported following dual inoculation of *Pseudomonas sp*ecies and *Glomus* sp., which additionally increased the growth of the host plant in an additive manner (Meyer and Linderman 1986).

One major problem in characterizing effects of bacteria on AM fungi is the difficulty of identifying the active bacteria against a consortium of biological community of immense diversity. There are no doubts that mycorrhizosphere bacteria have a number of potential effects on AM fungi. The challenge then remains whether effects demonstrated under laboratory conditions can be reproduced in the field.

Earthworms, an important soil organism, contribute to the maintenance of soil properties and aid in the cleanup of contaminated soil. Earthworms accumulate

heavy metals from soil and thus can withstand metal toxicity (Morgan et al. 1989). Interaction between earthworm and mycorrhiza results in rapid remediation of heavy metal contaminated soils. Earthworm activities result in rapid colonization rate in tropical plants (Yu et al. 2005). This interaction significantly increased the amount of Cd removed from the soil, and this was attributed to the production of phytohormones by earthworms, which may have stimulated mycorrhizal infection. Earthworms also contribute to the effective dispersal of the fungi propagules through their feeding habits. Gange (1993) showed that earthworm casts contain more than ten times the number of infective mycorrhizal propagule in surrounding soils. On the other hand, earthworms may also contribute to the disconnection of mycorrhizal fungi from plant root as they feed and burrow through the soil (Ma et al. 2006). The combined effect of earthworm and mycorrhiza on soil remediation is complex; the mechanism involved in this relationship is not fully understood. However, Lebron et al. (1998) argue that the relationship depends on the plant species the fungi colonize.

Resultant Effect of Degradation on Soil Health

Arbuscular mycorrhizal fungi influence microbial population and activity and consequently nutrient dynamics in the soil through the release of organic compounds. AM fungi may directly or indirectly contribute to soil C and N dynamics. The activity of AM fungus in the mycorrhizosphere could be a source of different soil enzymes required for biochemical reactions (Dubey and Fulekar 2011). Kothari et al. (1990) reported increased soil enzymatic activities such as phosphatases and dehydrogenases when AM fungus was inoculated in soil to aid remediation. Most of the organic phosphorus (Po) mineralizing activity occurs at the rhizosphere where phosphatases released from plant roots (Helal and Dressler 1989) and soil microorganisms (Asmar et al. 1995) are present. Researchers in this field assert that the soil alkaline phosphatase (ALP) activity increases due to the presence of mycorrhizal hyphae and AM mycelia may have P mineralizing activity (Jayachandran et al. 1992; Tarafdar and Marshner 1994). Acid phosphatase activity ACP (Fox and Comerford 1992) as well as AM colonization (Joner and Jakobsen 1995) can be induced by P deficiency.

Mycorrhiza establishment changes both quantitatively and qualitatively the microbial populations in the rhizosphere. Plants grown in mycorrhizospheric soil are often more competitive and better able to tolerate environmental stresses than normally grown plants, potentially enhancing pollutant availability and plant tolerance (Gaur and Adholeya 2004). Mycorrhizosphere development process by the inoculation of mycorrhiza has been reported to modify the quality and abundance of rhizospheric microflora and alter overall rhizosphere microbial activity which may be responsible for the bioremediation in the contaminated soil (Khan 2006) mycorrhizosphere provide the better water and nutrient acquisition by the plants.

Conclusions

The hydrocarbons and related compounds containing DDT, dioxins, and PAHs are among the major organic pollutants. As a result of the low polarity, these compounds are soluble in fat (i.e., lipophilic) and also are poorly soluble in water and persistent in the environment. Notably, organic substances can be produced by human society and discharged into the environment as sewage, storm water, and industrial waste. Pesticides are major sources of pollutants from agricultural inputs into soil and watercourses. Mycorrhizas, literally means fungus root, occur in a specialized plant organ where intimate contact results from synchronized plant-fungus development. Mycorrhiza-plant relationship has profound beneficial effects on host plant nutrition and physiology. In fact, arbuscular mycorrhizal associations enhance plant acquisition of nutrients by increasing the effective surface area of the root system. Mycorrhiza fungi are known to be indirectly associated with bioremediation processes due to the so-called mycorrhizosphere effect. AM fungi have a large surface area, which can enable mycorrhizal fungi to adsorb considerable amounts of pollutants from soil. In context with pollutant's degradation effects on soil health, AM fungi have the capacity to influence microbial population and activity and consequently nutrient dynamics in the soil through the release of organic compounds and directly or indirectly contributing to soil C and N dynamics.

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Remediation of Mine Tailings and Fly Ash Dumpsites: Role of Poaceae Family Members and Aromatic Grasses

Ilika Ghosh, Manosij Ghosh, and Anita Mukherjee

Abstract Phytoremediation is an established technique for amelioration of soil contaminated with complex mixtures of heavy metals of anthropogenic origin. Coal fly ash and mine tailings include a conglomerate of heavy metals such as Cr, Pb, Hg, As, Ni, Cd, Cu, Mn, and Fe depending on the source of coal/ore and cause large-scale ecotoxicity. Unreclaimed mine tailing sites and coal fly ash dumpsites are a world-wide problem, presenting a source of contamination for nearby communities. The disposal sites are subject to erosion and are major causes of air pollution. Phytoremediation using plants for in situ stabilization and immobilization of these heavy metal-contaminated sites has gained momentum in the past few decades due to its cost-effectiveness and environmental sustainability. In this regard, the use of grasses is of prime importance due to their rapid growth, large biomass, resistance to phytotoxicity, and genotoxicity by heavy metals as compared to herbs, shrubs, and trees. Phytostabilization by the compact root system of grasses retards the formation, mobility, and bioavailability of hazardous leachates by high uptake and accumulation of the complex mixtures of heavy metals within them. Such grasses prevent natural succession by weeds and other plants leading to safe grazing by animals. Among the members of Poaceae, aromatic grasses are economically important plants due to their essential oil production. They rank higher than edible grasses, which are susceptible to heavy metal contamination in their edible parts. Various biochemical and molecular mechanisms govern the ADME (absorption, distribution, metabolism, excretion) of heavy metal contaminants in grasses growing in mine tailings and fly ash dumpsites. Metal-binding phytochelatins, metallothioneins, and antioxidant enzymes have key functions in these mechanisms. This chapter encompasses the role of members of

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Poaceae and aromatic grasses in phytoremediation of mine soil and coal fly ash with emphasis on their biochemical and molecular mechanisms.

Keywords Poaceae • Aromatic grass • Phytoremediation • Fly ash • Mine tailings • Heavy metals

Introduction

The inception of urbanization and industrialization has brought about enormous global economic development and improvement of human life. Mining activities and coal-based thermal power generation are indispensable aspects of such progress that enable civilizations to flourish. Nevertheless, the worldwide environmental ramifications of the by-products accompanied with mining and power generation cannot be ignored. The closure of mining operations leads to the occurrence of mine tailings forming hazardous industrial wastes (EPA 2015; Mendez and Maier 2008). Fly ash, being the most abundant component of coal incineration residue, produced during power generation, has tremendous ecotoxicological effects in all biological systems (Carlson and Adriano 1993; Borm 1997). Mine tailings comprise heavy metals (HMs), which are the most abundant components along with metalloids, polycyclic aromatic hydrocarbons (PAHs), explosive materials, and radioactive substances (Mendez and Maier 2008). Fly ash consists of crystalline, irregularly shaped particles containing oxides of essential and nonessential HMs and unburnt residual carbon with an average particle size of 257.86 μm (Ghosh et al. 2015) and high surface-area-to-mass ratio (Ctvrtnickova et al. 2009). Each year, millions of tons of HMs mainly Pb, Zn, Cd, Cu, Mn, As, Mo, Hg, and Fe are generated by mine tailings and fly ash dumpsites (Nriagu and Pacyna 1988; Moore and Luoma 1990; Dwivedi et al. 2012). Among the above metals, Fe, Cu, Mg, Zn, and Mn are the essential micronutrients, although they create toxic consequences at elevated concentrations (White and Brown 2010). The other metals Cd, As, Pb, and Hg are nonessential and hence have detrimental effects at relatively low concentrations (Cappa and Pilon-Smits 2014). Therefore, Pb, Zn, Cd, As, Cu, and Hg are termed as metals of tremendous environmental concern due to their ability to jeopardize ecosystems through the food chain/web (Clemens 2006; Verbruggen et al. 2009). These HM contaminants occur in elevated concentrations in both mine tailings and fly ash dumpsites. Mine tailings spread across vast areas of land rendering them barren. The contaminants can migrate to distant places by soil erosion and water-mediated runoff (Schwegler 2006). Fly ash dumpsites occur as large areas of damp or dry ash ponds, and the particles migrate to far off areas by water leachate formation and eolian dispersion, respectively (Chakraborty and Mukherjee 2009). Therefore, both abandoned mine wastelands and fly ash ponds provide the most challenging environments for flora and fauna to thrive. The magnitude of damage is highest in plant, animal, and human communities living around the tailings and fly ash dumpsites. The hazardous effects of such sites are well

documented in all biological systems from prokaryotes to higher eukaryotes (Borm 1997; Babula et al. 2008; Chakraborty et al. 2009, Chakraborty and Mukherjee 2011). The conventional modes of riverine and marine disposal of mine wastes cause aquatic toxicity leading to biomagnification of contaminants to affect human life through the food web (Grimalt et al. 1999). The practice of keeping fly ash landfills wet to prevent airborne particle migration in turn leads to the formation of toxic water leachates that are rich in HMs (Sikka and Kansal 1995). Therefore, it is of urgent need to resort to safe methods of mine tailing reclamation and fly ash dumpsite remediation. Hence, phytoremediation is the best cost-effective alternative technology of ecological engineering that utilizes the ability of a number of plant species to take up, accumulate, metabolize, and detoxify contaminants (particularly HMs) from soil to mitigate such environmental hazards (Cunningham et al. 1995).

Ideal plants for phytoremediation should have the potential to grow and spread over vast areas rapidly, have high biomass, tolerate and accumulate high concentrations of HMs in their roots or shoots, and metabolize them for detoxification with their innate physiological, biochemical, and molecular mechanisms (Cunningham and Ow 1996). In this context, members of the family Poaceae are of prime importance as they suit all the above criteria. Moreover, grasses have an added advantage of an elaborate root system with underground rhizomatous stems that enable them to immobilize contaminants in the soil, prevent water leachate formation and soil erosion, and grow rapidly over contaminated regions to form a robust green cover (Pandey and Singh 2014; Chakraborty and Mukherjee 2011). This in turn facilitates safe grazing by animals and provides an aesthetic landscape. Aromatic grasses, on the other hand, are the best candidates for phytoremediation of a range of contaminated areas due to the advantageous feature of essential oil production (Gupta et al. 2013). Remarkably, the essential oils are free of any contamination and are produced in larger amounts under abiotic stress (Verma et al. 2014). Based on the level of HM tolerance, phytoremediating grasses can be divided into the categories of accumulator, hyperaccumulator, and excluders. Accumulators can survive despite the concentration of HMs in the aerial parts. Compared to accumulator plants, hyperaccumulator plants take up relatively higher concentrations of HMs by the roots and concentrate them in their aerial plants without any harm to the plant body (Rascio and Navarilzzo 2011). Excluder plants accumulate HMs within their roots and stall their uptake and transport into the shoots (Ali et al. 2013; Malik and Biswas 2012).

The phytoremediating potential of both aromatic and nonaromatic grasses growing in mine tailings and fly ash dumpsites can be better understood by studying the details of absorption, distribution, metabolism, and excretion (ADME), which indicates HM uptake, transport, breakdown, and detoxification, respectively, within the plant body. This process is appropriately regulated by various physiological mechanisms involving cellular ultrastructural changes, altered xylem and phloem transport, changes in photosynthetic and respiratory machinery, symbiotic rhizosphere microbial associations, phytoextraction, phytostabilization, rhizofiltration, phytodegradation, phytovolatilization, and vacuolar compartmentalization (Hall 2002; EPA 1997). These physiological mechanisms comprise the first line of defense for innate HM resistance. Various reports confirm the ability of HMs to generate reactive oxygen

species (ROS), which is the underlying reason behind their toxic effects in cellular organelles and macromolecules (Agrawal et al. 2011; Nagajyoti et al. 2010). In grasses, ROS-induced oxidative stress is alleviated by a series of biochemical defense mechanisms mainly involving enzymatic and nonenzymatic antioxidants, organic acids, and phytohormones. Such biochemical responses can be activated in grasses under HM stress (Shah et al. 2001; Schützendübel and Polle 2002). On the molecular level, novel ways of HM detoxification and maintenance of HM ion homeostasis have been adapted by grasses by the synthesis of HM-chelating peptides phytochelatins (PCs) and metallothioneins (MTs) (Clemens 2001; Lal 2010; Yang et al. 2005b). Expression of quantitative trait loci that code for genes controlling HM uptake and genetic variations for phenotypic changes also occurs in a number of grasses exposed to HMs (Yun et al. 2015). Alterations in the expression pattern of numerous genes regulating the synthesis of phytochelatins and metallothioneins take place under HM stress for increased synthesis of PCs and MTs with increasing concentrations of HM ions within the cells (Cobbett 2000). Substantial progress has been made in the study of phytoremediation of HM-contaminated areas for revegetation and restoration of ecosystems. In recent years, the study of molecular mechanisms of phytoremediation by grasses is steadily gaining popularity for their HM-resistant characteristics, and further such studies are needed in aromatic grasses. This chapter provides a thorough insight into the remediation of mine tailings and fly ash dumpsites using grasses and aromatic grasses emphasizing on the physiological, biochemical, and molecular mechanisms of HM tolerance within the plant body.

Heavy Metal-Contaminated Areas and Their Ecotoxicological Implications

Mine Tailings

Mine tailings are abandoned mining lands, which are left behind after the economically valuable metal fractions are completely separated from an ore. They occur as vast areas of unreclaimed unfertile land and pose worldwide ecological health risks. The most difficult environmental challenge of mining activities is the management of mine tailings. There are innumerable such sites around the world, and the USA alone has 500,000 abandoned mine sites (Carrillo-Chavez et al. 2003; EPA 2015). Mine tailings give rise to problems such as soil erosion-induced migration of contaminants from spoil dump slopes, increased sedimentation loads on catchment areas of rivers near the mining area, and contamination and degradation of soil in surrounding areas (Verma et al. 2012). These areas contain complex mixtures of HMs such as Fe, Mn, Pb, Zn, Cu, Ni, Cr, etc. depending on the source of ore. The HMs individually are known to cause a range of toxic effects affecting various metabolic pathways through different mechanisms in all biological systems. Mine waste disposal is conventionally carried out by riverine discharge (Dold 2014). Additionally, marine disposal involves the drainage of the wastes into deep stratified waters of oceans that are

below the euphotic zone to prevent mixing with the surface water (Dold et al. 2011). Both the methods are highly undesirable as they cause direct toxicity of the aquatic ecosystem; bioaccumulation of HMs along the food web, eventually affecting human health, decreased biodiversity and altered benthic habitats (Grimalt et al. 1999).

Toxicity of mine wastes in different biological systems is well documented (Babula et al. 2008). Pb/Zn mine tailings increased the mortality of the earthworms *Pheretima guillelmi* and *Eisenia fetida* (Ma et al. 2002). Similar results were observed in *E. fetida* exposed to abandoned uranium mine soil (Antunes et al. 2008). Abandoned mine wastes cause genotoxic responses in *Allium cepa* as studied by the cytogenetic endpoints of mitotic index and chromosome aberration (Geras'kin et al. 2011; Geremias et al. 2010). Studies in terrestrial plants such as *Spinacia oleracea*, aquatic plants such as *Nymphaea* sp. and *Spirodela polyrhiza*, and numerous aquatic algae, viz., *Scenedesmus* sp., *Micrococcus* sp., *Chlorella* sp., *Scenedesmus* sp., *Cladophora* sp., etc., demonstrated reduced growth ratio, chlorosis, high proline content, and increased activity of antioxidant defense mechanisms (Kilic and Donmez 2007; Notton and Hewitt 1974; Pandey et al. 2005; Travieso et al. 1999; Vernay et al. 2007). Pb mine tailings also caused detrimental responses in *Vicia faba* which can be attributed to the generation of oxidative stress (Probst et al. 2009). Human beings working and living in the vicinity of mine tailings are at high risk of HM-induced health effects. Reports indicate elevated levels of toxic HMs from the mines in their blood and urine leading to a variety of toxic responses mainly affecting the respiratory, digestive, and excretory systems (Bodénan et al. 2004; Colín-Torres et al. 2014; Krysiak and Karczewska 2007; Lim et al. 2008; Schonfeld et al. 2014).

Fly Ash Dumpsites

Thermal power plants utilizing coal contribute approximately 40% of the global electricity production (Montes-Hernandez et al. 2009) and produce large amounts of fly ash as a by-product (15–30% of the total residues) of pulverized coal combustion (Carlson and Adriano 1993). Moreover, low-grade lignite coal incineration magnifies the content of HMs in the fly ash to exacerbate its hazardous effects (Baba et al. 2008; Baba and Türkman 2001; Pandey et al. 2009). Recent estimates reveal a yearly global fly ash production of 600 million tons, covering about 3235 km^2 of land (~799,386 acres) by the end of 2015 (Pandey and Singh 2012). Approximately 10% of the global fly ash production is contributed by the USA and Europe, while India and China contribute about 18% each. India meets about 80% (80,201.45 MW) of its energy demands from such thermal power plants resulting in its emergence as one of the largest producers of fly ash in the world. In India, fly ash dumpsites cover nearly 65,000 acres of land producing more than 120 million tons every year which is estimated to rise to 150–170 million tons per year at the termination of the 11th 5-year plan (Pandey et al. 2009; Dwivedi et al. 2012). Despite such enormous production, only 30% of global and less than 20% of Indian fly ash production is utilized annually (Jala and Goyal 2006; Haynes 2009). Dry fly ash dumping sites promote the

migration of the particles to distant regions and polluting large areas of land and air. Therefore, to prevent air pollution, these landfills are usually kept wet to form large fly ash ponds resulting in runoff of toxic fly ash leachate containing complex mixtures of HMs into the water table and surrounding water bodies (Sikka and Kansal 1995; Chakraborty and Mukherjee 2009). Fly ash comprises essential and nonessential HMs such as Zn, Pb, Cu, Ni, Cd, As, Cr, Hg, Se, Mn, Si, Co, B, F, Fe, Ca, Mg, Na, K, and Al, radioactive substances, and aromatic hydrocarbons (Scotti et al. 1999; Rai 1987; Patham et al. 2003; Ruwei et al. 2013). It has both crystalline and amorphous phases (Gupta et al. 2002) with an average particle size of 257.86 μm with irregular shapes and sizes as studied by scanning electron microscopy (Ghosh et al. 2015). Airborne fly ash particles have an average diameter of <10 μm with high surface-area-to-mass ratio (Petruzzelli et al. 1987). Fly ash leachate is highly alkaline with a pH range of 8.5–12.5 and high salinity (Singh and Yunus 2000). The composition, pH, and particle size are all dependent on the physicochemical properties of the parent coal and its processing (Gupta et al. 2002).

The alkaline pH of fly ash and absence of nitrogen and phosphorus jeopardize the physicochemical character of soil, although it is rich in essential and nonessential minerals (Gupta et al. 2002). Deposition of fly ash on leaves of plants inhibits normal physiological processes of transpiration and photosynthesis. Fly ash particles accumulate in guard cells and prevent stomatal closure to prevent transpiration (Krajickova and Majstrick 1984). On the other hand, high foliar deposition creates thick barriers that occlude water vapor loss, impede light exposure, and reduce photosynthetic rate (Gupta et al. 2002). Numerous studies have established the toxic effects of fly ash in plant systems due to the high concentrations of HMs, which can be attributed to the generation of reactive oxygen species (Ali et al. 2004; Sinha et al. 2005; Valko et al. 2006). The toxicological effects of fly ash include genotoxicity to aquatic organisms such as *Palaemonetes pugio* (grass shrimp) (Kuzmick et al. 2007) and terrestrial plants such as *A. cepa* (Chakraborty and Mukherjee 2009, 2011; Chakraborty et al. 2009; Ghosh et al. 2012; Ghosh et al. 2015) and *Nicotiana tabacum* (Chakraborty and Mukherjee 2009), oxidative stress in the leguminous tree *Prosopis juliflora* (Sinha et al. 2005) and freshwater fish *Channa punctata* (Ali et al. 2004), cytotoxicity in Chinese hamster ovarian cells (Garrett et al. 1981), pulmonary toxicity with impaired alveolar macrophage functions and bronchiolar and alveolar epithelium hyperplasia in mice (Kirchner et al. 1983), severe fibrosis in Sprague-Dawley rats (Schreider et al. 1985) mutagenicity by Ames test and sister chromatid exchange in human peripheral blood lymphocytes (Kleinjans et al. 1989), genotoxicity in human peripheral blood and lymphocytes (Chakraborty and Mukherjee 2009), and red blood cell hemolysis (Gormley et al. 1979; Liu et al. 1987). Fly ash exposure among workers caused chronic bronchitis, emphysema, massive fibrosis, and lung and stomach cancer (Borm 1997).

Hence, both mine tailings and fly ash dumpsites engender tremendous ecotoxicological effects involving all biological systems. In view of their toxic responses, it is prudent to carry out ecological restoration programs using phytoremediating plants to revegetate these areas. In this context, HM accumulator, hyperaccumulator, and excluder grasses are being widely used to form a green cover over such vast areas of barren land. These grasses hold the soil tightly within their root systems to

immobilize contaminants. In the process, they prevent migration of contaminants by soil erosion, water leachate formation, and wind dispersal leading to the alleviation of land, water, and air pollution, respectively. Therefore, phytoremediation, using grasses, takes advantage of the characteristic features of the family for HM cleanup.

Poaceae and Its Use in Phytoremediation

Characteristics of the Family Poaceae

Family Poaceae is one of the largest and nearly ubiquitous monocotyledonous angiosperm families, consisting of approximately 650–765 genera and more than 6000 species. The family characters have been described in literature (Prain 1903; Watson 1990; Soreng and Davis 1998). They are usually characterized by elaborate fibrous or adventitious root systems; underground rhizomatous stems with nodes and internodes; simple, alternate, exstipulate, sessile leaves with parallel venation; and an open, tubular sheath at the leaf base that surrounds the internodes. The inflorescence is a compound spike with bracteates and sessile, incomplete, bisexual or unisexual, zygomorphic, hypogynous, homochlamydeous flowers. Membranous scales called lodicules represent the perianth. The androecium contains 3–6 stamens with long filaments, dithecous, and versatile anthers, and the gynoecium consists of monocarpellary unilocular single ovule with basal placentation, short style that can be present or absent, bifid stigma, and superior ovary. Fruits are usually called caryopsis with the pericarp fused with the seed coat. Seeds have one cotyledon called scutellum, which remains embedded within the endosperm. Most grasses contain phytoliths with silica in the epidermal cells (Twiss et al. 1969). The above characteristic features of the family impart resistance to a wide range of environmental extremities including HM toxicity. Therefore, they are used for green capping of large areas of fly ash ponds and mine tailings for environmental restoration and ecological remediation programs. Members of the family Poaceae can be segregated into the categories of aromatic and nonaromatic grasses on the basis of essential oil production. Aromatic grasses are characterized by the production of copious amounts of essential oils in their roots or leaves or both, whereas nonaromatic grasses are usually pasture grasses (*Cynodon dactylon*) or food crops (*Triticum aestivum*, *Zea mays*, *Oryza sativa*, *Hordeum vulgare*) or cash crops (*Saccharum* sp.) and tree members (*Dendrocalamus* sp. and *Bambusa* sp.). The widely cultivated aromatic grasses belong to the genera of *Vetiveria* sp. (vetiver grass) and *Cymbopogon* sp. (lemongrass). The phytoremediating potential of nonaromatic and aromatic grasses is described in the succeeding sections.

Phytoremediation by Nonaromatic Grasses

Over the past few decades, there is an exponential growth in the use of members of the family Poaceae for the clearance of complex mixtures of HMs from mine tailings and fly ash dumpsites. A wide array of grass species is employed for the

phytoremediation of HM-contaminated soil. Early reports of HM tolerance of grasses for phytoremediation authenticate the resistance of *Agrostis tenuis* and *A. stolonifera* to high levels of Cu, Pb, Zn, and Ni in mine tailings (Bradshaw 1952; Gregory and Bradshaw 1965; Jowett 1958, 1964). In contrast to shrubs and trees, grasses produce large biomass, grow rapidly, and have innate resistance to biotic and abiotic stresses (Chu 2008; Gilbert 2000; Loch 2000; Xia et al. 1999; Ye et al. 2000). The profuse, compact, fibrous, rhizomatous root systems with high surface area form mesh-like soil coverings to retard the mobilization of HMs and their hazardous leachates by a process known as phytostabilization, which involves the uptake and accumulation of HMs into the roots. Hence, they prevent the occurrence of natural succession by other HM-susceptible weeds and herbs to facilitate safe grazing by animals and provide an aesthetic landscape (Chakraborty and Mukherjee 2011). Grasses have proved to be excellent cost-effective plants for the restoration and revegetation of HM-contaminated soil due to their rapid HM uptake and clearance potential and low maintenance requirements (Truong 2004). Conversely, trees and shrubs have long generation time which is a major impediment for the identification and selection of HM-tolerant species for eventual usage for phytoremediation (Dickinson et al. 1991).

Grasses are widely used to mitigate HM pollution from abandoned mine sites for ecological restoration. *Lolium multiflorum*, a HM-tolerant grass with an extensive root system, grown in mine tailings containing high concentrations of Cu, Zn, Pb, and Mn exhibited high HM extraction, bioaccumulation, phytostabilization, and rapid growth (Mugica-Alvarez et al. 2015). It forms a vegetative cover to prevent soil erosion and contamination of distant areas. Other reports reveal the ability of the grasses *C. dactylon*, *Paspalum notatum*, and *Imperata cylindrica* to stabilize Pb/Zn mine tailings (Shu et al. 2004). Similar results were observed in *Piptatherum miliaceum* exposed to Pb and Zn, showing hyperaccumulation of Pb and efficient extraction of Zn from mine tailings (García et al. 2004). *Poa pratensis* and *Phragmites communis* growing in Mn mine tailings (Liu et al. 2006) and *Phragmites australis* grown in Cu mine wastes (Chiu et al. 2006) showed considerable HM tolerance and accumulation for revegetation.

Fly ash dumpsites are most effectively remediated by plants belonging to the family Poaceae. Reports have established the ability of *Agropyron elongatum*, *Festuca arundinacea* (Mulhern et al. 1989), *F. rubra* and *Calamagrostis epigejos* (Mitrovic et al. 2008), *T. aestivum* (Kalra et al. 1997), *O. sativa* (Mishra et al. 2007), and *Lolium perenne* (Matsi and Keramidas 1999) to resist the hazardous effects of HMs in fly ash and produce high yield. *H. vulgare* and *Sorghum bicolour* seedlings were highly resistant to high proportions of fly ash treatments in media and soil as seen by successful germination and increased seed weight (Bilski et al. 2011). Other studies indicate the usage of grasses such as *Miscanthus x giganteus* for effective rehabilitation of fly ash dumpsites (Técher et al. 2012b). These grasses can be utilized for assisted phytoremediation for quick green capping of fly ash ponds (Pandey and Singh 2014). Naturally growing species of grasses such as *Saccharum munja* (Pandey et al. 2012), *Saccharum spontaneum* (Maiti and Jaiswal 2008; Pandey and Singh 2014; Pandey et al. 2015), and *S. ravennae* (Rau et al. 2009) colonize fly ash landfills at a rapid rate with high bioaccumulation of HMs and low translocation

into the shoots, for efficient HM stabilization. Along with *Saccharum* sp., other grasses such as *C. dactylon*, *S. bengalense*, *Dactyloctenium aegyptium*, *Cyperus esculentus*, *Typha latifolia*, *Fimbristylis bisumbellata*, *Phragmites karka*, and *Eragrostis nutans* are natural colonizers of fly ash landfills (Maiti and Jaiswal 2008; Maiti et al. 2005; Pandey et al. 2012). All the above grasses work together for HM clearance and create suitable soil conditions for other susceptible plant species to thrive after complete remediation by them (Pandey et al. 2015).

In recent years, field-based experiments involving grasses for phytoremediation are being carried out to assess the extent to which each plant species can perform HM cleanup. The advantageous features of grasses fulfill the objectives of HM clearance from contaminated soil; control of land, water, and air pollution; landscape improvement; and overall environmental safety. In this context, the choice of appropriate grass species for assisted phytoremediation is a vital factor. Native species that naturally colonize the contaminated sites can be utilized in larger quantities for rapid HM clearance. Crop rotation can also accelerate the restoration of soil fertility for ecological succession.

Phytoremediation by Aromatic Grasses

Aromatic grasses possess all the characteristics of nonaromatic members of Poaceae for excellent phytoremediation. They are superior candidates than other Poaceae species as they are perennial, unpalatable, and cost effective; have minimal water requirements; tolerate extreme stresses (drought, salinity, pH variation, HM toxicity); and render substantial economic returns in the form of essential oil production (Gupta et al. 2013). They also check air pollution by stalling dust flow especially in fly ash dumpsites and provide a shady environment or green capping over the land providing moisture conservation for other native plants to thrive after the remediation process. The microbial associations with their roots induce nutrient bioavailability and optimal nitrogen levels for other plant species to grow and reproduce. After extraction of essential oils, the solid wastes can be recycled back to the contaminated zones to enhance fertility (Verma et al. 2014). Along with high HM uptake and clearance, the phytoremediating edible grasses may pass on the contaminants along the food chain/web through their edible parts. Conversely, aromatic grasses are nonedible due to their silica phytoliths and therefore pose no risk of toxicity to other organisms of the food chain/web. Moreover, animals do not destroy these grasses due to their copious essential oils or secondary metabolites (Gupta et al. 2013). The essential oils of these grasses are excluded from HM contamination as they are extracted through hydro-distillation (Lal et al. 2013; Verma et al. 2014; Zheljazkov et al. 2006). These essential oils are of immense industrial importance. They are used in perfumes, soaps, cosmetics, insect repellents, aromatherapy, and medicines (Quitans-Junior et al. 2008; Rao 1999). Various independent studies have reported the use of the aromatic plants such as *Mentha piperita, Lavandula angustifolia, Matricaria recutita, Thymus vulgaris, Salvia officinalis,*

Mentha arvensis, Ocimum basilicum, and L. officinalis for remediation of soil from HM toxicity without affecting the yield or quality of their essential oils (Zheljakov and Nielsen 1996a, b; Zheljakov et al. 2006; Kumar and Patra 2012; Lydakis-Simantiris et al. 2012; Angelova et al. 2015). Following this principle, the use of aromatic grasses has gained popularity in phytoremediation programs due to the aforementioned advantageous features. Interestingly, it has been found that the essential oil production of aromatic grasses is enhanced under abiotic stress due to elevated synthesis of secondary metabolites to alleviate such stress (Farooqi et al. 1999). For instance, the essential oils extracted from *Vetiveria zizanioides*, a highly resistant aromatic grass, grown in HM-contaminated areas showed increased yield, slight changes in the chemical composition, and negligible contamination with HMs (Prasad et al. 2014; Danh et al. 2011). Similarly, other high-value aromatic grasses such as *Cymbopogon citratus*, *C. martini*, *C. flexuosus*, and *C. winterianus* perform uptake and stabilization of HMs with high essential oil and biomass production (Lal et al. 2008, 2013; Kumar and Maiti 2015; Patra et al. 2015). The essential oils occur in the roots of *V. zizanioides*; leaves of *C. winterianus*, *C. citratus*, and *C. flexuosus*; and the leaves and inflorescence of *C. martini*. In case of *V. zizanioides*, essential oil production in roots remains unaffected by HMs due to the activation of HM-chelating and compartmentalization mechanisms (Andra et al. 2009a; Andra et al. 2010). In all the above grasses, the accumulation of HMs mainly occurs in the roots as seen by low translocation into the shoots indicating their phytostabilization ability (Danh et al. 2009; Das and Maiti 2009; Ghosh et al. 2015; Kumar and Maiti 2015). Hence, the essential oils produced in the leaves are excluded from HM contamination.

The revegetation of abandoned mine tailings and mine overburden sites can be successfully carried out by assisted phytoremediation techniques using aromatic grasses. This cost-effective process is a fairly nascent ecological engineering technology, which takes advantage of the abiotic stress-resistant characteristics of aromatic grasses for HM clearance and restoration of soil fertility. *V. zizanioides* is the most extensively used aromatic grass due to its high tolerance to a wide variety of HMs such as Cd, Pb, Zn, Cr, As, Ni, Hg, Cu, and Se as well as temperature variations, soil salinity, and acidity (Danh et al. 2009). It is both xerophytic and hydrophytic and cosmopolitan in distribution (Sinha et al. 2013). Although it is a HM stabilizer plant, studies show that its roots and shoots can accumulate more than approximately five times the amount of Cr and Zn levels present in the soil (Truong 1999). Numerous reports over the past decade have elucidated the ability of *V. zizanioides* for rehabilitation of mine tailing and overburden sites involving ores of Pb/Zn (Chiu et al. 2006; Shu et al. 2002a; Pang et al. 2003), Cr/asbestos (Kumar and Maiti 2015), Pb (Meeinkuirt et al. 2013), Cu (Das and Maiti 2009), Fe (Roongtanakiat et al. 2008), and Cu and Au (Knoll 1997; Radloff et al. 1995; Truong 1999). A variety of non-fertile cultivars of vetiver such as Sunshine, Fort Polk, Vallonia, Boucard, Huffman, Monto, and Haiti grow successfully on the steep slopes of abandoned mines and, as a result, avert soil erosion (Joy 2009). DNA fingerprinting involving RAPD (random amplified polymorphic DNA) analyses revealed stark similarities in the genotypes of these cultivars which are

all resistant to soil erosion due to their tight, dense, mesh-like arrangement of root systems (Adams and Dafforn 1997). This data also shows that all the above cultivars have evolved from the Sunshine genotype due to their similar RAPD profiles (Adams and Dafforn 1997; Adams 2000; Joy 2009). Along with *V. zizanioides*, aromatic grasses belonging to the genus *Cymbopogon* sp. have also been studied for their tolerance to high concentrations of HMs yielding positive results. *C. citratus* accumulated HMs in their roots exhibiting phytostabilizing potential for remediation of Cr/asbestos mine wastes with the assistance of organic manures (Kumar and Maiti 2015). Similarly, this grass can be used for the rehabilitation of coal mine overburden dumps (Sen et al. 2014) and Cu mine wastes (Das and Maiti 2009). Other species such as *C. flexuosus* is tolerant to Cr (Patra et al. 2015), Pb, Hg, and Cd (Handique and Handique 2009), and *C. winterianus* is resistant to Cd toxicity (Boruah et al. 2000). In all the aforementioned reports, the accumulation of HMs was highest in the roots of the plant, which is similar to *V. zizanioides*. Hence, both the aromatic grasses show similar phytoremediation mechanisms for revegetation of mine wastelands.

Works on the phytoremediating ability of aromatic grasses on fly ash are limited. The use of aromatic grasses such as *C. flexuosus*, *C. winterianus*, *C. martini*, and *V. zizanioides* has been proposed for the remediation of fly ash landfills (Verma et al. 2014; Pandey 2015). Pioneering studies employing *V. zizanioides* and *C. martini* grown in fly ash-treated soil generated high yield due to higher availability of the essential plant nutrients and resistance to HM toxicity (Adholeya et al. 1997; Sharma et al. 2001). Studies by Chakraborty and Mukherjee (2011) and Ghosh et al. (2015), using *V. zizanioides* grown in different proportions of garden soil amended with fly ash, for 3 and 18 months, respectively, revealed higher bioaccumulation of HMs in the roots than that of shoots and low translocation into the shoots. Both the studies showed a mesh-like dense root system of the grass that tightly bound the fly ash-amended soil to itself for immobilization of HM contaminants within the fly ash particle aggregates. These observations established the ability of the grass to prevent the lightweight fly ash particles to get carried away by wind, hence checking air pollution. Moreover, it creates a vegetative cover to impede the formation of toxic fly ash leachate into groundwater and nearby water bodies. The stabilization of HMs within the roots also renders safe grazing of the shoots by animals, therefore preventing the HMs to contaminate the ecosystem through the food chain. Genotoxicity studies showed no DNA damage in this grass grown in soil amended with fly ash for 3 months as analyzed by alkaline comet assay (Chakraborty and Mukherjee 2011). Similarly, the model plant *A. cepa* was used to study the genotoxic potential of soil amended with different proportions of fly ash before and after remediation with *V. zizanioides* over a period of 18 months (Ghosh et al. 2015). This revealed a marked decrease in the genotoxic ability of the remediated soil after 18 months of exposure to vetiver system compared to the non-exposed soil. Therefore, it can be ascertained that aromatic grasses can successfully remove hazardous HMs from fly ash and alleviate its ecotoxicological effects without any phytotoxic effects on their own plant body.

Therefore, aromatic grasses offer ecological restoration and socioeconomic benefits in the process of restoration of HM-contaminated sites. Ecological engineering strategies using these grasses will prove advantageous over other members of Poaceae for ecological restoration, alleviation of ecotoxicity, and protection of human and animal life from HM hazards.

ADME of Heavy Metal Contaminants in Grasses

The key process of ADME within the plant body in phytoremediating grasses governs the fate of HMs. Grasses take up, transport, metabolize, and excrete HMs from contaminated soil by a concordant assemblage of physiological, biochemical, and molecular processes. Mainly with the aid of root exudates and symbiotic microorganisms in the rhizosphere, the extensive root systems of grasses accumulate HMs from abandoned mine wastes and coal fly ash dumpsites (Rau et al. 2009; Lorestani et al. 2011). Enhanced uptake of HMs is facilitated by the process of bioactivation which involves the mobilization of HMs in the soil as most HMs are not absorbed easily by the roots due to their low mobility. Hence, in order to combat low bioavailability of HMs, grasses form symbiotic microbial associations that help them secrete root exudates of protons and organic acids, which decrease the pH of soil to increase metal dissolution, and metal-chelating amino acids/peptides that bind to HMs in the rhizosphere for enhanced uptake by the roots (Yang et al. 2005a). Under normal circumstances, water and essential inorganic minerals are taken up, transported through the xylem, and transpired by the leaves. Two main physiological processes of phytoextraction and phytostabilization occur during phytoremediation, where HMs are either stored in the roots or translocated to the shoots, respectively (Evangelou and Deram 2014). These aspects have been elucidated in the next section. Therefore, ADME involves the enhanced bioactivation of HMs from the rhizosphere into the plasma membranes of root cells; distribution by metal transporters into apoplasts/symplasts and binding to cell walls; metabolism by innate biochemical pathways, metal chelators, and various ligands in the cytoplasm; and detoxification by transpiration or sequestration into vacuoles (Yang et al. 2005a). Figure 1 demonstrates an overview of the fate of HMs within the plant body of a grass.

HM uptake is regulated by the concentration of HMs within the plant body and plant biomass (Roongtanakiat 2009). Based on HM uptake, grasses fall within the categories of accumulators and excluders (Sinha et al. 2004). Accumulator grasses concentrate the HM contaminants in their aerial parts and metabolize them into nontoxic inert forms within the shoot tissues. Hyperaccumulator grasses perform the uptake of toxic HM ions at levels more than thousand ppm and store them in their shoots with a shoot: root-metal-ion ratio greater than unity (Roongtanakiat 2009). The excluders on the other hand restrict the uptake of HMs within the root systems (Tangahu et al. 2011). In these processes, a range of structures situated within the plasma membrane of root cells come into play, viz., proton pumps, protein channels, and co- and antitransporters (Tangahu et al. 2011). Numerous stud-

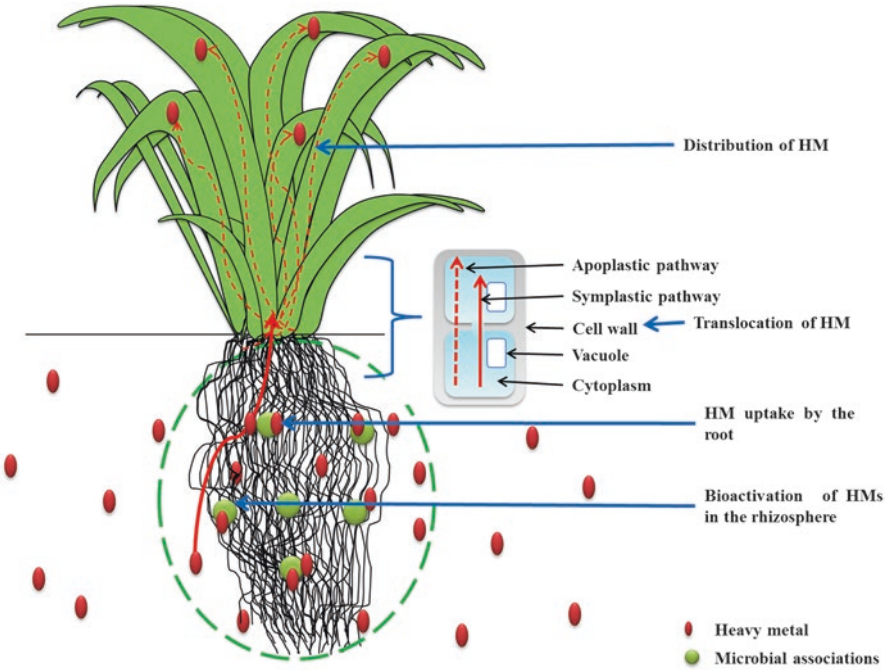

Fig. 1 Diagrammatic representation of ADME of heavy metal (HM) contaminants within the plant body of grass HMs are taken up from the rhizosphere by bioactivation with the aid of symbiotic microorganisms and root exudates, translocated from roots to shoots via apoplastic/symplastic transport, metabolized within the plant cells, and detoxified by vacuolar compartmentalization within roots or shoots

ies have highlighted the secretion of phytosiderophores as a characteristic feature of all members of Poaceae for HM mobilization and bioactivation in the rhizosphere (Lone et al. 2008). Cereal crops such as *T. aestivum*, *O. sativa*, *H. vulgare*, and *Z. mays* mainly release them during Fe deficiency for enhanced mobilization of Zn, Fe, Mn, and Cu in the soil (Romheld 1991). Root exudates of organic acids are important HM-chelating substances within the rhizosphere. The roots of *H. vulgare* and *Z. mays* and avenic acid released organic acids such as mugeneic and deoxymugeneic acids by *Avena sativa* (Welch and Norvell 1993), which facilitate the mobilization of HM contaminants. Symbiotic microbial associations such as arbuscular mycorrhiza and nitrogen-fixing bacteria help in the phytoextraction and degradation of HM pollutants (Lone et al. 2008). The root cell wall micropores also facilitate passive HM uptake for their degradation within the root cells (Hinchman et al. 1995). High uptake and accumulation of HMs such as Cd, Zn, Pb, Cu, Cr, and As from mine wasters and fly ash from field as well as in pot-scale experiments were observed in the roots of the grasses *Paspalum distichum* and *C. dactylon* (Shu et al. 2002b), *Miscanthus x giganteus* (Nsanganwimana et al. 2015), *V. zizanioides* (Pang et al. 2003; Chantachon et al. 2004; Rotkittikhun et al. 2007;

Andra et al. 2009a; Chakraborty and Mukherjee 2011; Ghosh et al. 2015), *C. flexuosus* (Handique and Handique 2009; Patra et al. 2015), *Echinochloa crus-galli* and *O. sativa* (Sultana et al. 2015), *T. aestivum* (Singh et al. 2010), *Thysanolaena maxima* (Rotkittikhun et al. 2007), and *Deschampsia cespitosa* (Mehes-Smith and Nkongolo 2015). These results specify the ability of these grasses for the functions of phytoextraction and phytostabilization, where the HM contaminants are sequestered within the shoots and roots, respectively.

The distribution of HMs from the roots to the aerial parts is measured by the parameter of translocation factor (TF), which is denoted by the ratio of HM concentration between shoot and root. *C. citratus* (Israila et al. 2015), *Sorghum bicolor* (Soudek et al. 2014), *Z. mays* (Rosas-Castor et al. 2014), *L. perenne* (Pricop et al. 2010), and *Poa pratensis* and *Festuca arundinacea* (Bosiacki and Zieleźiński 2011) exposed to complex mixtures of HMs performed the distribution of HMs from roots to shoots as seen by TF values greater than or equal to unity. Translocation takes place through the xylem and phloem via apoplastic/symplastic and ion exchange routes (Yang et al. 2005a). Different transporter proteins located in the plasma membrane and vacuole tonoplast regulate such distribution of HMs within the plant body (Krämer et al. 2007). HMs are taken up by the roots and translocated into the shoots by hyperaccumulator plants. On the other hand, such root to shoot translocation is not carried out by phytostabilizing grasses, where HMs are restricted to the roots. HMs are distributed into various parts such as trichome, cell wall, epidermis, mesophyll, and vacuoles (Krämer et al. 2000). Hence, hyperaccumulator grasses have evolved many classes of metal transporter proteins, which are involved in metal uptake, distribution, and homeostasis (Manara 2012). These transporters are good candidates for the careful control of intracellular levels of HMs. This occurs at the transcriptional level by the control of initiation rates and differential mRNA splicing or at the posttranscriptional level by targeting mRNA stability (Yang et al. 2005b). The primary transporters are CPx-type ATPase, P_{1B}-type ATPases, Nramp (natural resistance-associated macrophage protein), CDF (cation diffusion facilitator), ZIP (zinc ion permease), ABC (ATP-binding cassette), and HMA and CaCA (calcium calmodulin) families of proteins (Manara 2012); among which CPx-type ATPases take part in the overall metal ion homeostasis, distribution, and tolerance (Williams et al. 2000). CPx-type ATPases and Nramp have been studied in *O. sativa*, where OsNramp1 and OsNramp2 genes were found to code for these protein transporters for Mn (Belouchi et al. 1997). Additionally, the P_{1B}-type ATPases regulate Cu transport in *O. sativa* (Lee et al. 2007). Studies revealed substantial downregulation of amino acid and peptide transporter genes in *O. sativa* upon Cu stress (Sudo et al. 2008). The upregulation and downregulation of these transporter genes signify the pattern of HM distribution from root to aerial parts.

The processes of HM metabolism and detoxification occur conjointly in quick succession. HMs are metabolized within the root and shoot tissues by different metabolic pathways and biochemical processes as well as HM-chelating ligands and proteins. The end product of such metabolism is the formation of simpler nontoxic forms of complex mixtures of HM contaminants (Yang et al. 2005a). Phytochelatins and metallothioneins are important chelating peptides that bind HMs to form pep-

tide-HM complexes that are sequestered into vacuoles with the assistance of transporter proteins such as ABC transporters (Clemens 2001). Phytohormones such as auxins, salicylic acid, jasmonic acid, abscisic acid, and ethylene promote the mitigation of HM stress (Viehweger 2014). The detailed role of these biochemical processes and HM chelators is elucidated in the upcoming sections.

HM detoxification occurs by phytovolatilization in many plant species where water-soluble HMs are excreted in the form of vapors from the leaves by transpiration through the vascular system (Malik and Biswas 2012). Along with phytovolatilization, the chelated HMs are removed by intracellular compartmentalization within specialized structures such as vacuoles (Jabeen et al. 2009). During this process, the expression of vacuolar HM ion transporters such as TgMTP1 is enhanced for the amplification of HM sequestration within the vacuoles (Persans et al. 2001).

Therefore, the ADME of HMs encompasses all the mechanisms of phytoremediation to minimize HM exposure and sustain the physiological concentration of essential HMs for environmental cleanup.

Mechanisms of Phytoremediation

In the past few decades, remarkable progress has been made to unravel the mechanisms of phytoremediation at the physiological, biochemical, and molecular levels. Investigations on the tolerance of grasses to toxic concentrations of HMs are being carried out in pot-scale and field-based studies. A conglomerate of mechanisms takes place within the plant body for its protection from HM toxicity during remediation of soil. Both hyperaccumulator and tolerant grasses growing in mine tailings and fly ash dumpsites behave as solar-driven pumps for their ability to extract and concentrate HMs from the contaminated sites (Boros et al. 2014). The physiological, biochemical, and molecular mechanisms of phytoremediation in members of Poaceae and aromatic grasses are elucidated in the following sections.

Physiological Mechanisms

Various physiological processes are effective during ADME of HMs, for their elimination. Hyperaccumulator and resistant plants achieve HM protection for phytoremediation using the two main physiological strategies of avoidance and tolerance (Hall 2002). These strategies are used by grasses to assist in the remediation of HM contaminants from mine tailings and fly ash dumpsites involving the direct contribution of basic physiological processes of photosynthesis, respiration, mineral nutrition, transpiration, and symbiotic microbial associations (Emamverdian et al. 2015). Ultrastructural changes take place in individual plant cells where the integrity of cellular structures and their functions are altered for

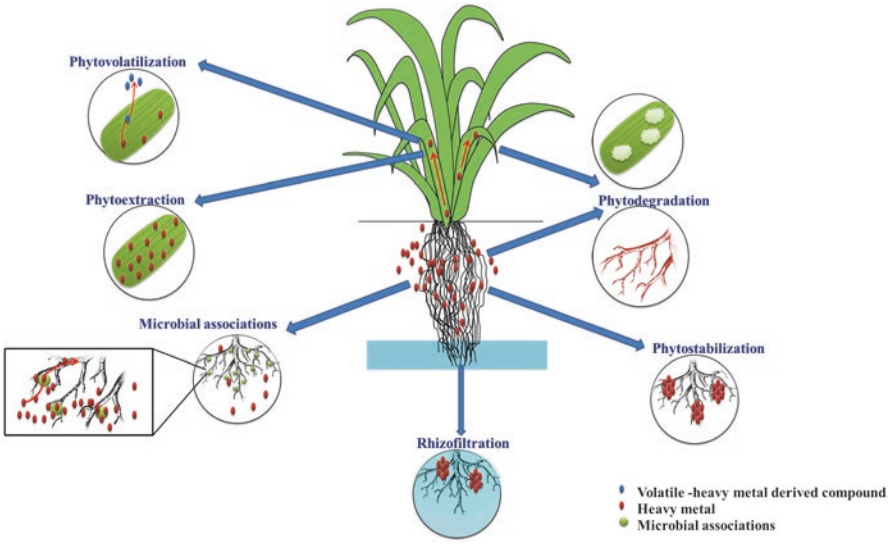

Fig. 2 Diagrammatic representation of the physiological mechanisms underlying grass-mediated remediation of heavy metals (HMs). Grasses remove HMs by their innate physiological mechanisms of rhizofiltration, microbial associations, phytoextraction, phytostabilization, phytodegradation, and phytovolatilization for remediation of HMs from soil without any phytotoxic responses

HM stress resistance. These physiological defenses come into play during the five broadly classified physiological mechanisms of phytoremediation-phytoextraction, phytodegradation, phytostabilization, rhizofiltration, and phytovolatilization (Fig. 2). These mechanisms cumulatively reduce the HM mass in the contaminated soil and its leachates in water bodies (EPA 1997). Furthermore, compartmentalization is the final physiological stage of HM sequestration inside intracellular compartments.

Ultrastructural Changes

The uptake of HMs is avoided by the alteration of membrane permeability and changes in the HM binding ability of the cell wall (Kvesitadze et al. 2001). Tolerance to HMs is exhibited by cellular compartmentalization and alterations in cell membrane structure. Significant amounts of HMs accumulate at the plasma membrane-cell wall interface, which can presumably be the main site of metal tolerance (Krzesłowska 2011). Ion passage by passive diffusion across membranes is inhibited by alterations in the molecular arrangement of membranes. A fall in free ion activity on the cell wall biomolecules rather than decreased diffusion across the cell wall is effective in avoiding HM uptake (Verkleij and Schat 1990). Increased HM binding ability of the cell wall by its pectin and protein fractions imparts resistance to HMs.

Binding of HMs to the cell wall occurs electrostatically by stable bond formation depending on the metal's coordination chemistry. HM binding stimulates modifications in the cell wall architecture increasing its capacity to accumulate metal ions and decreasing its permeability for HM migration into the protoplast (Krzesłowska 2011). The increase in low-methylesterified pectins or polysaccharides is an important alteration in the cell wall structure that promotes the binding of divalent and trivalent metal ions (Verkleij and Schat 1990). Pioneering studies in HM-tolerant grasses *T. aestivum* (Hossain et al. 2006; Tabuchi and Matsumoto 2001) and *Z. mays* (Schmohl and Horst 2000; Schmohl et al. 2000) have shown an increase in pectin level and low-methylesterified pectin fraction of the cell wall when exposed to Al. Similarly, in *C. dactylon*, the Al-resistant cultivars showed 33% higher accumulation of Al in the cell wall compared to the Al-sensitive cultivars (Ramgareeb et al. 2004). Cd exposure promoted increased synthesis of the cell wall polysaccharides, pectins, and hemicelluloses in *O. sativa* imparting higher metal resistance (Xiong et al. 2009). Zn resistance is directly correlated with increased Zn binding to the cell wall of *A. tenuis* (Turner and Marshall 1972). Transmission electron microscopy (TEM) studies in *Z. mays* exposed to Al and Zn revealed membranous configurations of myelin figure associations with the metals and the polysaccharide callose of the cell walls, which was confirmed by immunocytochemistry (Vázquez 2001). These results suggest an avoidance mechanism by altered HM binding to the cell wall. Within the cell, electrons are obtained from cytochrome P450 of the respiratory electron transport system for the activation of oxygen during HM stress (Kvesitadze et al. 2001). This requires coordination between the mitochondria and endoplasmic reticulum, which results in accumulation of microsomes and enhanced close contact between mitochondria and microsomal membranes (Gordeziani et al. 1999).

Changes in Basic Physiological Processes

The process of photosynthesis is highly sensitive to HMs in higher plants. Degradation of photosynthetic pigments is observed in plants growing in high concentrations of HMs, which causes alterations in the light-harvesting protein complexes. This creates a deficiency in the light-harvesting capacity, which ultimately culminates into impaired photosynthetic machinery of the plant (Mazhoudi et al. 1997). Zhang et al. (2014a) studied the effect of Cd-rich soil in *V. zizanioides* (vetiver grass) and *Pennisetum americanum* × *Pennisetum purpureum* (bana grass). The two grasses showed no change in chlorophyll content and rate of photosynthesis upon Cd stress affirming their ability to remediate Cd-rich soil without affecting their photosynthetic machinery. Translocation of metals from root to shoot is mainly controlled by leaf transpiration (Adhikary 2015). In the same study, the rate of leaf transpiration and water content increased in bana grass indicating the translocation of metals from root to shoot, while it remained unchanged in vetiver grass corroborating its phytostabilizing activity. In terms of mineral nutrition, the translocation factors of the essential metals were analyzed. The results showed a decrease in Fe concentration in the roots of vetiver grass and no change in the translocation factors

of Fe, Cu, Zn, Mn, Ca, Mg, and K. On the other hand, a decrease in Fe and Mn concentration and translocation factors of K and Zn took place in the roots of bana grass (Zhang et al. 2014b). Similarly, vetiver grass grown in lower concentrations of Pb/Zn mine tailing proportions showed no alterations in leaf chlorophyll content, photosynthetic photochemical activity, photosynthetic rate, and water potential (Pang et al. 2003). *Pennisetum purpureum* × *P. typhoideum* (king grass) grown in Cd-rich soil showed significantly higher chlorophyll content in young leaves and no change in mature leaves (Zhang et al. 2014b). Among the photosynthetic parameters, the net photosynthetic rate (Amax), light saturation point (LSP), and light compensation point (LCP) were enhanced. The ability of the plant to utilize the highest and lowest levels of light is reflected in terms of LSP and LCP, which measure the relationship between photosynthesis and light levels (Zhou et al. 2010; Chen et al. 2011). Increased LSP and LCP indicates enhanced and reduced light use efficacy at high and low light levels, respectively. LCP denotes the light value when the rate of CO_2 fixed during photosynthesis is equal to the rate of CO_2 released during respiration and photorespiration (Nunes et al. 2009). An increase in LCP reveals higher respiration and Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) oxygenase activity as well as lower RuBisCO carboxylase activity (Nunes et al. 2009). The mineral nutrition pattern in response to Cd showed increased concentration of Zn, Mg, and Ca in roots; Zn, Cu, Mg, and Ca in stems; and Zn, Mg, and K in leaves, whereas a decrease in the concentration of Mn and Ca took place in stems and leaves, respectively (Zhang et al. 2014b). Increased concentrations of Mg, Zn, Cu, and Ca is a defense response to Cd stress because enhanced Mg in chloroplasts protects against the binding of Cd to chlorophyll, and increased Ca concentration prevents the replacement of Ca by Cd in proteins to combat Cd-induced inhibition of photosynthesis (Faller et al. 2005; Küpper and Kochain 2010). The above findings demonstrate the relationship between photosynthesis, respiration, and mineral nutrition processes for phytoremediation. The results inspire further elucidation of the protective mechanisms that get activated during HM stress for the continuation of normal cellular process in these plants.

Microbial Associations

The microbial consortia of the rhizosphere symbiotically interact with the roots of phytoremediating grasses to enhance HM uptake (Emamverdian et al. 2015). It has been hypothesized that inoculation of HM-contaminated soil with nitrogen-fixing bacteria and arbuscular mycorrhizae facilitates soil remediation and overall ecosystem reconstruction (Wong 2003). Numerous studies have confirmed the remediating potential of grasses in symbiosis with mycorrhiza and nitrogen-fixing bacteria. These microorganisms enhance HM uptake by the excretion of organic compounds, which increase bioavailability and root absorption of HMs (Meharg 2003). They also alter the chemical properties of HMs directly influencing HM solubility (Sabir et al. 2015). Mycorrhizae such as ericoid and ectomycorrhizal fungi associated with the roots of *C. dactylon* help in phytostabilization by excreting organic acids that chelate HMs in the rhizosphere. These fungi transform the HMs into nontoxic forms

and facilitate higher uptake by the roots (Meharg 2003). The hyphae of most mycorrhizal fungi contain HM-binding polyphosphate that can bind more than 60% metals and retain them in the apoplast of their cell walls or vacuolar compartments (Bücking and Heyser 1999; Tam 1995; Hall 2002; Yang et al. 2005a). Similarly, arbuscular mycorrhizal (AM) symbioses utilize the plant photosynthetic assimilations and in return enhance mineral nutrition status in plants (Upadhyaya et al. 2010). They chelate HMs from the soil matrix by forming phosphate-metal complexes or producing glycoproteins inside the hyphae (de Andrade and da Silveira 2008; Garg and Chandel 2010). They also release heat shock proteins and glutathione (Hildebrandt et al. 2007), decrease pH of the contaminated soil (Bano and Ashfaq 2013), and sequester HMs within cortical cells, mycelium, cell wall, vacuole, and other organelles (Turnau 1998; Hall 2002). *L. perenne,* a HM-tolerant grass, having AM associations, showed a decreased translocation of Ni, Zn, and Cd from roots to shoots confirming the phytostabilization potential of AM symbiosis (Takács et al. 2001). Similar results were observed in *Z. mays* associated with the AM fungus *Glomus mosseae*, which facilitated the binding of the HMs Cu and Pb with organic matter and absorbed them into its organs, hence stalling metal uptake (Huang et al. 2005). On the other hand, the AM fungi *G. verruciforme* and *G. etunicatum* prevented ROS damage in *T. aestivum* exposed to Cd with high activity of the antioxidant enzymes ascorbate peroxidase (APX) and glutathione peroxidase (GPX) in roots and shoots (Abad and Khara 2007). The AM fungus *G. intraradices*, in association with *Z. mays* growing under Cr stress, induced a rise in chlorophyll content exhibiting its ability to protect the photosynthetic machinery (Rahmaty and Khara 2011). Similarly, vetiver grass roots colonized with *G. mosseae* in Pb-contaminated soil showed increased chlorophyll content and plant biomass and decreased levels of thiols (Punamiya et al. 2010). These results corroborate the high phytoextraction capacity of vetiver grass. Symbiotic AM fungi consortium comprising of *G. intraradices*, *G. albidum*, *G. diaphanum*, and *G. claroideum* in symbiotic association with *H. vulgare* exposed to Pb showed enhanced uptake of Pb, which was greater in roots than that of shoots, affirming high immobilization of Pb by the AM association (Arias et al. 2015). In a study by Rau et al. 2009, the rhizobacteria of *S. ravennae* growing in a fly ash dumpsite are protected against toxic concentrations of HMs as seen by improved seedling establishment, plant weight, and shoot length, hence promoting plant growth. In *Z. mays* grown in soil overlying coal fly ash, colonization with the AM fungi *G. mosseae* and *G. versiforme* increased crop yield and protected the plants from excess Na accumulation proving the ability of the fungi to promote crop establishment in soils overlying coal fly ash dumpsites (Bi et al. 2003). Hence, microbial symbiosis with the phytoremediating members of Poaceae and aromatic grasses enhances their ability to remediate HM-contaminated soil.

Phytoextraction

Phytoextraction is the process by which HM-accumulating plants transport and concentrate HMs from contaminated soil into the harvestable aerial parts (Sabir et al. 2015). Hyperaccumulator plants use this mechanism to extract HMs, which can be

recovered by harvesting these plants. This mechanism takes place either by the natural physiological ability of the plants, which is termed as continuous phytoextraction, or by induced phytoextraction by the artificial addition of chelating agents such as ethylene diamine tetraacetate (EDTA) (Andra et al. 2009a), ethylenediamine disuccinate (EDDS) (Andra et al. 2011), and other organic amendments (Shutcha et al. 2010). Phytoextraction of Pb, Zn, and Cd mine wastes is performed by *V. zizanioides* (Chantachon et al. 2004; Chen et al. 2012b; Roongtanakiat and Sanoh 2011; Shu et al. 2004; Wilde et al. 2005), *F. arundinacea*, and *L. perenne* (Pricop et al. 2010); Ni by *P. pratensis* and *F. arundinacea* (Bosiacki and Zieleziński 2011); Cd and Zn by *Eleusine indica* (Garba et al. 2013) and Zn by *H. vulgare* (Ebbs and Kochian 1998) and *Lygeum spartum* (Conesa et al. 2007); and Pb and Zn by the natural colonizers of mine tailings *C. intybus* and *C. dactylon* (Del Rio-Celestino et al. 2006).

Phytostabilization

HM-tolerant plants perform phytostabilization to limit HM mobility and bioavailability in the soil by mainly using their root systems and rhizosphere microorganisms, thereby preventing further environmental degradation through airborne spread or leaching into the groundwater table (EPA 2000). The major benefit of this mechanism over phytoextraction is that the disposal of toxic HMs from the resultant plant biomass is not required (Jadia and Fulekar 2009). In this regard, grasses are the most suitable plants due to their elaborate fibrous or rhizomatous root systems that penetrate deep into the soil and create a firm initial ground cover. They form a protective barrier between the contaminated soil and the environment and check the distribution of toxic contaminants to distant areas by soil erosion (Raskin and Ensley 2000). The root systems of grasses perform HM stabilization by accumulation and sorption on the root surfaces or complexation with root exudated such as organic acids, enhanced metal reduction, and metal precipitation into sparingly soluble forms (Wong 2003). The roots also form symbiotic associations with heterotrophic microbial communities in and around the rhizosphere that promotes plant growth in spite of toxic concentrations of HM in the soil (Mendez et al. 2007). These properties prevent the HM uptake into shoots resulting in safe grazing by animals to impede the transfer of these HMs into the food chain (Ghosh et al. 2015). The large surface area and deep penetrability of the root system of *Sorghum* sp. facilitated the phytostabilization of Zn, Cu, Cd, Ni, and Pb (Jadia and Fulekar 2008). Cu-contaminated soil can be remediated using the phytostabilization mechanism of grasses such as *Rendlia altera*, *C. dactylon*, and *Monocymbium ceresiiforme* (Shutcha et al. 2010). Among the aromatic grasses, *V. zizanioides* is the most efficient for phytostabilization of a vast range of HMs such as As, Cd, Cr, Cu, Hg, Ni, Pb, Se, and Zn (Danh et al. 2009). In a study by Ghosh et al. 2015, the HM stabilizing activity of *V. zizanioides* in garden soil amended with fly ash was validated by higher bioaccumulation in roots than that of shoots. The translocation of almost all the HMs present in fly ash from root to shoot was low confirming the restriction of HM contaminants to the root system. Similar results were observed in the same plant grown in fly ash-amended garden soil for a shorter period of time (Chakraborty

and Mukherjee 2011). Phytostabilization potential of *Thysanolaena maxima* and *V. zizanioides* was also observed in Pb mine tailings (Meeinkuirt et al. 2013). *C. citratus* and *V. zizanioides* stabilize chromite-asbestos mine wastes containing Cr, Ni, Mn, Zn, and Cu (Kumar and Maiti 2015) as well as and Cu mine tailings (Das and Maiti 2009). *Piptatherum miliaceum* root systems immobilize large quantities of Cu, Pb, and Zn from mine wastes (Conesa et al. 2006). Studies have shown the phytostabilization of Pb/Zn and Cu mine wastes by the grasses *F. ovina* and *F. rubra* (Pichtel and Salt 1998) and *A. tenuis* (Smith and Bradshaw 1979). Studies on the phytostabilization of fly ash dumpsites are rare. Recently, seven grasses, viz., *Andropogon schirensis, Eragrostis racemosa, Hyparrhenia diplandra, Loudetia simplex, Monocymbium ceresiiforme, Trachypogon spicatus*, and *Tristachya bequaertii*, have been identified to perform successful phytoremediation of Cu-rich soil and are interesting Poaceae candidates for the phytostabilization and reclamation of Cu mine tailings in tropical countries (Boisson et al. 2015).

Phytovolatilization

Phytovolatilization occurs when HMs are taken up by plants from soil and volatilized into the atmosphere by transpiration from foliar parts (Cunningham et al. 1995; Malik and Biswas 2012). In this process harmful HM ionic forms are converted to their less-toxic elemental forms. After release, these elemental forms are further subjected to a more effective and rapid breakdown by photodegradation (Elekes 2014). This technique is effective for the remediation of As, Hg, and Se, although its prominent limiting factor is that the HM contaminants are not completely removed and only transferred from soil to the atmosphere where it can remain redeposited if it does not undergo photodegradation (Ali et al. 2013). However, some researchers confirmed that HMs released by phytovolatilization are diluted and dispersed in the atmosphere posing no risk to the environment (Lin et al. 2000; Meagher et al. 2000).

Rhizofiltration

Phytofiltration or rhizofiltration is similar to phytostabilization in that the roots absorb, precipitate, and concentrate toxic HMs from contaminated water (Dushenkov et al. 1995). Coal fly ash leachate is a potential source of toxic HM contamination of the water table and other water bodies. The cereal crops *Secale cereale*, *S. bicolor*, and *Z. mays* remove Pb from water by this mechanism (Dushenkov et al. 1995; Raskin and Ensley 2000). The same HM is accumulated in large amounts by numerous grasses such as *A. tenuis, P. pratensis, A. palustris, Eragrostis curvula, L. perenne, F. ovina, P. trivialis, F. rubra, F. arundinacea, Eremochloa ophiuroides, Buchloe dactyloides, Panicum virgatum, P. amarum*, *and C. dactylon* (Dushenkov et al. 1995). Both terrestrial and aquatic plants use this mechanism, and the HMs are solely concentrated within the root system. Hence, non-hyperaccumulator plants can be used. Grasses are therefore preferred for their fibrous and long root system that provides larger root area and rapidly regenerate their roots in water after pruning (Jadia and Fulekar 2009).

Phytodegradation

Plants break down contaminants by their metabolic processes and rhizospheric associations with soil microorganisms. Plant enzymes secreted into the rhizosphere or within the tissues assist in the metabolism of contaminants and their transformation into nontoxic forms. Enzymes such as dehalogenase, peroxidase, nitrilase, nitroreductase, and laccase have been isolated from soils and plant sediments (Jabeen et al. 2009). This process involves AM and nitrogen-fixing bacterial associations with the roots of plants (Reichenauer and Germida 2008). *Miscanthus x giganteus* root exudates containing polyphenolic (caffeic, gallic, and chlorogenic acids) and flavonoid (rutin, quercetin, and catechin) compounds induce biostimulation of rhizoshpheric microorganisms that specifically act upon organic and inorganic contaminants by promoting bacterial activity and diversity which leads to phytodegradation (Nsanganwimana et al. 2014; Técher et al. 2011, 2012a, b). Phytodegradation also occurs for the breakdown of organic compounds such as herbicides, chlorinated solvents, insecticides, and inorganic nutrients (Schnoor 2000).

Intracellular Compartmentalization

After failure of the avoidance and tolerance strategies, the final physiological protective stage is the sequestration of HMs by compartmentalization inside various intracellular compartments as a physiological detoxification mechanism (Jabeen et al. 2009). Vacuoles are the main storage sites of HMs that restrict them to sensitive cellular components and account for approximately 90% of the cell volume (Vögeli-Lange and Wagner 1990). Specific peptides such as phytochelatins (PCs) and metallothioneins (MTs) and metal-chelating organic acids (OAs) form complexes with HMs in the cytosol (Manara 2012). These complexes are sequestered into vacuoles by specific metal transporters such as ABC, CDF, HMA, CaCA, and Nramp (Manara 2012). The detailed role of organic acids, PCs, and MTs in phytoremediation is explained in the later sections. Vacuolar compartmentalization limits the free concentration of HM ions in the cytosol by forcing them into a confined space with the assistance of the aforementioned metal transporter proteins (Tong et al. 2004). Increase in the number of vacuoles with peripheral arrangement of nuclei and other organelles is a characteristic feature of HM-affected cells (Jabeen et al. 2009). Studies in *Z. mays* exposed to Cu revealed increased vacuole formation and higher metal accumulation in the roots than that of shoots (Ouzounidou et al. 1995). The ultrastructural studies showed that majority of root cells remained unaffected by Cu stress indicating a resistance mechanism by sequestration within the vacuoles (Ouzounidou et al. 1995). Compartmentalization of Zn in the leaves of *H. vulgare* was observed by the isolation of intact vacuoles (Brune et al. 1994). *F. rubra*, a HM-tolerant grass, showed increased vacuolar volume fraction in meristematic cells when exposed to Zn (Davies et al. 1991). These findings confirm the intracellular accumulation of HMs in vacuoles as a mechanism of detoxification in grasses.

The above physiological defense responses have been observed in a wide variety of accumulator and tolerant grasses. From the present literature survey, it can be confirmed that aromatic grasses are excellent candidates for remediation of HM-contaminated soil in terms of their pronounced physiological defense responses. When all the above physiological mechanisms are exhausted, the biochemical mechanisms are activated to combat HM toxicity.

Biochemical Mechanisms

Grasses growing in areas rich in complex mixtures of heavy metals (HMs) undergo alterations in their biochemical pathways. After physiological defense responses, altered synthesis of various cellular biomolecules is the next step to tolerate HM toxicity. Such biochemical responses can occur as changes in the enzymes involved in photosynthesis and antioxidant defense system (Bhardwaj and Mascarenas 1989). HMs diffuse into the cells and affect the activity of regulatory enzymes involved in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation consequently altering the biosynthesis of ATP and other adenosine nucleotides (ADP, AMP, and GDP) (Bataynen et al. 1986). HMs present in fly ash and mine tailings generate reactive oxygen species (ROS) leading to impaired cellular redox homoeostasis within the plant body (Mourato et al. 2012). To combat such oxidative stress, grasses used in phytoremediation have evolved enzymatic and nonenzymatic antioxidants (Fig. 3) to scavenge the free radicals produced by HMs (Mittler et al. 2004). The

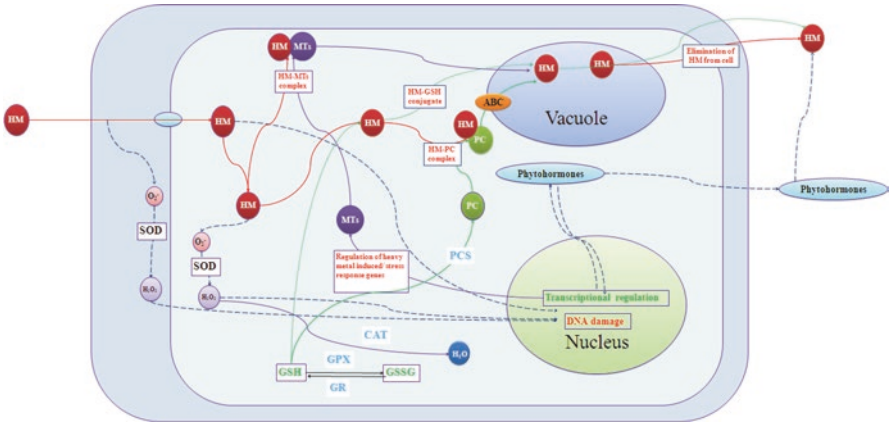

Fig. 3 Schematic representation of the biochemical and molecular pathways within a grass cell for heavy metal (HM) detoxification. HMs entering the cell are complexed with low molecular weight chelating peptides PCs and MTs. The PC biosynthetic pathway is overlapped with the GSH pathway, as GSH is a substrate for PCS protein synthesis that produces PC. MTs are synthesized by transcriptional regulation from mRNA. These HM-PC or HM-MT complexes are sequestered into vacuoles for elimination of HMs. The ROS generated by HMs is quenched by the enzymatic (SOD, CAT, GPX, GR) and nonenzymatic (GSH) antioxidants

enzymatic antioxidants include superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2), glutathione peroxidase (GPX, EC 1.11.1.9), and guaiacol peroxidase (GPOD, EC 1.11.1.7). The nonenzymatic antioxidants are glutathione (GSH), proline, ascorbate (AA), thiols, and phenolic compounds (polyphenols, flavonoids, tannins, and lignin) (Michalak 2006; Sharma et al. 2012; Sharma and Dietz 2006). Among the other biochemical mechanisms, low molecular weight HM-chelating organic acid (OA) anions of malate, oxalate, and citrate are exuded by plants from the root apices as metal chelators around the rhizosphere. Hormonal responses involve the release of hormones such as salicylic acid, jasmonic acid, abscisic acid, indole-3-acetic acid (IAA), and ethylene to mitigate HM toxicity (Dalvi and Bhalerao 2013; Viehweger 2014). The biochemical mechanisms of phytoremediation in grasses are elaborated below.

Antioxidant Defense Responses

The antioxidant defense mechanism is a major strategy developed by plants to cope up with excess ROS generated by HMs. In plants that do not perform phytoremediation, the antioxidant enzyme activities increase at lower HM concentration and show steady decrease with high HM concentration as the defense mechanism breaks down (Nadgórska-Socha et al. 2013). Enhanced synthesis of antioxidant enzymes at high concentrations of HMs has been observed in grasses used in phytoremediation of HM-contaminated areas to protect themselves from oxidative damage (Andra et al. 2011; Khatun et al. 2008). The activity of enzymatic and nonenzymatic antioxidants varies between plant species depending on the concentration of HMs entering the cells. The role of enzymatic and nonenzymatic antioxidants has been elucidated in the successive sections.

Enzymatic Antioxidants

Specific enzymes that are integral components of different metabolic pathways form the enzymatic antioxidant defense against excess ROS production by HMs. Synchronized alteration in the activities of SOD, CAT, APX, GPOD, GPX, and GR is a key biochemical mechanism of phytoremediation by grasses. Aromatic grasses growing in HM-rich soil also show high antioxidant levels that protect the plants from oxidative damage. The first line of defense against ROS is provided by SOD that can convert two O_2^- radicals to H_2O_2 and O_2. POD and CAT further catalyze H_2O_2 to H_2O, stalling the accumulation of O_2^- and H_2O_2 to inhibit membrane lipid peroxidation (Scandalios 1993). Hence, SOD is the catalyst in the dismutation of superoxide molecules to produce hydrogen peroxide and oxygen (Alscher et al. 2002). Oxidative stress stimulates a rise in the number of peroxisomes. CAT diffuses into the peroxisomes from other locations of the cell and scavenges H_2O_2 to break it down to water (Mittler 2002). All peroxidases (POD, EC 1.11.1) also scavenge H_2O_2 or check its

accumulation. They require electron donors such as guaiacol (GPOD), ascorbate (APX), and glutathione (GPX). APX is more effective in maintaining low H_2O_2 levels due to its higher affinity to it, while CAT can remove excess H_2O_2 from the peroxisomes.

Pang et al. (2003) reported enhanced activity of SOD, POD, and CAT in *V. zizanioides* growing in metalliferous mine tailings. The above enzyme activities differed in shoots and roots indicating marked differences in ROS scavenging machinery between different parts of the plant body. The same plant showed increased CAT activity in roots and shoots when grown in Pb-contaminated soil with higher activity in roots than that of shoots (Andra et al. 2009a). These results specify the metal ion accumulation pattern in different organs of the same plant, where higher antioxidant enzyme activity occurs in parts accumulating higher metal ions. Such a mechanism of differential enzyme activity facilitates quenching of large amounts of HM ions after their bulk uptake. In another study, the activities of SOD, CAT, and GPX were reported to be higher in both roots and shoots of vetiver grass grown in Pb-contaminated soil than that of the same soil amended with chelants such as ethylene diamine tetraacetate (EDTA) and ethylenediamine disuccinate (EDDS) (Andra et al. 2011). In the same study, the overall activity of GPX was higher than that of CAT in plants grown in both types of soils. Hence, chelating agents added to HM-contaminated soil bind to the free HM ions in the soil leading to their increased uptake in the plant body and mask their toxic effects by lowering oxidative stress levels compared to unamended soils. Higher GPX activity protects the cell from membrane lipid peroxidation caused by HM stress. Several independent studies in *H. vulgare*, a hyperaccumulator grass with high biomass, revealed high GPX activity with increasing concentration of Cd, Cu, Ni, and Hg (Halusková et al. 2009; Hossain et al. 2012). Increased SOD and CAT activities imparted Pb tolerance in the high biomass yielding turfgrass *Eremochloa ophiuroides* (Li et al. 2015). POD activity increased in *C. flexuosus* upon Cr stress imparting protection against Cr-induced phytotoxicity (Patra et al. 2015). High CAT and POD levels were also observed in vetiver grass exposed to Cd (Aibibu et al. 2010). In case of *Sorghum* sp. subjected to Cd stress, no change in APX activity was reported, whereas there was a significant rise in the POD and CAT activities (Soudek et al. 2014). Few studies explored the antioxidant status of the HM-tolerant grass *Saccharum* sp. growing in HM-contaminated environment. The activities of CAT and GR were inversely proportional to each other in response to Cd stress in sugarcane seedlings (Fornazier et al. 2002a), whereas callus culture showed increase in CAT activity (Fornazier et al. 2002b). The results of the above studies indicate that different tissues and organs of the same plant species respond differently to the same HM and further field studies are required to corroborate all antioxidant enzyme responses in *Saccharum* sp. Cd exposure also stimulated the activities of APX, GPOD, SOD, and GR in *Z. mays* (Ekmekci et al. 2008). Patra et al. (1994) established the tolerance of the grass *Chloris barbata* grown in Hg-contaminated dumpsite and evinced its co-tolerance to Cd and Zn as assessed by high CAT and POD activities when the Hg-tolerant grasses were exposed to Cd and Zn. *M. sacchariflorus*, a HM-resistant grass growing in mine tailings, showed increased activities of CAT, SOD, and POD

to alleviate oxidative stress when grown in Cd-rich soil (Zhang et al. 2015). Rise in SOD, CAT, POD, and APX activities was reported in *Arrhenatherum elatius* growing in metalliferous mine tailings containing high concentrations of Ni, Cu, Cd, Co, Mn, Pb, Cr, and Zn (Lu et al. 2013). Conversely, *L. perenne* growing in an abandoned Pb smelter contaminated by Cd, Pb, and Zn showed a fall in SOD activity (Bidar et al. 2007). These results are in accordance with the results of Wu et al. (2003), showing a decrease in SOD activity in *H. vulgare* in response to Cd toxicity. The roots of Cd-treated *P. australis*, a useful plant for phytoremediation of fly ash and mine wastes, showed high CAT, GPX, and GR content corroborating their property of phytostabilization (Iannelli et al. 2002). Therefore, the innate antioxidant potential of members of Poaceae and aromatic grasses validates their authenticity for HM cleanup.

Nonenzymatic Antioxidants

GSH, a nonprotein thiol, is regulated by the ascorbate-glutathione cycle, which comprises a vital group of reactions for detoxification of ROS. This cycle converts H_2O_2 generated by oxidative stress into water, using APX and GPX and other enzymes such as monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and dehydroascorbate reductase (DHAR, EC 1.8.5.1). MDHAR and DHAR result in the regeneration of reduced form of ascorbate (AsA) and GR, which in turn maintain the pool of GSH. GSH traps free radicals in aqueous phase and protects membranes as a chief function of the ascorbate-glutathione cycle (Gill et al. 2013). Along with HM chelation, complexation, compartmentalization, and detoxification, GSH and its metabolizing enzymes (GR, GST, GPX, dehydroascorbate reductase, glyoxalase I, and glyoxalase II) cumulatively and coordinately work to moderate ROS damage to the cellular machinery (Hossain et al. 2012). The increase in $NADP^+$/NADPH ratio is a marker for enhanced GR activity. *P. australis* roots, grown in the presence of Cd, showed enhanced synthesis of GSH, GST, and AsA along with a sharp increase in the ratio of $NADP^+$/NADPH showing efficient regulation of the ascorbate-glutathione cycle and their constituent metabolizing enzymes (Iannelli et al. 2002). High GSH content was found in *Arabis paniculata*, a Zn/Cd hyperaccumulator (Zeng et al. 2009). Al-tolerant *T. aestivum* showed increase in GST levels (Darkó et al. 2004). The perennial grass *Holcus lanatus* showed elevated GSH levels in resistance to As (Hartley-Whitaker et al. 2001). Such stimulation of GSH synthesis plays an important role in the maintenance of GSH/GSSG ratio in HM-tolerant grasses. On the other hand, *Sorghum* sp. roots and shoots showed a marked decrease in GST activity under Cd stress (Soudek et al. 2014). In another study, Cd negatively affected GST isozymes of rice roots (Zhang and Ge 2008). Hence, the extent of GSH synthesis in grasses tolerant to HMs depends on the plant species, duration of exposure, and age of the plant. *V. zizanioides*, grown in Pb-contaminated garden soil, exhibited increased total thiol level in both roots and shoots, which was reduced to a significant extent when grown in the same soil amended with EDTA that chelated the Pb ions in the soil rendering them inactive (Andra et al. 2009a).

Proline is also a ROS scavenger (singlet oxygen and hydroxyl radicals), which increases the antioxidant enzyme activities and maintains redox homeostasis (Matysik et al. 2002). It also plays an integral role in cell signaling pathways that regulate stress genes, reconstruction of chlorophyll, and regulation of intracellular pH (Khedr et al. 2003; Rastgoo et al. 2011). Elevated proline content is a nonenzymatic defense response to a wide array of biotic and abiotic stress including HM toxicity and oxidative stress (Szabados and Savouré 2010). High proline content was found in *C. flexuosus*, under Cr stress, which lead to the increased antioxidant enzyme activities and tolerance to Cr (Patra et al. 2015). In another study by Handique and Handique (2009), proline accumulation in *C. flexuosus* was high during short-term exposure to Pb, Hg, and Cd, which reduced after prolonged exposure. Such a pattern in proline accumulation can occur due to high concentration of HMs during the initial time period and subsequent tolerance to such a stress over a long period of time. *C. winterianus* showed similar tolerance to HMs, specifically, Pb and Hg by the same biochemical mechanism (Boruah et al. 2000). *V. zizanioides,* growing in Pb/Zn mine tailings, showed high proline content in the leaves and roots (Pang et al. 2003). Cu- and Zn-induced proline accumulation occurred in *T. aestivum*, where Cu was a stronger inducer of proline synthesis (Kumar et al. 2012a). Leskó and Simon-Sarkadi (2002) also showed enhanced proline accumulation in *T. aestivum* seedlings upon Cd stress. Similar results were found in *M. sacchariflorus* under Cd stress (Zhang et al. 2015). In recent years, the exogenous application of proline is gaining popularity. Grass species that actively accumulate HMs can be sprayed with proline on their foliar parts to alleviate HM toxicity within the plant system. The leaves of *L. perenne* sprayed with proline showed enhanced resistance to Ni toxicity (Shahid et al. 2014). Such studies have been carried out in other plant families such as Fabaceae, with desirable results (Hayat et al. 2013; Shahid et al. 2014).

Phenolics including flavonoids and phenylpropanoids, generated from the shikimate pathway, play key roles in H_2O_2 scavenging after they are oxidized by POD (Harborne 1989). Flavonoids are the most common phenolics forming the skeletal backbone of the class of polyphenolics called proanthocyanidins (or tannins) (Singer et al. 2003). The antioxidant activity of phenolics is imparted by their ability of chelate metals. Such a property is due to their chemical structure with at least one aromatic ring (C_6) and one or more hydroxyl groups. *T. aestivum* showed the induction of phenolic compound biosynthesis upon toxic Ni exposure (Michalak 2006).

Organic Acids (OAs)

All HM-tolerant plants modify their rhizospheres by the release of organic compounds from their root apices. These exudates mainly comprise low and high molecular weight OAs such as acetate, lactate, oxalate, succinate, malate, fumarate, isocitrate, citrate, and aconitate. They mediate the translocation and detoxification of HMs within the plant body. They enhance the bioavailability of HM cations allowing the entry of the OA-bound HMs into the root systems (Ross 1994). The influence of HMs is reflected by changes in the concentration of OAs that are

involved in pathways of primary plant metabolism, viz., respiration, photosynthesis, and production of ATP. Al tolerance by citrate has been reported in many plant tissues such as *Z. mays* and *T. aestivum* (Papernik et al. 2001; Pineros and Kochian 2001). Citrate has high affinity toward Fe, Cd, Co, Ni, and Zn showing strong chelating ability (Hossain et al. 2012). Delhaize and Ryan (1995) reported the exudation of malic acid from the radical apex of *T. aestivum* as a result of Al stress, hence increasing the tolerance of the plant to toxic concentrations of Al in the soil. Other grasses such as *Z. mays* and *L. perenne* release malate, oxalate, or citrate that chelate Al^{3+} cations from the rhizosphere and facilitate the entry of Al^{3+}-OA complexes into the roots and accumulate nontoxic Al oxalate in the leaves (Ma et al. 2001). In *T. aestivum* and *Z. mays*, Al-activated plasma membrane anion channels secrete oxalic acid from the roots and accumulate Al oxalate in the leaves (Ma et al. 2001). The Al-induced rapid secretion of malate in *S. cereale* occurred by the alteration in OA metabolism, whereas the same took place by the activation of an anion channel in the case of *T. aestivum* (Li et al. 2000). *C. dactylon* also showed the release of OA for phytostabilization of As, Zn, and Pb (Leung et al. 2007).

Hormonal Responses

Phytotoxicity by HMs can be attenuated with altered levels of endogenous phytohormones such as abscisic acid, ethylene, auxin, jasmonic acid, and salicylic acid. HM stress stimulates the mutual interaction of these hormones through a complex web of regulatory networks. Such hormonal expression varies between plant species, age of plants, and nature and intensity of HM toxicity in the soil (Glick and Stearns 2011). However, there is a paucity of information related to the variation in grass hormonal responses to HM toxicity. Pang et al. (2003) studied the role of abscisic acid (ABA), a plant stress hormone which is vital in terms of tolerance to environmental adversities. In *V. zizanioides* growing in abandoned Pb/Zn mine tailing sites, the authors reported a linear increment in the concentration of ABA in both roots and shoots with an increase in proportion of tailing and time of treatment. These results indicate the activation of a protective mechanism through ABA synthesis by *V. zizanioides* under HM stress. ABA also elevates the activities of antioxidant enzymes such as SOD, APX, and GR (Bellaire et al. 2000). The mechanism of action of ABA is to hinder the translocation of fluids leading to blocked HM ion uptake from roots to shoots (Munzuroğlu et al. 2008). Increased accumulation of ABA was also observed in leaves and roots of Tainung 67 (TNG67) cultivar of *O. sativa* under Cd stress (Hsu and Kao 2003). It was also found that exogenous application of ABA in Cd-sensitive Taichung Native 1 (TN1) cultivar of *O. sativa* resulted in increased Cd tolerance (Hsu and Kao 2003). These results prove the correlation of endogenous biosynthesis of ABA with HM tolerance in *O. sativa*. Similarly, germinating *T. aestivum* seedlings exposed to Hg, Cu, and Cd showed linear increase in endogenous levels of ABA with HM concentration. Hg promoted maximum ABA accumulation, while Cu induced least synthesis of the same (Munzuroğlu et al.

2008). Interestingly, ethylene plays a dual role during environmental stresses. It can alleviate as well as exacerbate the effects of abiotic stresses by changes in the amount of ethylene produced within the cell (Arshad and Frankenberger 2002). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, a precursor of ethylene, lowers the ethylene levels, which in turn combats growth suppression by the environmental stresses (Glick and Holguin 1998). Plants under HM stress produce more amount of ethylene that inhibits their growth. Hence, ACC deaminase plays a cardinal role in phytoremediation by inhibiting ethylene synthesis. Auxins such as IAA mitigate the phytotoxic effects of HMs (Frankenberger and Arshad 1995). IAA and ethylene work in concert with each other to combat the growth inhibitory activity of HMs. Exogenous application of IAA has been carried out in many dicotyledonous plants such as *Solanum nigrum* exposed to Cd (Ji et al. 2015), *Helianthus annuus* grown in HM-polluted soil (Liphadzi et al. 2006), *Picris divaricata* exposed to Pb (Du et al. 2011), and few grasses such as *V. zizanioides* exposed to Cu (Chen et al. 2012a), to ameliorate phytotoxicity and promote normal plant growth. Jasmonic acid, an important regulator of plant development, is also involved in biotic and abiotic stress responses. The role of jasmonic acid in abiotic stress tolerance is less elucidated than that of its role in biotic stress response. In a study by Cai et al. (2015), the phytohormones ABA, IAA, jasmonic acid, cytokinins, and biologically active gibberellins showed marked increase in *O. sativa* under Cd-induced abiotic stress. Further field studies are needed to uncover the underlying mechanisms of all phytohormone regulatory pathways in HM-tolerant grasses.

Grasses used in the clearance of toxic HMs from mine tailings and fly ash dumpsites combat HM stress by their enhanced antioxidant or chelating properties as a protective mechanism to minimize ROS-induced damage, leading to a successful HM accumulation and clearance (Aibibu et al. 2010). Essential HMs such as Cu, Zn, Mn, and Ni are required for physiological processes, while metals such as Cd, Hg, and Zn are nonessential and excess amount of essential HM and a small quantity of nonessential HMs can render substantial phytotoxicity. Numerous pot-scale laboratory studies are being carried out in accumulator plants to understand the precise biochemical mechanisms of tolerance to elevated HM accumulation without any metabolic alteration in the plant cell machinery. Such studies involving aromatic grasses are scarce. The curious property of essential oil production without any contamination with HM ions involves intricate biochemical pathways leading to the elimination of these ions. There is a dearth of direct field studies of biochemical mechanisms of HM tolerance in phytoremediation of mine tailings and fly ash dumpsites by grasses, and more such studies will facilitate better understanding of the altered biochemical pathways.

Molecular Mechanisms

The molecular mechanisms of phytoremediation are characterized by the synthesis of stress response-related amino acids, proteins, and cell signaling molecules (Dalcorso et al. 2008, 2010). Members of the family Poaceae and other aromatic

grasses have adapted novel ways of detoxification and maintenance of intracellular heavy metal (HM) ion homeostasis by the synthesis of cysteine (Cys) rich, low molecular weight thiol peptides, phytochelatins (PCs), and metallothioneins (MTs) to mitigate the detrimental effects of HM stress (Clemens 2001). Figure 3 provides a schematic representation of the molecular mechanisms of HM detoxification by PCs and MTs, within a plant cell. These HM ion-binding ligands protect the metabolically active sites of cells from toxic effects of metals (Zenk 1996). Their occurrence is ubiquitous in the living world, from fungi to many animal species (Vatamaniuk et al. 2001). On the genetic level, numerous genes are upregulated and downregulated in tandem for the synthesis of PCs and MTs during HM stress. The expression profile of such specific genes has been traced in many grasses using molecular tools. The expression of quantitative trait loci (QTL) is another potential molecular biomarker controlling genetic variation and phenotypic changes in a wide array of grasses that sequester HMs. The following sections elucidate the role of PCs, MTs, and QTL in phytoremediation.

Phytochelatins

PCs bind to HMs via sulfhydryl and carboxyl residues. They form high molecular weight complexes with metal ions due to their cysteine-rich structures, hence separating the ions from cellular metabolism (Salt and Rauser 1995). These complexes are transported into vacuoles by Mg-ATP-dependent carriers or ATP-binding cassette (ABC) transporters (Sytar et al. 2013; Emamverdian et al. 2015). PCs were discovered as Cd-binding peptides cadystin A and B in *Schizosaccharomyces pombe* (Grill et al. 1986) followed by algae and higher plants (Fischer et al. 2014; Grill et al. 1985). They are produced by a cytosolic protein, PC synthase (γ-glutamylcysteine dipeptidyl transpeptidase), as oligomers of glutathione (GSH) with a general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, ($n = 2–11$) (Grill et al. 1989). PC biosynthesis in phytoremediating grasses and aromatic grasses is triggered by HMs found in fly ash and mine soil including Cd, Hg, Ag, Cu, Ni, Au, Pb, As, and Zn, among which Cd ions most effectively stimulate the process. These metal ions bind to the enzyme PC synthase (γ-glutamylcysteine dipeptidyl transpeptidase) (PCS) leading to its activation and in turn facilitating the conversion of GSH to PC (Zenk 1996). Hence, the PC biosynthetic pathway is overlapped with the GSH pathway, where GSH is the substrate for PC synthesis (Cobbett 2000; Cobbett and Goldsbrough 2002). PCs are abbreviated as PC_1, PC_2, PC_3, PC_4, PC_5, and so on, depending on the number of transpeptidations of γ-Glu-Cys units from GSH (Cobbett 1999; Grill et al. 1989; Vatamaniuk et al. 2004). The most frequently used analytical techniques for the separation and characterization of PCs are liquid chromatography and mass spectrometry (Kozka et al. 2006; Mishra et al. 2006; Figueroa et al. 2007).

Among the aromatic grasses, the PCs of *V. zizanioides* L. grown in Pb-contaminated soil have been identified as PC_n ($n = 1–4$) (Andra et al. 2009a). In another study by Andra et al. (2010), PC_1, PC_2, PC_3, and PC_4 from root and PC_1 and PC_2 from shoot

tissues were identified in hydroponically cultured vetiver plants exposed to high concentrations of Pb. No PC_n were quantified in plants grown in media devoid of Pb. PC_1 was the most abundant PC in both root and shoot. Hence, it was proposed that PC_1 was the substrate for the synthesis of higher-order PC_n. This study is one of the pioneering investigations that affirmed the synthesis of PCs exclusively during HM stress in aromatic grasses. Along with PCs, PC-related peptides such as des Gly-PC_6 and iso-PC_5 (βAla) were also observed from the root tissues grown in the same conditions (Andra et al. 2009a). The identified PCs were observed to bind Pb as Pb-PC_1 complexes for successful removal of Pb. A novel study by Andra et al. (2009b) used vetiver grass grown in EDTA (a widely used chelating agent) supplemented Pb-contaminated soil. PC_1 was found in maximum concentration in plants growing in EDTA untreated Pb-contaminated soils, while its concentration was five times lower in the above soil supplemented with EDTA. The results of this study revealed complexes of Pb-PC_1 in Pb-contaminated soil and Pb-PC_1-EDTA in the same soil treated with EDTA. This confirmed that supplementation of EDTA in HM-contaminated soil can reverse the adverse effects of HM ions by formation of the ion-quenching complexes HM-EDTA and HM-PC_n-EDTA.

Identification of genes encoding metal-binding proteins facilitates improved understanding of the mechanism of phytoremediation in grasses. HMs induce upregulation of PCS genes leading to PCS protein production and subsequent PC synthesis and chelation of HM ions in PC-HM complexes (Cobbett and Goldsbrough 2002). Several studies have been carried out to identify the PCS genes in higher plants, to unravel the molecular mechanisms underlying PC synthesis. *AtPCS1, CAD1,* from *Arabidopsis thaliana* and *TaPCS1* from *T. aestivum* were among the first PCS genes identified after Cd stress (Cobbett 2000; Ha et al. 1999). The above genes encode 50–55 kDa proteins which are 40–50% identical to each other (Rea 2012). These findings lead to an upsurge in studies for the identification of PCS genes. *OsPCS1*, *OsPCS5*, and *OsPCS9* from *O. sativa* (Shen et al. 2010), *PaGCS* and *PaPCS* in *P. australis* (Zhao et al. 2014), *HvPCS1* from *H. vulgare*, *TmPCS1* from *T. monococcum*, *ZmPCS1* from *Z. mays*, and *CdPCS1* from *C. dactylon* were some of the identified genes from grasses (Loscos et al. 2006; Shen et al. 2010). Some PCS genes are constitutively expressed and induced by specific HM ions (Clemens et al. 1999), while others show no HM specificity (Cobbett 2000). In *O. sativa*, *OsPCS9* is induced by Cd^{2+} and Zn^{2+} and *OsPCS7* by Hg^{2+} and Pb^{2+} (Shen et al. 2010). The gene expression profiles of other grasses, especially aromatic grasses, which synthesize PCs, need to be studied for their improved implementation for phytoremediation.

Metallothioneins

Metallothioneins (MT) are low molecular weight, small, cysteine-rich cytoplasmic metal-binding proteins synthesized enzymatically from mRNA translation (Memon and Schröder 2009; Verkleij et al. 2003). They usually contain two HM-binding Cys-rich domains that impart a dumbbell-shaped conformation. The

basic invariant structure of these proteins contains Cys-Cys, Cys-X-Cys, and Cys-X-X-Cys (X denotes any amino acid) motifs (Yang et al. 2005b). The first evidence that plants contain not only PCs but also MTs as Cys-rich metal ligands came from the discovery of the Ec Type 4 MT protein that was purified from embryos of *T. aestivum* as a Zn-binding protein (Lane et al. 1987). While PCs mainly bind to Cd, MTs can also equally bind to a wider range of other metals including Zn, Cu, and As (Yang and Chu 2011). They follow the same principle of HM detoxification as PCs involving sequestration of metals, intracellular metal ion homeostasis, and adjustment of metal transport (Guo et al. 2013). Additionally, MTs play key roles in ROS scavenging (Wong et al. 2004), maintenance of redox homeostasis (Macovei et al. 2010), and DNA repair (Grennan 2011) to resist conditions of abiotic stresses such as osmotic, temperature, and nutrient stresses as well as biotic stresses such as viral infections (Yang and Chu 2011; Du et al. 2012; Manara 2012; Emamverdian et al. 2015).

MTs bind a variety of metal ions by mercaptide bonds between their numerous Cys residues and the metal ions. The arrangement of these Cys residues partially determines the metal-binding properties of the MT proteins (Cobbett and Goldsbrough 2002; Leszczyszyn et al. 2013). Based on the Cys arrangement, plant MTs have been grouped into four types, types 1 through 4 (MT_1, MT_2, MT_3, MT_4), each having specific functions. Majority of the MT genes encoding all four types of MTs have been identified from angiosperms including grasses such as *O. sativa*, *Z. mays*, *T. aestivum*, and *Saccharum* sp. (Cobbett and Goldsbrough 2002; Yang et al. 2005b). In *O. sativa*, *OsMT1a* gene encoding a MT_1 protein enables Zn homeostasis in the roots (Yang et al. 2009). Zn and Cu homeostasis is maintained by MT_3 and Zn storage by MT_4 in mature seeds of *H. vulgare* (Hegelund et al. 2012). *OsMT1e-P* gene, belonging to a multigene family consisting of 13 genes and 15 protein products, of a salt-tolerant variety of *O. sativa* encodes a type 1 MT which is responsible for Cu and Zn tolerance via ROS scavenging by ectopic expression in transgenic *N. tabacum* (Kumar et al. 2012b). AK062653 and AK062796 MT-like genes were up- and downregulated, respectively, in *O. sativa* leaves upon excess Cu stress (Sudo et al. 2008). OsMT1a, OsMT1b, OsMT1c, OsMT1d, OsMT1e, OsMT1f, and OsMT1g are the MT_1 genes; OsMT2a, OsMT2b, OsMT2c, and OsMT2d are the MT_2 genes; OsMT3a and OsMT4 are the MT_3 and MT_4 gene families, respectively, identified in *O. sativa* (Kumar et al. 2012b). It has also been found that several type 1 and type 2 MTs play direct roles in ROS scavenging in *O. sativa* in conditions of abiotic stress (Zhou et al. 2005). Specifically, the *OsMT1e-P* gene from *O. sativa* reduced oxidative stress resulting in tolerance to multiple abiotic stresses when expressed ectopically in *N. tabacum* (Kumar et al. 2012b). Hence, along with HM chelation, MT proteins suppress oxidative stress to prevent phytotoxicity and ultimately remove toxic HMs from soil without affecting the physiological conditions of the plants. Although studies based on the identification and characterization of MTs and MT synthesizing genes are in progress, detailed information is needed, as MTs are promising tools for phytoremediation due to their wide variety of HM-binding regions.

Quantitative Trait Loci

Metal-specific quantitative trait loci (QTL) control the uptake of HMs and create the foundation of genetic variation and phenotype of phytoremediating grasses. Identification of QTL is useful in plant breeding to select varieties with high uptake of essential heavy metals such as Zn and Fe and reduced uptake of toxic heavy metals such as Al, As, Cd, and Pb (White and Broadley 2005; Tuli et al. 2010; White and Brown 2010; Baxter and Dilkes 2012; Yun et al. 2015). These loci were identified in the genomes of a number of Poaceae genera. Yun et al. (2015) identified 25 QTL in populations derived from two perennial wildrye species *Leymus cinereus* and *L. triticoides* grown in soil with high content of As, Cd, Cu, Mo, Pb, Zn, and other trace elements. After alignment of the identified QTL with the *H. vulgare* genome sequence, a putative *phytochelatin synthase 2* gene (*PCS2*) was located in the aligned Cd QTL linkage group. *PCS2* functions during the transport of heavy metals (Briat 2010; Dave et al. 2013; Kamiya and Fujiwara 2011), especially Cd (Clemens et al. 1999; Guo et al. 2008; Wojas et al. 2010). The Zn QTL linkage group alignment revealed four MT genes that were downregulated with high Cd, Cu, and Zn exposure (Schiller et al. 2014). Differential expression of QTL occurred in rye species growing on phytotoxic soil than that of fertile soil (Yun et al. 2015). In *O. sativa*, QTL were identified for the control of As, Cd, and Pb uptake (Dasgupta et al. 2004; Zhang et al. 2008; Kashiwagi et al. 2009; Ueno et al. 2009; Ishikawa et al. 2010; Norton et al. 2010; Kuramata et al. 2013). In *T. aestivum*, Cu-tolerant QTL (Bálint et al. 2007) and Cd content regulating QTL were discovered (Wiebe et al. 2010). QTL controlling the uptake of Fe and Zn were found in *H. vulgare* (Lonergan et al. 2009), *T. aestivum* (Xu et al. 2011), and *Z. mays* (Šimić et al. 2012; Baxter et al. 2013), whereas QTL resistant to Al uptake were identified in *T. aestivum* (Sasaki et al. 2004), *H. vulgare* (Furukawa et al. 2007), and *Sorghum* sp. (Magalhaes et al. 2007). Therefore, studies on the expression profiles of QTL will facilitate the identification of PC and MT genes as well as other genes involved in HM tolerance.

The molecular mechanisms of phytoremediation are being explored over the past decade in various plants exposed to HM stress. However, PCs, MTs, and QTL of specifically aromatic grasses are rather unexplored and need further elucidation. In this regard, much more information is needed to comprehensively understand the function of every biomolecule involved in HM sequestration, metabolism, and clearance in grasses. The nature and stoichiometry of complexes responsible for HM chelation are being analyzed by various groups, and further studies are required for a detailed understanding of the specific HM-protein/thiol complexes that are formed in grasses growing in toxic HM-contaminated areas. On the other hand, creation of transgenic plants originally with high biomass and growth rate but lacking in PCs and MTs, genetically modified by incorporating PC and MT genes leading to overexpression of PCs and MTs, is promising developments for a successful phytoremediation. The identification, characterization, and expression profiles of PC and MT genes will clarify the different pathways involved in heavy metal tolerance and detoxification without any damage to the plant body.

Conclusions and Future Prospects

Heavy metal (HM) contamination by fly ash dumpsites and mine tailings is a persistent global issue that requires immediate mitigation. Conventional modes of cleanup of such sites often exacerbate the problems. Thus, phytoremediation is an environmentally safe and cost-effective method for restoration of such sites. Recent advances in ecological engineering facilitated the development of green capping of vast areas of mine tailings and fly ash dumpsites with various species of grasses that are ideal for restoration of environmental health. The mechanisms of such resistance to HMs include a conglomerate of physiological and biochemical pathways along with altered gene expression patterns, which need further studies. Moreover, there is a paucity of field-based reports for the analysis of the basic biochemical and molecular processes involved in HM clearance by grasses growing in soil containing complex mixtures of HMs. Aromatic grasses have proved to be the best plants for phytoremediation of HM-contaminated sites due to their industrial importance in terms of essential oil production, although studies on their mechanisms of phytoremediation are limited. Furthermore, the analysis of the secondary metabolite biosynthesis pathways synthesizing the components of essential oils is needed to understand the mechanisms behind the exclusion of essential oils from HM contamination. Genetic manipulations to create transgenic plants that suit the criteria for ideal hyperaccumulator plants are interesting aspects of assisted phytoremediation. The natural characters of grasses can be utilized to genetically engineer them into hyperaccumulators/excluders by the identification of genes regulating such processes. In this regard, identification of all genes responsible for PC and MT synthesis and regulation, and their expression patterns, needs to be analyzed in more details to induce their overexpression for HM chelation within the plant cell. Research on genetic engineering of plants for greater HM sequestration is in its nascent stage. Preliminary reports on raising the ability of model plants to perform increased vacuolar compartmentalization of HMs requiring a small number of transporters are available. This approach does not rely on increasing the synthesis of HM-chelating proteins; rather it is more specific to HM detoxification. Such manipulations in combination with transgenic approaches to enhance PC and MT synthesis in grasses and aromatic grasses can be beneficial for rapid HM detoxification and overall remediation of ecological health. Therefore, members of the family Poaceae, especially aromatic grasses, are ideal for the reclamation, restoration, and revegetation of abandoned fly ash landfills and mine sites.

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Bioremediation of Sulfide Mine Tailings: Response of Different Soil Fractions

H. Cortez, A. Ballester, F. González, M.L. Blázquez, and J.A. Muñoz

Abstract Bioremediation phenomena of soils contaminated with heavy metals have not been considered a key sustainability issue for the mining industry until recently. At least, this is what can be deduced from the huge amount of mining activities spread out worldwide. Nevertheless, mine wastes accumulated over long periods of time have a negative impact on the landscape and pose serious threats to ecosystems. Far from being solved, this issue is becoming more acute as the metalliferous mining industry is seriously affected by the cutoff grades decline of natural resources. The mining district of Sierra Cartagena-La Unión in southeast Spain, with a total area of 100 km^2, is a good example of poor mine practices. Metal extraction (Ag, Pb, and Zn) from sulfide mineral ores in this mining area dates back before Roman times. Consequently, large amounts of mining wastes have been accumulated over the centuries close to human settlements. Facts like this, underestimated in the past, could be a potential source of metal propagation with possible detrimental effects on human health. In this work, a bioremediation study has been accomplished in a metalliferous contaminated soil considering different particle size fractions. Each fraction, including the global material waste, has been chemically characterized using an ad hoc approach, followed by its mineralogical characterization. The investigation has been focused on the effect of bioaugmentation on metal mobilization and redistribution of heavy metals (Zn, Pb, Cu, Fe) among different soil fractions.

Keywords Mine tailing • Contaminated soil • Microcosms • Bioaugmentation • Soil fractions

Introduction

Environmental pollution is becoming a big concern in industrialized countries especially because of the long-term persistence of metals in soils. Unlike many organic compounds that become innocuous overtime, hazardous metals cannot be degraded

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neither biologically nor chemically. In the group of metals, heavy metals have p-articularly high relevance in contamination processes (Gosar 2004; Natarajan et al. 2006; Cortez et al. 2010). Soils contaminated with heavy metals are a source of potential threats to humans through the food chain from surface and subsurface waters, plants, and animals (Nriagu 1990). Another factor to take into account is the rapid human population growth that exerts enormous pressure on both underground reservoirs and natural soils. In some instances, urban areas have spread out colonizing natural spaces, which had supported industrial or mining activities.

The content of metals in soils is associated with natural processes and human activities; however, anthropogenic emissions of heavy metal exceed by far its mobilization by weathering processes. The main sources of soil pollution by heavy metals have been the subject of numerous literature revisions and may include, among others residual sludges, refineries, incinerators, military installations, urban and traffic residues, mining and metallurgical residues, fertilizers, and pesticides (Kelly et al. 1996; Norgate et al. 2007). In 1999, the number of soils potentially contaminated within the European Union was estimated to be between 300,000 and 1,500,000. Table 1 collects the progress in the treatment of contaminated sites (residues from industrial and military sources) in different European countries and from the point of view of identification and evaluation. In Spain, those percentages corresponded with 4902 identified sites and 370 evaluated sites.

According to the Spanish Geological and Mining Survey, most of industrial residues produced annually in Spain mainly originate from overburden mining exploitations (http://www.igme.es). Metallurgical processing of sulfidic ore deposits can produce tailings of discarding gangue material containing variable amounts of metallic minerals. Disposal of such metalliferous mine tailings can, therefore, mobilize metals into the environment.

Over the past 20 years, Europe has been witnessing big environmental disasters associated with the collapse of wastes stockpiles and tailings dams. The relatively recent accidents occurred in Aznalcóllar (Spain) (Álvarez-Ayuso et al. 2008) and in Baia Mare (Romania) (Gelencsér et al. 2011) have sped up an internal debate in EU countries in order to tackle environmental issues created by mining residues containing toxic metals and its remediation possibilities by natural processes.

Soils can be considered as living systems that respond in a fast way to external aggressions. In this sense, soils can cooperate to restore environmental impacts by modifying chemical species responsible for such damage. Clear evidences proved that geochemical and biological processes taking place beneath the soil surface could affect many contaminants. Soil differences related to geological, physiographic, and climatic aspects have an effect on the indigenous microbiota and that could modify the decontamination mechanism. Although these natural processes elapse for periods of time relatively long, natural attenuation is probably one of the cheapest and more efficient solutions that can be adopted in the decontamination of contaminated soils (Sinha et al. 2009; Juwarkar et al. 2010; Kavamura and Esposito 2010; Girma 2015).

Industrial dumping, particularly mining residues, contain elements potentially toxic and are an important source of pollution in natural systems. The ability of soils to attenuate these detrimental phenomena through natural processes (biological,

Table 1 Major progress in the treatment of contaminated sites in Western European countries (Prokop and Schamann 2000)

Phase	Germany	Austria	Belgium	Denmark	Spain	Finland	Holland	Sweden	Switzerland
Identification	95%	42%	90%	0%	*27%*	*72%*	34%	57%	70%
Evaluation	18%	0%	35%	20%	*2%*	*3%*	6%	9%	7%

chemical, and physical) can be used as a remediation technique in the treatment of contaminated soils (Peng et al. 2009; Johnson 2014). Bioremediation process has the potential to decontaminate soils based on the vast quantities of microorganism species present in terrestrial habitats with the ability to mobilize metals from labile to less available soil fractions (Ellis 2004). However, the effectiveness of this process depends on several factors among which the most relevant are the type and concentration of contaminants and the physicochemical, mineralogical, and microbiological characteristics of soil (Wang and Mulligan 2009; Díaz et al. 2015).

Heavy metals in soils can be more or less detrimental depending on its distribution in different soil fractions. Figure 1 depicts a general scheme of the relationship between distribution of metals in different soil fractions and bioavailability of metals in soil constituents. Metal (im)mobilization is strongly conditioned by microbiological processes and by the sorption characteristics of the different solid fractions present in the soil. The potential for mobilizing metals in natural environments is notably affected by metal partitioning among different soil fractions. In this way, heavy metals can be more or less harmful depending on its distribution in soils (Fig. 1a).

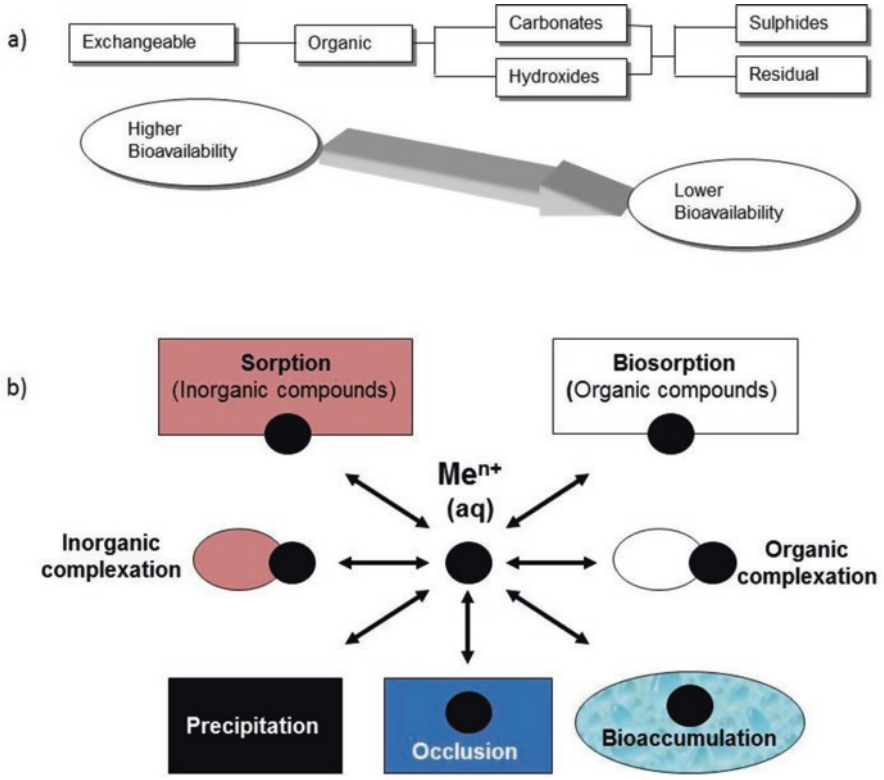

Fig. 1 Schemes of metal distribution in different soil fractions based on sequential extraction procedures (**a**) and interaction modes between metals and soil components (**b**) (Cortez et al. 2010)

Research in this field has been focused on metal-soil interactions and in remediation methods (Malik 2004; Gadd 2010; Brown and Calas 2011). The degree of immobilization of metals in soils is closely related with its partitioning between the different fractions and depends largely on the environmental conditions prevailing in natural ecosystems (pH, redox potential, salinity, metal concentration, microbial consortia, temperature, ecotoxic components, etc.) (Fig. 1b). Fluctuations of these environmental parameters can have a big influence on metal mobilization from different soil fractions: exchangeable (soluble fraction easily available), organic (metals bound to organic matter), carbonates and hydroxides (less-mobile metals, especially in alkaline soils), oxides-sulfates (metal associated to soils affected with conditions slightly oxidizing), sulfides (higher stability fraction – metals in this fraction could be solubilized in acid soils containing Fe- and S-oxidizing microbial populations), and residual (metals with the lowest mobilization rate). In this way, metals associated with the exchangeable soil fraction are more bioavailable than metals in the residual fraction.

Environmental risk assessment of heavy metals in terrestrial environments cannot be determined from soil global analyses. On the contrary, metals bioavailability greatly depends on its distribution between the different soil fractions. Thus, metal distribution in contaminated soils can be conveniently evaluated analytically by sequential extraction procedures. These analytical methods are based on the use of specific chemical reagents for dissolving preferentially metals from different solid soil fractions (interchangeable, organic, carbonates, oxides/hydroxides, sulfides, and residual) allowing the determination of metal partitioning in soils.

Typical microcosms are laboratory-scale models of natural systems and provide useful information in the short term on the biochemical processes involved. Bioaugmentation is, besides biostimulation, one of the existing possibilities to modify the soil decontamination process by increasing the indigenous microbial population of the ecosystem with the external addition of metabolically active microorganisms (Olaniran et al. 2006; Salinas-Martínez et al. 2008; Kang et al. 2016). In the present study, bioprocess was investigated, and the physicochemical changes provoked were monitored both in the aqueous phase (pH, ORP, metals in solution) and in the solid phase (metal distribution in different fractions).

The present work focused on environmental issues caused by toxic metal residues and its remediation possibilities by natural processes. A contaminated soil collected in a mining district in the southeast of the Iberian Peninsula will be characterized, and its behavior against the contaminants will be tested for two different soil fractions in microcosm tests. The speciation of the contaminants in the soils will be carried out following the ad hoc technical protocol shown in Fig. 2.

Background of the Case Study

Mining activities in the mining district of Sierra Cartagena-La Unión in the southeast of the Iberian Peninsula have been undertaken from more than 2500 years until practically 1991, when the extraction of metals was shut down as a consequence of

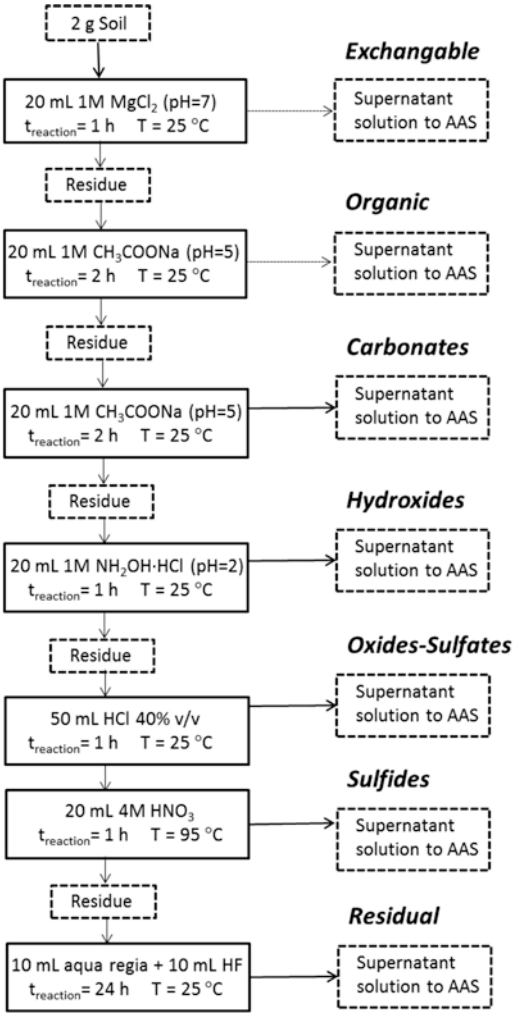

Fig. 2 *Ad hoc* sequential extraction procedure used in the present study for the analysis of the different soil fractions

economic and environmental issues (Robles-Arenas et al. 2006). Over this long period of time, large quantities of Pb-Zn tailings have been accumulated in the main mining area, with an extension of approximately 100 km^2.

Historically, more than 2000 abandoned mine waste accumulations have been recorded; among them are 12 open-pits, 3000 mineshafts, and hundreds of kilometers of underground mine galleries (Robles-Arenas 2007). All these mining operations and the accumulation of waste residues have swept away the natural vegetation cover from the soil. Likely, weathering processes have affected metal mobilization of these waste rock piles and, as a result of it, the entrance of ecotoxic elements present in the mineralization toward these ecosystems. On the other hand, the wind could have favored dust transportation to distant soils, which were not directly affected by surface drainages. In this study, sampling was done in two different locations several kilometers

Fig. 3 General view and detail of sampling sites: mine tailing (*left side*) and natural soil (*right side*)

away from each other with (Fig. 3) a contaminated soil (CS), a mine tailing collected from an overburden dump, and a non-contaminated natural soil.

Tailing samples used in this study were taken from the bottom of a mineral waste rock pile located nearby the small town of Llano del Beal and at 4.0 km from the mining town of La Unión (Autonomous Community of the Region of Murcia, Spain). As shown in Fig. 3 (above), the waste rock pile presented weathering overprints of a white precipitate rich in zinc (later identified as zinc sulfate) formed by the disturbance of the waste residue. In order to have a soil sample with less weathering grade, the topsoil was discarded. Nevertheless, soil samples from the topsoil and the bottom soil were characterized by SEM-EDS to have a better understanding of changes that could have occurred during its accumulation. The natural soil (NS) was sampled several kilometers from the mine tailing (CS) in an area apparently away from direct sources of pollution. The presence of a well-established vegetation cover in the natural soil could be used as soil pollution indicator. In this case, the vegetation cover was removed away before sampling (Fig. 3) and the natural soil was used for comparative purposes (Table 2).

The mineralogical characterization of the contaminated soil by X-ray diffraction is shown in Fig. 4. The main mineral constituents present in the CS are oxides (quartz, birnessite), sulfates (gypsum and potassium jarosite) and in less proportion sulfides (pyrite).

Table 2 Elemental composition of soils sampled

Sample	Fe (%)	Cu (%)	Zn (%)	Pb (%)
Contaminated soil (Chemical analysis)	26.09	0.017	0.66	0.77
Contaminated soil (Sequential extraction)	27.33	0.018	0.77	0.81
Natural soil	2.12	0.003	0.02	0.06

Elements were determined by chemical analysis (CS and NS) and by the sequential extraction procedure (CS) (please also refer to Fig. 2)

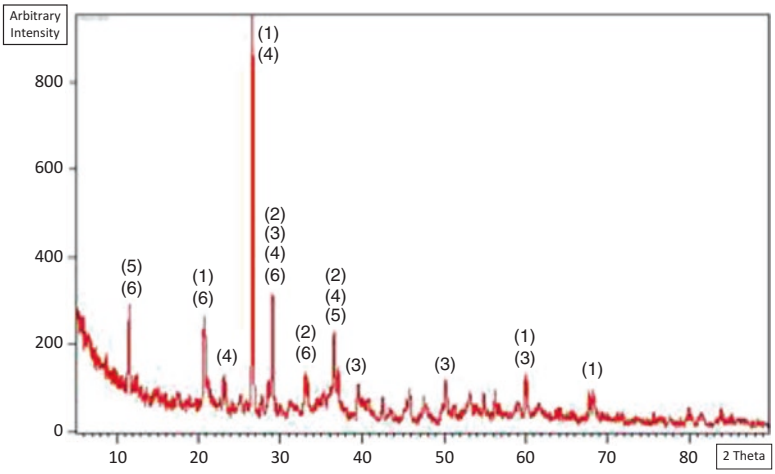

Fig. 4 Diffractogram of the contaminated soil sampled. Diffraction peaks correspond to (*1*) quartz (SiO_2), (*2*) pyrite (FeS_2), (*3*) potassium jarosite ($KFe_3(OH)_6(SO_4)_2$), (*4*) elemental sulfur (S°), (5) birnessite ($(Na_{0.3}Ca_{0.1}K_{0.1})(Mn^{4+},Mn^{3+})_2O_4 \cdot 1.5H_2O$), and (6) gypsum ($CaSO_4 \cdot 2H_2O$)

SEM-EDS Study of the Contaminated Soil Sample

It would be reasonable to assume that there should be a close relationship between the environmental impact caused by mine tailings accumulations on the host soil and the chemical composition of the mining residues abandoned. Based on this premise, the most surficial zone of the mine tailing was sampled (dashed circle in Fig. 3) and chemically characterized by scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDS). SEM-EDS study was performed on both the top cover and the underneath cover of the contaminated soil.

SEM observations of the top cover (Fig. 5) showed specific areas rich in mineral sulfides, mainly pyrite (FeS_2). The analysis of zone 3 (Table 3 and Fig. 5b) corresponds with the white areas in the EDS maps for sulfur shown in Fig. 5c. EDS analyses (zones 1 and 2) collected in Table 3 confirmed that the sample under study was mainly composed of a mixture of aluminosilicates and a phase rich in S, Fe, and O, possibly a basic iron sulfate formed by the attack of the mining tailing exposed to the natural elements.

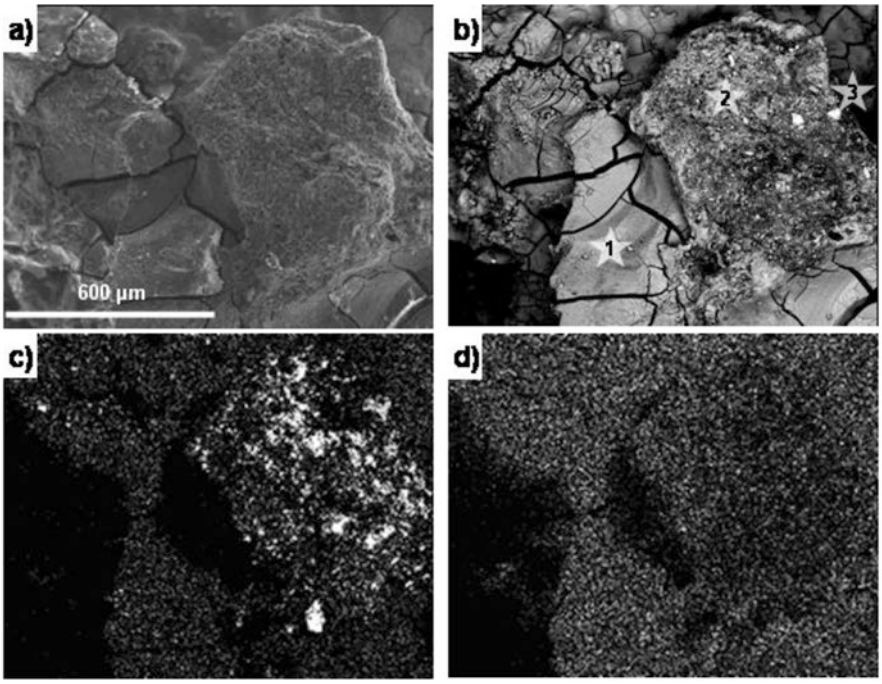

Fig. 5 SEM micrograph of the CS top cover: secondary (**a**) and backscattered electron images (**b**), with the three point analyses, and EDS maps of S (**c**) and Fe (**d**) (please also refer to shown in Table 3)

Table 3 EDS point analysis performed in zones shown in Figs. 5b and 6b

	O	Fe	S	Si	Al	Pb	Zn	Ti	Mg	K	Na	Mn
Top cover												
Zone 1	41.16	51.06	6.98	0.26					0.54			
Zone 2	46.82	22.56	4.47	12.93	9.05			0.43	2.51	1.23		
Zone 3		47.04	52.96									
Underneath cover												
Zone 1	37.72	50.40	5.85	2.65	1.24		0.95		0.89	0.29		
Zone 2	53.61	32.76	5.79	2.34	2.06	1.63			0.56	0.43	0.53	0.27

The underneath cover, unlike the top cover, contained less significant amounts of iron sulfides (Fig. 6). Nevertheless, enrichments of sulfur and heavy metals, such as Pb and Zn, were punctually found in this zone (Table 3). These findings point to the important role-play by the weathering of the top cover to mobilize these metallic elements toward deeper soil horizons. In fact, the chemical composition of the underneath cover in this zone rich in sulfur is very similar to the top cover, with significant amounts of basic sulfates and iron aluminosilicates (Fig. 6).

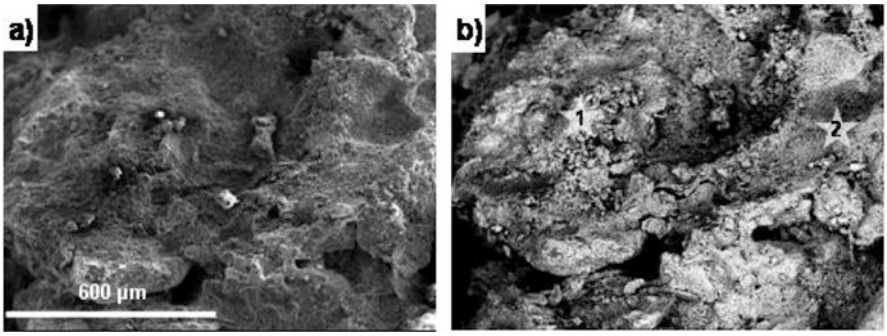

Fig. 6 SEM micrograph of the CS underneath cover in Fig. 3: secondary (**a**) and backscattered electron images (**b**), with the two point analyses (please also refer to Table 3)

Table 4 Metal and S-sulfate contents determined by chemical analysis of the soils sampled: contaminated soil (global and coarse and fine fractions) and natural soil

Soil sample	Fe (mg/kg soil)	Zn (mg/kg soil)	Pb (mg/kg soil)	Cu (mg/kg soil)	S (SO_4^{2-}) (mg/kg soil)
CS - Global	260,880	6650	7679	172	34,900
CS - Coarse fraction	275,131	4542	4503	168.5	15,984
CS - Fine fraction	330,787	6190	13,585	230.4	43,582
NS	21,173	231.6	612	29.8	n.d.

N.D. not detected

Materials and Methods

Contaminated Soil

The contaminated soil (CS) was tested in microcosms. The influence of bioaugmentation was investigated using two different soil particle sizes: a fine fraction (<100 µm) and a coarse fraction (100 < × < 1000 µm). Chemical analyses were performed in triplicate by acid digestion followed by determination of metal contaminants by atomic absorption spectrometry. The content of metals (Fe, Zn, Pb, and Cu) and S-sulfate both in the contaminated and natural soils are collected in Table 4. In addition, an ad hoc sequential extraction chemical analysis performed in triplicate and considering six soil fractions (exchangeable, organic, carbonates, hydroxides, sulfides, and residual) confirmed that the main soil contaminants were metal sulfides.

Microbial Cultures

The microorganisms used in the bioaugmentation process were isolated from the own contaminated soil. The Fe- and S-oxidizing ability of the strain was achieved by contact between the soil (10 g), 90 mL of 0 K nutrient medium (3.0 g/L

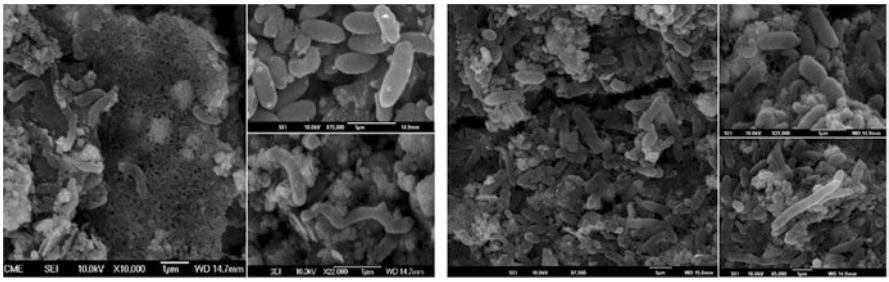

Fig. 7 Scanning electron micrographs of Fe-oxidizing (*left*) and S-oxidizing (*right*) microbial cells grown on the contaminated soil

$(NH_4)_2SO_4$; 0.5 g/L $MgSO_4 \cdot 7H_2O$; 0.1 g/L KCl; 0.1 g/L K_2HPO_4; 0.013 g/L $Ca(NO_3)_2 \cdot 4H_2O$) and either ferrous sulfate (1.0 g/L Fe^{2+}) or elemental sulfur (1.0 g), respectively. After positive bacterial growth, Fe- and S-oxidizing bacterial cultures were grown for 20 days in three consecutive transfers using sterilized CS. The morphology of bacterial cells of both strains is shown in Fig. 7.

Microcosms

The microcosm tests were performed in Erlenmeyer flasks, containing 10 g of contaminated soil and 90 mL of deionized water, in an orbital shaker at 150 rpm and 35 °C. The contaminated soil was tested both in sterilized conditions (control microcosm) or bioaugmented with 10 mL of a microbial strain of Fe-oxidizing or S-oxidizing microorganisms (microcosm Fe-ox and S-ox, respectively). Periodically, pH, ORP, and metal concentration (Fe and Zn) were measured in solution. After 49 days, the solid residues were dried and analyzed following a sequential extraction procedure similar as for the as-received contaminated soil.

Results and Discussion

pH and Redox Potential Measurements

pH and ORP are physicochemical parameters that can be used to predict microbial activity in microcosms. The evolution of both parameters is shown in Fig. 8. The evolution of pH in microcosms with both soil particle size fractions is depicted in Fig. 8a. It is clear from this figure that a bioaugmentation in the system with Fe- or S-oxidizing microorganisms had an immediate effect on this parameter due to the acidic conditions created in the inoculated microcosms by bacterial activity. No big differences were observed in pH evolution when comparing the two different

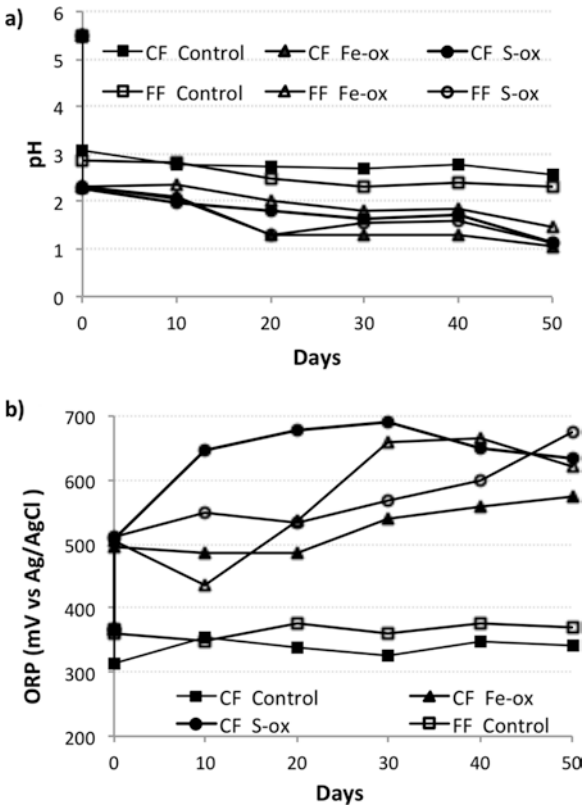

Fig. 8 Evolution of pH (**a**) and ORP (**b**) over time for the different soil fractions (CF, coarse fraction and FF, fine fraction) tested in non-inoculated microcosms (control) and microcosms inoculated with Fe-oxidizing (Fe-ox) or S-oxidizing (S-ox) microorganisms

bacterial cultures tested independently of the soil fraction used. However, there is a trend toward higher acid production for the coarse fraction compared to the fine soil fraction. This could be attributed to the higher content of pyrite in the coarse fraction and to a greater production of H_2SO_4 after biological oxidation of mineral sulfides mediated by S-oxidizing and Fe-oxidizing microorganisms, according to reactions 1, 2, 3, and 4 (Plumb et al. 2008; Schippers et al. 2010).

$$MeS + 2Fe^{3+} \rightarrow Me^{2+} + 2Fe^{2+} + S^{\circ} \quad (1)$$

$$S^{0} + H_2O + \tfrac{3}{2}O_2 \xrightarrow{\text{S-oxidizers}} H_2SO_4 \quad (2)$$

$$FeS_2 + \tfrac{7}{2}O_2 + H_2O \rightarrow FeSO_4 + H_2SO_4 \quad (3)$$

$$2Fe^{2+} + \tfrac{1}{2}O_2 + 2H^{+} \xrightarrow{\text{Fe-oxidizers}} 2Fe^{3+} + H_2O \quad (4)$$

The evolution of redox potential in microcosms with both soil particle size fractions is shown in Fig. 8b. ORP values in inoculated microcosms were much higher

than those in control tests for both the fine and coarse soil fractions. Bioaugmentation of the contaminated soil resulted in a higher bacterial activity when using S-oxidizing bacteria. Thus, in the microcosms with microbial activity, values of pH and ORP were close to 1.0 and 600 mV, respectively. By contrast, the values obtained in the microcosm control were 2.4 and 340 mV, respectively.

Metals Dissolution

The aggressive conditions generated by the microbial activity could promote the dissolution of metals from the soil. As shown in Fig. 9a, the iron concentration in the solution increased gradually and equally in microcosms with S-oxidizing microorganisms for both soil particle sizes. Probably the content of elemental sulfur detected by XRD in the as-received contaminated soil (Fig. 4) would be sufficient to stimulate the growth of the S-oxidizing bacterial population. Conversely, microcosms inoculated with Fe-oxidizing microorganisms showed a different behavior depending on the soil particle size. Higher iron dissolution was recorded for the coarse fraction compared to the fine fraction. The growth of Fe-oxidizing bacterial cultures in the sterilized contaminated soil should be regulated mainly by the content of pyrite. The lower amount of iron sulfides in the fine soil fraction would be responsible for the bacterial growth delay observed for this soil fraction. Furthermore, it cannot be discarded iron precipitation as hydroxysulfates favored by the preexisting jarosite in the fine soil fraction (<100 μm). Unlike inoculated microcosms, iron dissolution in microcosm control was practically insignificant.

Zinc dissolution started uniformly and instantly in all microcosms (Fig. 9b). In all cases, the zinc dissolution process proceeds in two stages: in the first stage, zinc is easily leached from the soil depending on the oxidizing conditions generated by the microbial activity, followed by a second stage where a plateau is more or less reached. The more marked differences were recorded in the first 10 days and are principally associated to the coarse soil fraction in inoculated microcosms with respect to the rest of tests. Again this would be an indication that the main carrier of metal sulfides in the contaminated soil is the coarse soil fraction.

Metal Distribution in Solid Residues

The chemical and biochemical reactions that took place in the microcosms with both particle size fractions affected the composition of both the aqueous and the solid phases. After 50 days, the new distribution of metal contaminants (Fe, Zn, and Pb) was determined in the solid residues in both the fine (<100 μm) and coarse (>100 μm) soil fractions. Residues were analyzed following an ad hoc sequential extraction procedure, slightly different from the EPA procedure (Brady et al. 1999). In the case of iron (Fig. 10), its mobilization from the sulfides to the oxide-sulfate soil fraction

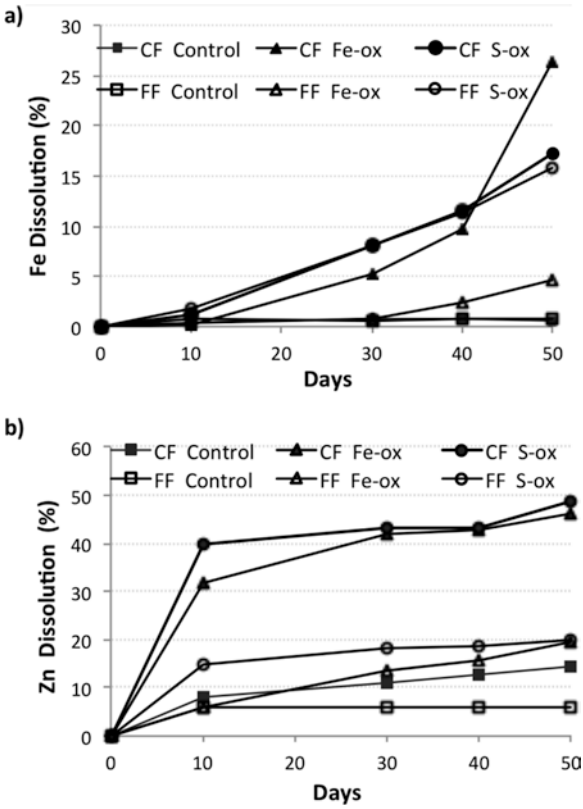

Fig. 9 Metals dissolved, (**a**) iron and (**b**) zinc, over time for the different soil fractions (CF, coarse fraction and FF, fine fraction) tested in non-inoculated microcosms (control) and microcosms inoculated with Fe-oxidizing (Fe-ox) or S-oxidizing (S-ox) microorganisms

and to the leached phase is quite evident because of the low iron concentration in the more labile soil fractions (exchangeable, organic, carbonates, and hydroxides). That is in agreement with the amounts of iron in the solution observed: the higher the microbial activity is, the higher the mobilization of iron and the more pronounced its decrease in the sulfide fraction for both soil particle sizes tested. On the other hand, zinc was easily mobilized from the more labile soil fractions to the aqueous phase (Fig. 10). This is an indication that the zinc weakly adsorbed can be dissolved from those soil fractions in mild environmental conditions of pH and ORP (Gleyzes et al. 2002; Poulton and Canfield 2005). That would be in agreement with the dissolution of Zn observed during the first stage in all microcosms (Fig. 9b). Since more oxidizing conditions are required in order to release zinc from the sulfides soil fraction, such effect is only detectable in the microcosms with microbiological activity, in agreement with the dissolution of Zn observed in Fig. 9b. Then, the absence of microbial activity in the microcosm control would clearly condition the dissolution of Zn from the less labile soil fractions for both soil particle sizes tested.

Unlike Fe and Zn, lead in the fine soil fraction (<100 μm) has a low bioavailability and is mainly associated to the oxide-sulfates either as plumbojarosite ($PbFe_6(SO_4)_4(OH)_{12}$) or anglesite ($PbSO_4$) (Fig. 10). By contrast, the content of lead

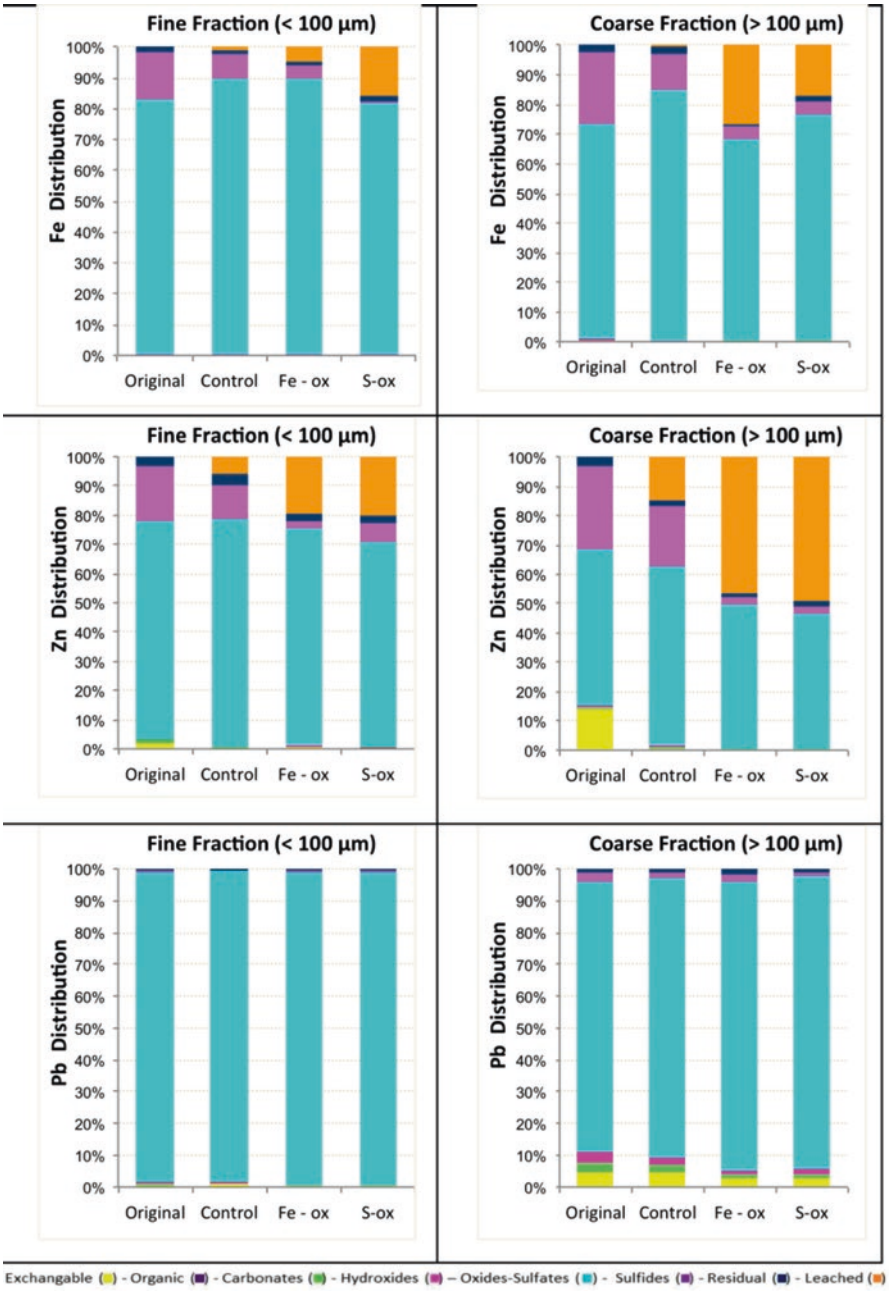

Fig. 10 Distribution of metals (iron, zinc, and lead) in the different soil fractions (determined by the ad hoc sequential extraction procedure shown in Fig. 2) for the residues collected from the different microcosms: fine (*left*) and coarse (*right*) fractions

in the coarse soil fraction is more bioavailable in more labile soil fractions, which decrease in the presence of microbial activity (microcosm Fe-ox and S-ox) but not for the microcosm without microbial activity (control). However, mobilization of Pb from the less bioavailable sulfide soil fraction occurred in all microcosms. This would be attributable to the dissolution of galena and to the incorporation of the oxidized compounds form to the oxide-sulfates fraction, which is highly stable under environmental conditions of microcosms (Lu et al. 2005; Romero et al. 2007). Finally, lead in the residual fraction did not show noticeable changes as compared to the other two metals (Fe and Zn) .

Conclusions

The present work focused on environmental issues caused by a mine tailing residue collected in a mining district in the southeast of the Iberian Peninsula and its remediation possibilities by natural processes. The influence of bioaugmentation was investigated in microcosm tests using two different soil particle sizes. Bioaugmentation with Fe- and S-oxidizing microorganisms favored metal mobilization with respect to the control test, with a better performance of the S-oxidizing bacterial culture. S-oxidizing microorganisms led to a higher mobilization of Fe and Zn from the sulfide fraction toward the aqueous phase. Conversely, Pb was mobilized, both in biotic and abiotic microcosms, toward the oxide-sulfates soil fraction. The speciation of metals in different soil fractions of the contaminated soil was determined by an ad hoc sequential extraction protocol. The main phases associated to the fine soil fraction (<100 μm) were gypsum and jarosite, while goethite was mainly present in the coarse soil fraction (>100 μm). This is an indication that the weathering products of the contaminated soil are mostly incorporated to the fine soil fraction.

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Remediation of Polluted Soils Using Hyperaccumulator Plants

Neerja Srivastava

Abstract The problem of pollution is continuously worsening due to a series of human activities, leading to intensification of the research dealing with the phytotoxicity of these contaminants and with the mechanisms used by plants to counter their harmful effects. Great interest has been gained by the behavior of hyperaccumulator plants growing on metalliferous soils, which accumulated heavy metals in leaves at concentrations several 100-folds higher than other plants. The aims of studying these heavy metal hyperaccumulator species have been to highlight physiological and molecular mechanisms underlying the hyperaccumulation ability, to discover the adaptive functions performed by hyperaccumulation in these plants, and to explore the possibility of using them as tools to remove metals from contaminated or natural metal-rich soils. However, in spite of important progress made in recent years by the numerous studies accomplished, the complexity of hyperaccumulation is far from being understood, and several aspects of this astonishing feature still await explanation.

Keywords Phytoremediation • Metal hyperaccumulators • Phytoextraction

Introduction

Trace elements (heavy metals and metalloids) are important environmental pollutants, and many of them are toxic even at very low concentrations. Pollution of the biosphere with trace elements has accelerated dramatically since the beginning of the Industrial Revolution (Padmavathiamma and Li 2007). The primary sources of this pollution are the burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, and sewage (Wei and Zhou 2008). In addition to sites contaminated by human activity, natural mineral deposits containing particularly large quantities of heavy metals are present in many regions of the globe. These areas often support characteristic plant species that thrive in these metal-enriched environments. While many species avoid the uptake of heavy metal

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forms of these soils, some of these species can accumulate significantly high concentrations of toxic metals to levels, which by far exceed the soil levels (Baker and Brooks 1989). It is known that the essential metals Fe, Mn, Zn, Cu, Mo, and Ni are taken up and accumulated by plants (Williams et al. 2000). Certain plants are also able to accumulate heavy metals, which have no known biological function. These include Cd, Cr, Pb, Co, Ag, and Hg (Baker and Brooks 1989). However, excessive accumulation of these heavy metals can be toxic to most plants. The ability to acquire a tolerance both against heavy metals and an accumulation to very high concentrations have evolved both independently and together in a number of different plant species (Baker and Walker 1990; Stearns et al. 2007).

Generally, there are three different types of plants that have developed three basic strategies for growing on contaminated and metalliferous soils (Baker and Walker 1990). Metal excluders effectively prevent metal from entering their aerial parts over a broad range of metal concentrations in the soil; however, they can still contain large amounts of metals in their roots. Metal indicators accumulate metals in their aboveground tissues, and the metal levels in the tissues of these plants generally reflect metal levels in the soil. Metal accumulators are usually referred to as hyperaccumulators that concentrate metals in their aboveground tissues to levels far exceeding those present in the soil or in non-accumulating species growing nearby. It has been proposed that a plant containing more than 0.1% of Ni, Co, Cu, Cr, and Pb or 1% of Zn on a dry weight basis is called a hyperaccumulator, irrespective of the metal concentration in the soil (Baker and Walker 1990). There are around 400 plant species known worldwide to accumulate metals in large amounts, and these species are of interest for potential use in phytoremediation of metal-contaminated soils (Brooks 1983; Memon and Yatazawa 1984; Baker et al. 2000; Pilon-Smits 2005). Information related to accumulator plants is most needed in four areas: first, the metal-accumulating ability of various species as a function of soil metal concentrations, physicochemical soil properties, and physiological state of the plant; second, the specificity of metal uptake, transport, and accumulation; third, the physiological, biochemical, and molecular mechanisms of accumulation; and fourth, the biological and evolutionary significance of metal accumulation (Memon and Schröder 2009).

Types of Pollutants

There are a variety of different pollutants, originated, in most cases, by human action. To facilitate the development of studies on decontamination techniques and also according to the different physical and chemical characteristics they present, the different types of contaminants were divided into two major classes: organic and inorganic. These two groups are further subdivided. Organic pollutants include various compounds such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), nitroaromatic (explosives), halogenated hydrocarbons, and chlorinated solvents. When compared to inorganic, the organic pollutants are relatively less toxic to plants because they are less reactive and do not accumulate readily. Many of these compounds are not only toxic or teratogenic but also carcinogenic. The

inorganic contaminants include heavy metals, such as mercury, lead, and cadmium, among others and nonmetallic compounds like arsenic and radionuclides like uranium, cesium, chromium, strontium, technetium, tritium, etc. Many metals are essential to growth and development of living forms. However, when in high concentrations, they become extremely toxic, leading the organism to oxidative stress with great production of harmful-free radicals, highly dangerous to cells and tissues. Some particularly reactive metals interfere in the structure and function of proteins and also cause the substitution of other essential nutrients (Garbisu et al. 2002; Taiz and Zeiger 2002; Pulford and Watson 2003). Many elemental pollutants penetrate the plant through regular systems of nutrient absorption. The plants protect themselves from these xenobiotics through degradation of endogenous toxic organic or sequestering them in the vacuoles (Meagher 2000). Different technologies of phytoremediation are compatible with a great number of pollutants. Constructed wetlands have been applied for many inorganics, including metals, nitrates, phosphates, and cyanides, as well as organics such as explosives and herbicides (Schnoor et al. 1995; Horne 2000; Jacobson et al. 2003). There is a special category of plants called hyperaccumulators, for they accumulate a considerable amount of toxic metals and radionuclides in their tissues (phytoextraction), keeping these compounds above the ground surface. This is the main goal of phytoremediation (Mello-Farias et al. 2011).

To physically remove metals from the contaminated site, the aboveground shoots of the hyperaccumulator plants are harvested and subsequently disposed of as hazardous wastes or treated for the recovery of the metals. Phytoremediation can be used to remove not only metals (e.g., Ag, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, Zn) (Juwarkar et al. 2010) but also radionuclides (e.g., 90Sr, 137Cs, 239Pu, 234U, 238U) (Cook et al. 2009; Fulekar et al. 2010; Hegazy and Emam 2010; Cerne et al. 2011) and certain organic compounds (i.e., petroleum hydrocarbons) (Abhilash et al. 2009; Gerhardt et al. 2009; Hussain et al. 2009; Perelo 2010; Megharaj et al. 2011). Plants growing in metal-contaminated environments can accumulate toxic metal ions and efficiently compartmentalize them into various plant parts. Several studies indicated that the partitioning of heavy metals at the whole plant level can broadly be divided into three categories. For instance, Chaney and Giordano classified Mn, Zn, Cd, B, Mo, and Se as elements, which were readily translocated to the plant shoots; Ni, Co, and Cu were intermediate, and Cr, Pb, and Hg were translocated to the lowest extent (Alloway 1995; Zitka et al. 2013).

Inorganics

The absorption of any metal by plants depends on the metal relative bioavailability in the contaminated array. Changes in the soil chemistry, such as decreased pH, may increase the availability of many metals for the absorption by the roots. Many plants can absorb significant levels of metals in some soil conditions. Changes in rhizosphere microbial status (e.g., presence of mycorrhizae) can also have profound effects (positive or negative) on the uptake of metals by the roots. The general consensus of researchers in this area, however, is that phytoremediation, especially for

heavy metals, will only be economically viable through the use of hyperaccumulators. The research in the past two decades has shown that certain specialized plants have the ability to accumulate more than 3% (dry weight) of heavy metals and over 25% (dry weight) in sap/latex with no apparent damage to the plant (Baker and Brooks 1989; Baker et al. 1994a, Huang and Cunningham 1996). The mechanisms that govern this tolerance and absorption of excessive concentrations of metals in leaves were the subject of active research and vary according to the element (Cunningham and Lee 1995; Huang and Cunningham 1996). The mechanisms of tolerance include the accumulation of Zn in cell walls; Ni associated with the pectin in large cells; Ni, Co, and Zn being chelated by malic acid; phytochelatin associated to Zn; Ni chelation by citrate; and Co associated with oxalate crystals calcium in plant tissues. Knowledge of the mechanisms of tolerance will aid in identifying the genetic characteristics necessary for the transfer of metal tolerance of plants capable of producing greater biomass with deeper rooting. It was suggested that in some cases, the resulting biomass rich in metals (biominery) could be incinerated and have metals economically recycled. This "biomineration" of metals can also be applied as a mining technique for metals with significant economic value (Robinson et al. 1998). Another type of inorganic compounds that may be susceptible to phytoremediation is radionuclides. The presence of radionuclides in soil and water poses serious risk to human health. These contaminants come from the explosion of atomic bombs or nuclear power plant accidents such as Chernobyl, Ukraine, and more recently in Fukushima, Japan. The selection of an appropriate cleaning technology of these contaminated areas is based on the environmental chemistry of each element, character of deposition, and rate of radioactive decay. A variety of physicochemical methods are available, like soil washing, ion exchange, leaching with chelating agents, flocculation, and osmosis-ultrafiltration. Recently there has been increasing interest in the use of biological methods to remove radionuclides (Dushenkov 2003). Negri and Hinchman (2000) reported data in the use of plants for the treatment of 3H, U, Pu, 137Cs, and 90Sr.

Organics

More recently, with the development of the pesticide industry, the metabolic capacity of the plant system began to be assessed. The most modern herbicides are based on the selectivity of crops due to metabolic differences between species of plants. This capability, often created by man, is the cornerstone of the highly profitable market of herbicides. "Desirable" plants rapidly metabolize the herbicide compound in a nontoxic one, while "undesirable" herbs do not and are therefore dead. This mechanism, developed by the natural selection of plants, proves to be potentially exploitable in the remediation of contaminated soils. This ability of plants to detoxify xenobiotics is widely recognized and with current utility. Besides, plants generally have a metabolic system with differences in the efficiency of degradation of toxic compounds when compared to microorganisms, what makes the union of these two distinct systems in the rhizosphere, an ideal situation for a more efficient

phytoremediation. Recent research includes plant selection, alternative patterns of rooting, the composition of exudates produced by the plant and its effect on microbial communities, exudation of specific compounds inducing specific metabolic pathways, and inoculation with rhizosphere microorganisms capable of degrading xenobiotics efficiently (Langenbach 1994). The plants and their roots can create an environment in the soil which is rich in microbial activity, able to change the availability of organic contaminants or increase the degradation of certain organic compounds, such as hydrocarbons derived from petroleum. Siciliano et al. (2003) evaluated the impact of microbial remediation on soil mass and the capacity of microbial community to degrade hydrocarbons in order to determine whether phytoremediation treatments increase the metabolic potential of microbial community by altering its taxonomic structure. It was found that the best remediation system to reduce hydrocarbons in the soil was obtained by increasing the population of bacteria-containing genes for the catabolism of hydrocarbons in the rhizosphere community, thus demonstrating the importance of using microorganisms in phytoremediation. However, it is necessary to identify the species of suitable plants that can beneficially alter microbial diversity for soil remediation. According to Pires et al. (2003), the absorption of herbicides by plants is affected by the compound's chemical properties, environmental conditions, and the characteristics of plant species. Actually, the probability of a plant being phytoremediator depends on the type of pollutant; plants should be tested to detect that one with the greater resistance to a specific pollutant. Esteve-Núnez et al. (2001) evaluated trinitrotoluene (TNT) and found that its chemical structure influences its biodegradability. According to these authors, the oxygenated metabolism for aromatic compounds by bacteria does not occur in TNT because of its chemical properties generating compounds not metabolized by microorganisms. However, anaerobic processes have advantages because of the absence of oxygen. Therefore, the use of fungi for the bioremediation of TNT has generated considerable interest. Esteve-Núnez et al. (2001) concluded that the remediation of TNT by these organisms is a very valid process, and the rhizoremediation by microbes able to colonize the rhizosphere of plants will provide a fast and efficient mechanism for the removal of this pollutant. There are some contaminants called persistent organic pollutants (POPs) that resist long in the soil. Some examples are dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB), dioxins, etc. Research has shown that a variety of plants can remove persistent compounds, transporting them to aerial plant tissues (Coutinho and Barbosa 2007). It is important to highlight that, due to variety of contaminants, the study of pollutant is very important to generate an effective phytoremediation. Most current methods of cleaning metals and volatile organic compounds not on the soil surface are coarse, expensive, and physically destructive (Baker et al. 1994). The remediation by conventional methods of engineering often costs 50–500 dollars per ton of soil, and certain specialized techniques can cost up to US$ 1000 (Cunningham and Ow 1996). Phytoremediation associated with biotechnology is an emerging technology that promises a viable remediation when pollutants (a) are close to the surface, (b) are relatively nonleachable, and (c) have little immediate risk to the environment (Cunningham and Lee 1995). The results are more effective in slightly or moderately polluted areas. For heavy contamination, the decontamination time is too long

Table 1 Summary of phytoremediated chemicals (Nwoko 2010)

Types	Chemicals treated	Reference
Phytoaccumulation/ extraction	Cd, Cr, Pb, Ni, Zn, radionuclides BTEX, penta chlorophenol, and short-chained aliphatic compounds	Horne (2000), Blaylock and Huang (2000)
Phytodegradation/ transformation	Nitrobenzene, nitroethane, nitrotoluene, atrazine, chlorinated solvent, for example, DDT, chloroform etc.)	Schnoor et al. (1995), Jacobson et al. (2003)
Phytostabilization	Heavy metals in ponds, phenols, and chlorinated solvents	McCutcheon and Schnoor (2003), Newman et al. (1997)
Phytostimulation	Polycyclicaromatic hydrocarbon, BTEX, PCB, and Tetrachloroethane	Hutchinson et al. (2003), Olson et al. (2003)
Phytovolatilization	Chlorinated solvent, Hg, and Se	Terry et al. (1995)
Phytofiltration	Heavy metals, organics, radionuclides, and plant nutrients	Horne (2000), Nwoko et al. (2004)

BTEX benzene, toluene, ethyl benzene, xylenes, *PCB* Polychlorinated biphenyl

(Robinson et al. 1998). The combination of metal hyperaccumulation and degradation or increased sequestration of organic compounds with greater biomass and deeper rooting systems can result in a powerful technology of phytoremediation that will provide cheaper, permanent, and intrusive remediation. Table 1 shows a summary of the techniques applied to the different types of phytoremediated compounds (Mello-Farias et al. 2011).

Pollutant Remediation Strategies

The overall objective of any soil pollutants remediation approach is to create a final solution that is protective of human health and the environment (Martin and Ruby 2004). Remediation is generally subject to an array of regulatory requirements and can also be based on assessments of human health and ecological risks where no legislated standards exist or where standards are advisory. The regulatory authorities will normally accept remediation strategies that center on reducing metal bioavailability only if reduced bioavailability is equated with reduced risk and if the bioavailability reductions are demonstrated to be long term (Martin and Ruby 2004). For heavy metal-contaminated soils, the physical and chemical form of the heavy metal contaminant in soil strongly influences the selection of the appropriate remediation treatment approach. Information about the physical characteristics of the site and the type and level of contamination at the site must be obtained to enable accurate assessment of site contamination and remedial alternatives. The contamination in the soil should be characterized to establish the type, amount, and distribution of heavy metals in the soil. Once the site has been characterized, the desired level of each metal in soil must be determined. This is done by comparison of

observed heavy metal concentrations with soil quality standards for a particular regulatory domain or by performance of a site-specific risk assessment. Remediation goals for heavy metals may be set as total metal concentration or as leachable metal in soil or as some combination of these. Several technologies exist for the remediation of metal-contaminated soil. Gupta et al. (2000) have classified remediation technologies of contaminated soils into three categories of hazard-alleviating measures: (i) gentle in situ remediation, (ii) in situ harsh soil restrictive measures, and (iii) in situ or ex situ harsh soil destructive measures. The goal of the last two harsh alleviating measures is to avert hazards either to man, plant, or animal, while the main goal of gentle in situ remediation is to restore the malfunctionality of soil (soil fertility), which allows a safe use of the soil. At present, a variety of approaches have been suggested for remediating contaminated soils. USEPA (2007) has broadly classified remediation technologies for contaminated soils into (i) source control and (ii) containment remedies. Source control involves in situ and ex situ treatment technologies for sources of contamination. In situ or in place means that the contaminated soil is treated in its original place, unmoved, and unexcavated, remaining at the site or in the subsurface. In situ treatment technologies treat or remove the contaminant from soil without excavation or removal of the soil. Ex situ means that the contaminated soil is moved, excavated, or removed from the site or subsurface. Implementation of ex situ remedies requires excavation or removal of the contaminated soil. Containment remedies involve the construction of vertical engineered barriers (VEB), caps, and liners used to prevent the migration of contaminants. Another classification places remediation technologies for heavy metal-contaminated soils under five categories of general approaches to remediation (Table 2): isolation, immobilization, toxicity reduction, physical separation, and extraction (GWRTAC 1997). In practice, it may be more convenient to employ hybrid of two or more of these approaches for more cost-effectiveness. The key factors that may influence the applicability and selection of any of the available remediation technologies are (i) cost, (ii) long-term effectiveness/permanence, (iii) commercial availability, (iv) general acceptance, (v) applicability to high metal concentrations, (vi) applicability to mixed wastes (heavy metals and organics), (vii) toxicity reduction, (viii) mobility reduction, and (ix) volume reduction (Wuana and Okieimen 2011).

Table 2 Technologies for remediation of heavy metal-contaminated soils

Category	Remediation technologies
Isolation	(i) Capping (ii) subsurface barriers
Immobilization	(i) Solidification/stabilization (ii) vitrification (iii) chemical treatment
Toxicity and/or Mobility reduction	(i) Chemical treatment (ii) permeable treatment walls (iii) biological treatment bioaccumulation, phytoremediation (phytoextraction, phytostabili-zation, and rhizofiltration), bioleaching, and biochemical processes
Physical separation and extraction	(i) Soil washing, pyrometallurgical extraction, in situ soil flushing, and electrokinetic treatment

Phytoremediation

Phytoremediation is a newly evolving field of science and technology that uses plants to clean up polluted soil, water, or air (Salt et al. 1998; Rugh et al. 1999; Meagher et al. 2000). With the help of genetic engineering, plants can be used to extract, sequester, and/or detoxify a wide variety of environmental contaminants. This field is generating great excitement because phytoremediation techniques may offer the only effective means of restoring the hundreds of thousands of square miles of land and water that have been polluted by human activities. Currently, cleanup methods, such as physically removing contaminated soil from a site and burying it elsewhere, are generally too costly and environmentally destructive to be applied on the imposing scale that is now required. The principles behind phytoremediation may also improve the utility of traditionally marginal lands for agriculture and forestry. It is important to distinguish between the phytoremediation of elemental and organic pollutants at the outset. Elemental pollutants are essentially immutable by any biological or physical process short of nuclear fission and fusion, and thus their remediation presents special scientific and technical problems. Elemental pollutants include toxic heavy metals and radionuclides, such as arsenic, cadmium, cesium, chromium, lead, mercury, strontium, technetium, tritium, and uranium. With a few notable exceptions, the best scenarios for the phytoremediation of elemental pollutants involve plants extracting and translocating a toxic cation or oxyanion to aboveground tissues for later harvest, converting the element to a less toxic chemical species (i.e., transformation), or at the very least sequestering the element in roots to prevent leaching from the site. For organic pollutants, the goal of phytoremediation is to completely mineralize them into relatively nontoxic constituents, such as carbon dioxide, nitrate, chlorine, and ammonia (Cunningham et al. 1996). Organic pollutants that are potentially important targets for phytoremediation include polychlorinated biphenyls (PCBs) such as dioxin, polycyclic aromatic hydrocarbons (PAHs) such as benzoapyrene, nitroaromatics such as trinitrotoluene (TNT), and linear halogenated hydrocarbons such as trichloroethylene (TCE). Many of these compounds are not only toxic and teratogenic but also carcinogenic. Pollutants can be remediated in plants through several natural biophysical and biochemical processes: adsorption, transport and translocation, hyperaccumulation, or transformation and mineralization. For example, many elemental pollutants enter plants through nutrient transport systems. The degradation of endogenous toxic organics or their sequestration in vacuoles also protects plants from toxic xenobiotics. In many cases, the overexpression of existing plant genes or transgenic expression of bacterial or animal genes is required to enhance these natural properties (Meagher 2000).

Types of Phytoremediation Strategies

Techniques of phytoremediation include phytoextraction (or phytoaccumulation), phytofiltration, phytostabilization, phytovolatilization, and phytodegradation (Alkorta et al. 2004) (Table 3).

Table 3 Summary of the different techniques of phytoremediation (Ali et al. 2013)

Technique	Description
Phytoextraction	Accumulation of pollutants in harvestable biomass i.e., shoots
Phytofiltration	Sequestration of pollutants from contaminated waters by plants
Phytostabilization	Limiting the mobility and bioavailability of pollutants in soil by plant roots
Phytovolatilization	Conversion of pollutants to volatile form and their subsequent release to the atmosphere
Phytodegradation	Degradation of organic xenobiotics by plant enzymes within plant tissues
Rhizodegradation	Degradation of organic xenobiotics in the rhizosphere by rhizospheric microorganisms
Phytodesalination	Removal of excess salts from saline soils by halophytes

Phytoextraction

Phytoextraction (also known as phytoaccumulation, phytoabsorption, or phytosequestration) is the uptake of contaminants from soil or water by plant roots and their translocation to and accumulation in aboveground biomass, i.e., shoots (Sekara et al. 2005; Yoon et al. 2006; Rafati et al. 2011). Metal translocation to shoots is a crucial biochemical process and is desirable in an effective phytoextraction because the harvest of root biomass is generally not feasible (Zacchini et al. 2009; Tangahu et al. 2011).

Phytofiltration

Phytofiltration is the removal of pollutants from contaminated surface waters or wastewaters by plants (Mukhopadhyay and Maiti 2010). Phytofiltration may be rhizofiltration (use of plant roots) or blastofiltration (use of seedlings) or caulofiltration (use of excised plant shoots; Latin caulis = shoot) (Mesjasz-Przybylowicz et al. 2004). In phytofiltration, the contaminants are absorbed or adsorbed, and thus their movement to underground waters is minimized.

Phytostabilization

Phytostabilization or phytoimmobilization is the use of certain plants for stabilization of contaminants in contaminated soils (Singh 2012). This technique is used to reduce the mobility and bioavailability of pollutants in the environment, thus preventing their migration to groundwater or their entry into the food chain (Erakhrumen 2007). Plants can immobilize heavy metals in soils through sorption by roots, precipitation, complexation, or metal valence reduction in rhizosphere (Barcelo and Poschenrieder 2003; Ghosh and Singh 2005; Yoon et al. 2006; Wuana and Okieimen 2011). Metals of different valences vary in toxicity. By excreting special redox enzymes, plants skillfully convert hazardous metals to a relatively less toxic state and decrease possible metal stress and damage. For example, reduction of Cr(VI) to Cr(III) is widely studied, the latter being both less mobile and less toxic (Wu et al. 2010). Phytostabilization limits the accumulation of heavy metals in biota and

minimizes their leaching into underground waters. However, phytostabilization is not a permanent solution because the heavy metals remain in the soil; only their movement is limited. Actually, it is a management strategy for stabilizing (inactivating) potentially toxic contaminants (Vangronsveld et al. 2009).

Phytovolatilization

Phytovolatilization is the uptake of pollutants from soil by plants, their conversion to volatile form, and subsequent release into the atmosphere. This technique can be used for organic pollutants and some heavy metals like Hg and Se. However, its use is limited by the fact that it does not remove the pollutant completely; only it is transferred from one segment (soil) to another (atmosphere) from where it can be redeposited. Phytovolatilization is the most controversial of phytoremediation technologies (Padmavathiamma and Li 2007).

Phytodegradation

Phytodegradation is the degradation of organic pollutants by plants with the help of enzymes such as dehalogenase and oxygenase; it is not dependent on rhizospheric microorganisms (Vishnoi and Srivastava 2008). Plants can accumulate organic xenobiotics from polluted environments and detoxify them through their metabolic activities. From this point of view, green plants can be regarded as "green liver" for the biosphere. Phytodegradation is limited to the removal of organic pollutants only because heavy metals are nonbiodegradable. Recently, scientists have shown their interest in studying phytodegradation of various organic pollutants including synthetic herbicides and insecticides. Some studies have reported the use of genetically modified plants (e.g., transgenic poplars) for this purpose (Doty et al. 2007).

Rhizodegradation

Rhizodegradation refers to the breakdown of organic pollutants in the soil by microorganisms in the rhizosphere (Mukhopadhyay and Maiti 2010). Rhizosphere extends about 1.0 mm around the root and is under the influence of the plant (Pilon-Smits 2005). The main reason for the enhanced degradation of pollutants in the rhizosphere is likely the increase in the numbers and metabolic activities of the microbes. Plants can stimulate microbial activity about 10–100 times higher in the rhizosphere by the secretion of exudates containing carbohydrates, amino acids, and flavonoids. The release of nutrient-containing exudates by plant roots provides carbon and nitrogen sources to the soil microbes and creates a nutrient-rich environment in which microbial activity is stimulated. In addition to secreting organic substrates for facilitating the growth and activities of rhizospheric microorganisms, plants also release certain enzymes capable of degrading organic contaminants in soils (Kuiper et al. 2004; Yadav et al. 2010).

Phytodesalination

It is a recently reported and emerging technique (Zorrig et al. 2012). Phytodesalination refers to the use of halophytic plants for removal of salts from salt-affected soils in order to enable them for supporting normal plant growth (Manousaki and Kalogerakis 2011; Sakai et al. 2012). Halophytic plants have been suggested to be naturally better adapted to cope with heavy metals compared to glycophytic plants (Manousaki and Kalogerakis 2011). According to estimation, two halophytes, *Suaeda maritima* and *Sesuvium portulacastrum*, could remove 504 and 474 kg of sodium chloride, respectively, from 1.0 ha of saline soil in a period of 4 months. Therefore, *S. maritima* and *S. portulacastrum* could be successfully used to accumulate NaCl from highly saline soils and enable them for crop production after a few repeated cultivation and harvest (Ravindran et al. 2007). Another study has reported accumulation of about 1.0 t ha^{-1} of Na+ ions in the aboveground biomass of the obligate halophyte *S. portulacastrum* cultivated on a salinized soil. The resultant decrease in salinity and sodicity of the phytodesalinized soil significantly reduced the negative effects on the growth of the test culture of the glycophytic crop, *Hordeum vulgare* (Rabhi et al. 2010).

Phytoextraction Through Hyperaccumulation

Hyperaccumulators are species capable of accumulating metals at levels 100-fold greater than those typically measured in common non-accumulator plants. A plant is classified as a hyperaccumulator for heavy metals when it meets following four criteria:

1. Transfer factor or shoot/root quotient (level of heavy metal in the shoot divide by level of heavy metal in the root) is more than 1.0.
2. Extraction coefficient (level of heavy metal in the shoot divide by total level of heavy metal in the growth medium) is more than1.0.
3. Higher levels of heavy metals of 10–500 times the levels in normal or uncontaminated plants.
4. Metal accumulation exceeding a threshold value of shoot metal concentration of 1% or 10,000 (Zn, Mn), 0.1% or 1000 mg/kg (Ni, Co, Cr, Cu, Pb, and Al), 0.01% or 100 mg/kg (Cd and Se) of the dry weight plant biomass (Dalvi and Bhalerao 2013).

Heavy Metal Uptake, Translocation, and Accumulation Within Plant

To know the avoidance and tolerance mechanism in detail, it is essential to study the heavy metal uptake in plants. Accumulation of metal is a function of uptake capacity and intracellular binding sites. Mobilization of metals, uptake from soil, compartmentation and sequestration, xylem loading, distribution in aerial parts, and storage in leaf cells are the main steps involved in accumulation of metal in plants.

At every level, concentration, affinities of chelating molecules, and selectivity of transport activities affect metal accumulation rates (Clemens et al. 2002). Quantity and intensity factor along with reaction kinetics govern the transfer of heavy metals in plants (Brummer et al. 1986). For hyperaccumulation, the plant must possess the ability to solubilize metals from the soil, take up metal using specific ion transporter proteins, and detoxify metal effects on cellular processes by chelation and compartmentation, thereby translocating metal even to sensitive regions of the plant, such as leaves, where many important metabolic processes occur (Rajakaruna et al. 2006).

Mobilization of Metals

Metals in soil mostly exist as insoluble bound fraction and need to be mobilized into the solution to make it available for plants. Natural hyperaccumulators solubilize the soil-bound metals by secretion of root exudates, which causes acidification of rhizosphere (Mahmood 2010) and metal chelation by secretion of mugenic and aveic acid (Salt et al. 1995). Though root exudates are important in metal mobilization and uptake, still its complete mechanism is not clear.

Root Uptake

Bioavailable metal enters in plant either through intercellular spaces (apoplastic pathway) or by crossing plasma membrane (symplastic pathway) (Peer et al. 2006). The extracellular negatively charged sites (COO-) of the root cell walls where most of the metal ions are adsorbed act as initial barrier for metal translocation. Additionally impermeable suberin layers in the cell wall also reduced transport of metals from root apoplast to root xylem (Lasat 2002; Taiz and Zeiger 2002). Therefore, to cross this barrier and to reach the xylem, metals must move symplastically. Ghosh and Singh (2005) stated that inward movement of metals during symplastic pathway is possible due to strong electrochemical gradient provided by negative resting potential of 170 mV. Symplastic pathway is an energy-dependent process mediated by specific or generic metal ion carriers or channels (Peer et al. 2006).

Root to Shoot Transport

Subsequent to metal uptake into the root symplasm, three processes govern the movement of metals from the root into the xylem: sequestration of metals inside root cells, symplastic transport into the stele, and release into the xylem. The xylem loading is a tightly regulated process mediated by membrane transport proteins, which remain to be identified (Clemens et al. 2002). Under normal condition, the high cation exchange capacity of xylem cell walls restricts the further transport of metal ions, while in hyperaccumulators complexation of metals with low-molecular-weight chelators allows easy translocation to shoot (Mahmood 2010).

Metal Unloading, Trafficking, and Storage in Leaves

Mahmood (2010) indicated that transportation and distribution of metal in leaves occur via apoplast or symplast. Trafficking of metals occurs inside every plant cell, maintaining the concentrations within the specific physiological ranges in each organelle and ensuring delivery of metals to metal requiring proteins (Clemens et al. 2002). In hyperaccumulators metal complexing with organic ligands provides high metal tolerance (Peer et al. 2006). In the leaf tissues, metals are sequestered in extracellular or subcellular compartments. The cell types where metals are deposited vary with the metal as well as with the plant species.

Sequestration

Sequestration in plant vacuole, which prevents free concentration of metal ions in cytosol, is the final step of metal accumulation (Tong et al. 2004). Peer et al. (2006) indicated that metals may remain in cell wall due to interaction of polyvalent cations with negative charge sites (Dalvi and Bhalerao 2013). In fact, the use of hyperaccumulators opens a new branch of bioremediation technology termed as phytoremediation – an eco-friendly and scientific approach to remove, extract, or inactivate metal ions in the soil using plants (Chaney 1983, 1993; Cunningham and Berti 1993; Baker et al. 1994; Cunningham et al. 1995; Raskin 1996; McGrath 1998; Salt et al. 1998; Lasat 2002). Metal hyperaccumulators are natural or purposely engineered for hyperaccumulation (Shah and Nongkynrih 2007).

Hyperaccumulator Plants

Natural Hyperaccumulators

Nearly 450 hyperaccumulator plants also known as metallophytes have been described belonging to a wide range of taxa, ranging from annual herbs to perennial, geographically distributed in all continents, both in temperate and tropical environments (Table 4). Notable centers of distribution are for Ni, New Caledonia, Cuba, SE Asia, Brazil, Southern Europe, and Asia Minor; Zn and Pb, NW Europe; and Co and Cu, South-Central Africa. Some families and genera are particularly well represented, e.g., for Ni, *Brassicaceae* (*Alyssum* and *Thlaspi*), *Euphorbiaceae* (*Phyllanthus*), *Leucocroton*, and *Asteraceae* (*Senecio*, *Pentacalia*); Zn, *Brassicaceae* (*Thlaspi*); Cu and Co, *Lamiaceae* and *Scrophulariaceae* (Baker and Brooks 1989; Chaney et al. 1997; Brooks 1998; Clemens 2001; Broadhurst et al. 2004; Gratão et al. 2005; Prasad 2005; Vinterhalter and Vinterhalter 2005). Interestingly 75% of the identified hyperaccumulators accumulate Ni and are termed as nickelophilous plants (Baker and Brooks 1989; Prasad 2005). Of the wide range of families of vascular plants, the natural hyperaccumulating plant species are well represented by the members of *Brassicaceae* (Reeves and Baker 2000; Prasad and Freitas 2003; Gratão

Table 4 Major natural plant metal-hyperaccumulator species and their bioaccumulation potential

Metals	Plant species	Amount [g $kg^{-1(d.m.)}$]	Reference
As	*Pteris vittata*	22.6	Ma et al. (2001)
Cd	*Thlaspi caerulescens*	10.0	Lombi et al. (2001a, b)
Cr	*Salsola kali*	2.9	Gardea-Torresday et al. (2005)
Co	*Haumaniastrum robertii*	10.2	Brooks (1977)
Cu	*Ipomea alpina*	12.3	Baker and Walker (1990)
Pb	*Thlaspi rotundifolium*	0.13–8.2	Reeves and Brooks (1983)
Mn	*Phytolacca acinosa*	19.3	Xue et al. (2004)
Ni	*Alyssum betolonni*	>10.0	Morrison et al. (1980)
Se	*Brassica juncea*	2.0	Orser et al. (1999)
Zn	*Thlaspi caerulescens*	30.0	Baker and Walker (1990)

et al. 2005). Natural hyperaccumulators can grow in their natural habitat alone, have slow growth and low biomass, and very often are selective for an individual metal (Kamnev and Van der Lelie 2000; Clemens et al. 2002*).* Among the earliest known natural metal hyperaccumulators are a group of small, weedy alpine flowers called Alpine pennycress (*Thlaspi* spp.), which lack the standard pathogen defense mechanism. *Thlaspi* spp. exhibit large interspecific and intraspecific variations which make it an important plant to study hyperaccumulation. Predominantly *Thlaspi* grow on nickel-contaminated sites and accumulate about 3% of its d.m. as metal. Other than *T. arvense* (non-accumulator species), various species of *Thlaspi* are known to hyperaccumulate more than one metal. *T. caerulescens* accumulate Cd, Ni, Pb, and Zn; *T. goesingense* and *T. ochroleucum* accumulate Ni and Zn, and *T. rotundifolium* accumulates Ni, Pb, and Zn (Baker and Brooks 1989; Baker and Walker 1990; Kramer et al. 1996; Prasad 2005). *Thlaspi caerulescens* has a remarkable capacity to accumulate extremely high levels of nonlabile zinc and cadmium in its shoots, 39.6 g (Zn) $kg^{-1(d.m.)}$ and 10.0 g (Cd) $kg^{-1(d.m.)}$, and has been the subject of intense research to gain a better understanding of heavy metal hyperaccumulation and tolerance mechanisms (Baker and Walker 1990; Lasat 2002). It has also been used as a source of genes for developing plant species better suited for the phytoremediation of metal-contaminated soils (Lombi et al. 2001a). Kramer (2005) demonstrated that an altered tonoplast Zn transport in root cells stimulated Zn uptake in leaf thus playing a role in Zn hyperaccumulation in *T. caerulescens*. *Pistia stratiotes* is used for phytoremediation of wastewater or natural water bodies polluted with heavy metals. The species exhibit different patterns of response to Ag, Cd, Cr, Cu, Hg, Ni, Pb, and Zn. A 5.0 mM concentration of each of these metals resulted in distinct levels of growth inhibition and biomass production in *P. stratiotes*, with almost all the elements being accumulated at high concentrations in the root system. The plant species exhibited highest tolerance index (the ratio of the d.m. of plant in polluted soil at a particular level of metal to the d.m. of the same plant in nonpolluted soil at zero level of metal) to Zn and lowest to Hg (Odjegba and Fasidi 2004; Rabie 2005). According to Bennicelli et al. (2003), the water hyperaccumulator fern *Azolla caroliniana* wild. (*Azollaceae*) accumulates high amounts of Hg and Cr with

the capacity to purify waters polluted by these metals. *Spartina* are threefold more tolerant to Hg than tobacco plants, owing to their ability to absorb organic Hg and transform it into an inorganic form (Hg^{+}, Hg^{2+}). The inorganic Hg then accumulates in the underground parts of the plant and is retransferred to the soil by diffusion and permeation, indicating that this natural hypertolerance toward Hg could be used in the phytoremediation of Hg-polluted environment (Tian et al. 2004). A Cr-hyperaccumulator plant species *Salsola kali* has recently been reported to accumulate 0.6–2.9 g (Cr) $kg^{-1(d.m.)}$ of hexavalent Cr in aerial parts of the plant suggesting its potential as a new option for phytoremediation of Cr-contaminated soil (Gardea-Torresday et al. 2005). The oldest natural hyperaccumulator reported for Pb is *Thlaspi rotundifolium*. It can accumulate 0.13–8.2 g (Pb) $kg^{-1(d.m.)}$ in leaves (Reeves and Brooks 1983). *Helianthus annuus* has been known to concentrate Pb in its leaf and stem and can be used as a hyperaccumulator to restore abandoned mines and factory sites contaminated with elevated Pb levels (Boonyapookana et al. 2005). Recently another Pb-hyperaccumulating species *Hemidesmus indicus* has been reported to accumulate Pb in roots and shoots proving itself to be a potential candidate for Pb removal from soil (Chandra Shekhar et al. 2005). The hyperaccumulator *Sesbania drummondii* is shown to accumulate Pb as lead acetate in roots and leaves, lead sulfate in leaves, and lead sulfide in root and shoot both (Sharma et al. 2004) indicating its ability to biotransform lead nitrate to lead acetate and lead sulfate in its tissues. This complexation of Pb with acetate and sulfate perhaps forms a part of Pb-detoxification strategy in these plants (Sharma et al. 2004). Arsenic accumulation has been demonstrated in *Lemna gibba*, and the species is warranted to be a preliminary bioindicator for As. It is used to monitor removal of As in mine tailing waters because of its high accumulation capacity (Mkandawire and Dudel 2005). Pickering et al. (2000) studied accumulation of As in *B. juncea*. These workers reported that As^{5+} is transported as a phosphate analogue in roots, which subsequently is reduced to As^{3+} in shoots and stored as As^{3+}-tris-thiolate. *Pteris vittata* can also hyperaccumulate As from naturally contaminated soils but is suitable for phytoremediation only in the moderately contaminated soils (Ma et al. 2001; Bondada and Ma 2003; Caille et al. 2004). In addition, *P. vittata*, *P. cretica*, *P. longifolia*, and *P. umbrosa* are also able to hyperaccumulate As to a similar extent (Zhao et al. 2002a). *Astragalus bisulcatus* and the perennial *Stanleya pinnata* (*Brassicaceae*) grow on seleniferous soils and hyperaccumulate Se in their shoots. Parker et al. (2003) determined that 16 diverse populations of *S. pinnata* each absorb selenate preferentially over sulfate. Most of the Se in *S. pinnata* shoots occur in the form of soluble amino acids that may serve as direct precursors of volatile forms of Se such as dimethylselenide. The most widely studied Se hyperaccumulator is *Brassica juncea* which concentrates nearly 2.0 g (Se) $kg^{-1(d.m.)}$ in leaves (Orser et al. 1999). Plant species differ considerably in their normal Mn leaf concentration: 0.30–5.0 g (Mn) $kg^{-1(d.m.)}$ (Clarkson 1988; Ducic and Polle 2005). Recently a new Mn-hyperaccumulator plant *Phytolacca acinosa* has been identified (Xue et al. 2004). This perennial herb can accumulate 19.3 g (Mn) $kg^{-1(d.m.)}$ when grown on Mn-rich soil. Authors warranted that *P. acinosa* grows rapidly, has substantial biomass, is widely distributed with broad ecological amplitude, and has potential for use in phytoremediation of Mn-contaminated soils (Xue et al. 2004). *Brassica oleracea var. acephala* and

Iberis intermedia are natural hyperaccumulators for thallium. These two species have been studied for mobility of Tl and its uptake (Al-Najar et al. 2005). Unusually high accumulation of Tl, 0.4 and 1.5%(d.m.) in *Iberis intermedia* and *Biscutella laevigata*, respectively, are reported (Anderson et al. 1999).

Transgenic Hyperaccumulators

Though several natural hyperaccumulators are known, the plants ideal for green technology to clean up soil should possess multiple traits. They must have deep roots, rapid growth, and high biomass, be easily harvested, and must tolerate and accumulate a large range of heavy metals in their aboveground parts. No plant is known to have all of the above traits. The development of engineered plants (transgenic) harboring the required traits for bioremediation is perhaps the only alternative. Overexpression and introduction of hyperaccumulating genes into a non-hyperaccumulator plant could be a possible way to enhance metal uptake and accumulation, tolerance, and detoxification process (Clemens et al. 2002). The overexpression of a gene encoding a rate-limiting gene product would be expected to lead a faster overall rate of the pathway and to more efficient phytoremediation (Pilon-Smits and Pilon 2001). Besides this, the repression of an endogenous gene, by inserting a gene of reverse orientation (antisense technology), can also result in enhanced metal uptake by plants. Several reports on bioengineered plant tolerant to the presence of toxic levels of metals like Se (Berken et al. 2002), Cd (Kawashima et al. 2004), As (Lee et al. 2003a), Zn, Cr, Cu, Pb (Bennett et al. 2003), etc. have appeared in the literature in the recent years. In most of the studies, the overexpression of the genes encoding for the enzymes of S metabolism, glutathione, phytochelatin synthase, ACC deaminase, Hg^{2+}-reductase, arsenate reductase, aldolase/aldehyde reductase, enzymes of histidine biosynthesis, and metallothionein (MT) genes have been carried out. The engineering of transporter genes to manipulate the transport of metal ions inside the cell has also been exploited effectively, and a combination of some of these genes in rapidly growing plant species have led to a few promising results (Lee et al. 2003a, b; Song et al. 2004; Verret et al. 2004). A well-known example of transgenic metal hyperaccumulator is *Brassica juncea*, which overexpress ATP sulfurylase. It shows higher uptake of Se and enhanced Se tolerance compared to wild type when grown in the presence of selenate in either hydroponic conditions or in soil (Pilon-Smits et al. 1999; Van Huysen et al. 2004). These transgenic plants can also tolerate Cd, Zn, Cu, Hg, and As (III, IV). Transgenic Indian mustard overexpressing cystathionine gamma synthase (CGS) had low shoot Se concentration with enhanced Se volatilization rate as well as Se tolerance than the wild-type plants grown either hydroponically or in soil (Van Huysen et al. 2003, 2004). *Astragalus bisulcatus* is a seleniferous plant and accumulates Se, but it has a slow growth rate. SeCys is the form in which Se has deleterious effects that result from the coupling of selenide with *O*-acetyl-Ser in a reaction catalyzed by the action of Cys-synthase (Terry et al. 2000). Since Se toxicity stems mostly from the incorporation of SeCys into proteins in the place of Cys, it has been shown that overexpressing the enzyme selenocysteine

methyltransferase (SMT) that specifically methylates selenocysteine (SeCys) to produce the non-protein amino acid methylselenocysteine (MetSeCys) in *A. bisculatus* led to a reduction in the intracellular concentrations of SeCys and selenomethionine (SeMet), thus preventing their incorrect insertion into protein, thereby increasing tolerance to Se compounds, in particular selenite (Le Duc et al. 2004). In an attempt to improve the potential for removal of metals using plants, Brewer et al. (1999) created somatic hybrids between *Thlaspi caerulescens* (Zn hyperaccumulator) and *Brassica napus*. Accumulation of high levels of Zn was observed in hybrids, which otherwise are toxic for *B. napus.* Somatic hybrids obtained from *T. caerulescens* and *B. juncea* have been shown to remove significant amounts of Pb (Gleba et al. 1999). *Arabidopsis* plants transformed with an *E. coli* gene *Znt A* that encodes for Pb^{2+}, Cd^{2+}, and Zn^{2+} transport had improved resistance to Pb^{2+} and Cd^{2+} (Lee et al. 2003b), and the ZntA was located at the plasma membrane (Lee et al. 2003b). Expression of a pea (*Pisum sativum*) MT gene *PsMTA* in *Arabidopsis thaliana* accumulated more Cu (severalfold in some plants) in the roots of transformed plants as compared to controls (Evans et al. 1992). Similarly when a type 2 MT gene, *tyMT*, cloned from *Typha latifolia*, a wetland plant with constitutional tolerance was introduced into *A. thaliana*; the transgenic plant showed an increased tolerance to both Cu^{2+} and Cd^{2+} (Zhang et al. 2004). The introduction of metallothionein proteins AtMT2a and AtMT3 from *A. thaliana* as fluorescent protein-fused forms into the guard cells of *Vicia faba* resulted in transgenic plants which had guard cells protected from degradation upon exposure to Cd brought about by reducing the reactive oxygen species. The authors concluded that the Cd stays bound to the MT in the cytoplasm and is not sequestered into the vacuole instead it gets detoxified by phytochelatins (PCs) in *V. faba* cells (Lee et al. 2003b). Transgenic tomato plants expressing the bacterial gene 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase showed enhanced metal accumulation and tolerance levels for a range of heavy metals (Cd, Cu, Ni, Mg, Pb, and Zn) than untransformed plants (Grichko et al. 2000). The expression of partial peptides from the C terminus of the TcHMA4 (the *Thlaspi* heavy metal ATPase) protein, which contains numerous possible heavy metal-binding His and Cys repeat residues, confers an extremely high level of Cd tolerance and hyperaccumulation in yeast. The possibilities for enhancing the metal tolerance and phytoremediation potential of higher plants via expression of TcHMA4 hold great potential in metal remediation studies (Papoyan and Kochian 2004). Arsenic poisoning is a serious problem (Dondon et al. 2005), and Dhankher et al. (2002) examined the effects of co-expressing two bacterial genes for arsenate reductase (arsC) and gamma-glutamylcysteine synthetase (gamma-ECS) in *Arabidopsis* plants. They observed that plants expressing SRS1p/ArsC and ACT2p/gamma-ECS together showed substantially greater arsenic tolerance than wild-type plants or plants expressing gamma-ECS alone. In addition, when grown on arsenic, these plants accumulated four- to 17-fold greater fresh shoot mass and accumulated two- to threefold more arsenic per gram of tissue than wild-type plants or plants expressing gamma-ECS or ArsC alone. Other than plants, the potential model phytoremediators for As now include various genotypes of transgenic trees, e.g., *Austromyrtus bidwillii* (Sjaan et al. 2002). Trees are ideal in the remediation of heavy metals as they can withstand and accumulate higher concentration of pollutants owing to their large biomass and size, can reach a

huge area and great depths for their extensive rootings, and can stabilize an area. They prevent erosion and the spread of the contaminant because of their perennial presence (Sykes et al. 1999). Researchers are now trying to extend this technology to cottonwood trees, which are potential effective remediators (Dondon et al. 2005). Once the trees accumulate sufficient quantities of arsenic, they could be harvested and removed from the site, taking with them large quantities of As (Sykes et al. 1999; Che et al. 2003; Dondon et al. 2005; Shah and Nongkynrih 2007).

Mechanisms of Metal Hyperaccumulation

Considerable progress has been made in understanding the mechanisms of metal hyperaccumulation at the physiological and molecular levels, although the full picture is far from complete. A genetic study using crosses between the Zn hyperaccumulator *Arabidopsis halleri* and the non-accumulator *Arabidopsis petraea* showed that Zn hyperaccumulation and tolerance are independent traits and that tolerance appears to be controlled by a single major gene (Macnair et al. 1999). There is evidence that hyperaccumulation of Zn and Cd by *T. caerulescens* involves enhanced root uptake of the metals (Lasat et al. 1996; Lombi et al. 2001b; Zhao et al. 2002b; Assuncao et al. 2001). Several Zn transporter cDNAs have recently been cloned from *T. caerulescens* (Pence et al. 2000; Assuncao et al. 2001). These transporters belong to the ZIP family (zinc-regulated transporter/iron-regulated transporter like proteins) (Maser et al. 2001). ZNT1 and ZNT2 are highly expressed in the roots of *T. caerulescens*, and expression is barely responsive to the Zn status in the plants. Through functional complementation in yeast, ZNT1 was shown to mediate high-affinity uptake of Zn2þ as well as low-affinity uptake of Cd2þ (Pence et al. 2000). It appears that increased Zn influx in *T. caerulescens* may be a result of the minimal downregulation of ZNT1 and ZNT2 gene expression, even when intracellular zinc levels are high. Thus, specific alterations in Zn-responsive elements (e.g., transcriptional activators) possibly play an important role in Zn hyperaccumulation in this plant (Pence et al. 2000). In the case of Cd, the superior ability of the southern French ecotype of *T. caerulescens* to accumulate Cd cannot be explained by the Zn transport pathway (Lombi et al. 2001b; Zhao et al. 2002b) but may be related to an enhanced expression of IRT1 (Lombi et al. 2002a). IRT1 is essential for Fe acquisition by the roots of *Arabidopsis thaliana* and can also mediate high-affinity Cd2þ uptake (Vert et al. 2002; Connolly et al. 2002). As happens in the case for arsenic non-hyperaccumulating plants, arsenate uptake is mediated by phosphate transporters in the arsenic hyperaccumulator *P. vittata* (Wang et al. 2002). Enhanced root-to-shoot transport is another key component of metal/metalloid hyperaccumulation. This may be achieved by a reduced sequestration of the metal in the root vacuoles (Lasat et al. 1998) or by enhanced xylem loading although there has been little progress in research on this aspect. In the Ni hyperaccumulator *Alyssum lesbiacum*, exposure to Ni elicited a large increase in the concentration of

histidine in the xylem sap (Kramer et al. 1996). This response may explain the enhanced Ni tolerance of roots of *A. lesbiacum* as well as the enhanced root-to-shoot transport of Ni. Hypertolerance is essential for the hyperaccumulation phenotype to occur in natural hyperaccumulators. Hypertolerance is achieved by internal detoxification and probably involves compartmentation and complexation. There is evidence that metals and metalloids are sequestered in leaf vacuoles in Zn, Cd, Ni, and As hyperaccumulators (Vazquez et al. 1994; Kupper et al. 1999, 2001; Kramer et al. 2000; Lombi et al. 2002b). Metal transporter genes, which encode putative vacuolar ion transport proteins, have been cloned from *T. caerulescens* (ZTP1) (Assuncao et al. 2001) and from the Ni hyperaccumulator *Thlaspi goesingense* (TgMTP) (Persans et al. 2001). These transporters belong to the cation diffusion facilitator (CDF) family, recently renamed the cation-efflux (CE) family (Maser et al. 2001). ZTP1 was found to be highly expressed in *T. caerulescens*, predominantly in leaves but also in roots, and the expression was hardly responsive to Zn treatments (Assuncao et al. 2001). ZTP1 from *T. caerulescens* is an orthologue of the ZAT gene cloned from *Arabidopsis thaliana*, and the latter when overexpressed in *A. thaliana* increased the plant's tolerance to Zn (Van der Zaal et al. 1999). TgMTP1 transcripts are highly expressed in *T. goesingense* compared with orthologues in several non-hyperaccumulator plants (Persans et al. 2001). In addition, heterologous expression of TgMTP1 in the yeast mutants cot1 and zrc1, which lack the equivalent CE members COT1 and ZRC1, suppressed the sensitivity of these mutants to Ni, Cd, and Co. These studies suggest that enhanced tonoplast transport of metals into vacuoles possibly has an important role in metal hypertolerance in hyperaccumulator plants. If there is only a single major gene responsible for metal tolerance in hyperaccumulators (MacNair et al. 1999), an enhanced vacuolar transport could well be the action point. With regard to complexation, Ni may be complexed by organic acids, particularly citrate in some Ni hyperaccumulators (Brooks 1998), or by histidine in roots and xylem saps of *A. lesbiacum* (Kramer et al. 1996). In *T. caerulescens*, Zn was found to be coordinated with histidine in roots and with organic acids or uncomplexed in shoots (Salt et al. 1999). In *Arabidopsis halleri*, Zn was predominantly coordinated with malate in shoots and with malate, citrate, and phosphate in roots (Sarret et al. 2002). There is strong evidence that phytochelatins (PCs) are essential for constitutive tolerance to Cd in non-hyperaccumulator plants (Cobbett and Goldsbrough 2002). However, recent studies showed that PCs are not involved in the hypertolerance to Cd in *T. caerulescens* (Ebbs et al. 2002; Schat et al. 2002). Detoxification of arsenate generally involves a reduction of arsenate to arsenite, followed by complexation with thiols, particularly PCs, in non-hyperaccumulator species (Meharg and Hartley-Whitaker 2002). In *P. vittata*, however, current evidence indicates that the main storage form of arsenic in the shoots is inorganic arsenite, noncomplexed with thiols (Ma et al. 2001; Wang et al. 2002; Francesconi et al. 2002) and is probably sequestered in the vacuoles (Lombi et al. 2002b). In the Se hyperaccumulator *Astragalus bisculatus*, Se is assimilated into Se-methyl-selenocysteine, which is incorporated into proteins, resulting in the hyperaccumulation of Se (Neuhierl and Bock 1996; McGrath and Zhao 2003).

Conclusions and Perspectives

Extensive progress has been made in characterizing the soil chemistry needed for phytoremediation and physiology of plants that hyperaccumulate and hypertolerate metals. It is increasingly clear that hypertolerance is fundamental to hyperaccumulation, and high rates of uptake and translocation are observed in hyperaccumulator plants. Fundamental characterization of mechanisms and cloning of genes required for phytoremediation have begun with mercuric ion reductase. Improved hyperaccumulator plants and agronomic technology to increase the annual rate of phytoextraction and to allow recycling of toxic soil metals accumulated in plant biomass are very likely to support commercial environmental remediation, which society can afford in contrast with present practices. Many opportunities have been identified for research and development to improve the efficiency of phytoremediation. Although progress is being made, more knowledge of the molecular mechanisms responsible for hyperaccumulation is necessary before the traits can be transferred to high biomass plants, and phytoextraction for soil remediation can be optimized. In addition, the practical aspects of the use of hyperaccumulating plants need further research.

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Metal Bioaccumulation by Plants in Roadside Soils: Perspectives for Bioindication and Phytoremediation

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Abstract Traffic-related metal pollution is a serious worldwide concern. Roadside soils are constantly subjected to the deposition of metals released by tailpipe gases, vehicle parts, and road infrastructure components. These metals, including platinum group elements from catalytic converters, constitute a threat to surrounding ecosystems that frequently comprise pasture and agricultural lands. Due to the capacity of plants to tolerate and accumulate metals, the study of the vegetation growing in soils adjacent to roads is important to understand their role as bioindicators of traffic-related metal pollution and infer their potential for the phytoremediation of roadside areas. This chapter reviews the main sources of metals in roadside soils and dusts, and the bioaccumulation of metals in plants growing alongside roads presenting different traffic loads and climatic conditions. The pertaining literature is discussed with a particular emphasis on the suitability of the assessed plant species to indicate and mitigate traffic-related metal pollution.

Keywords Phytoremediation • Soil pollution • Heavy metals • Platinum group elements • Roadside soils • Traffic pollution

Traffic-Related Metal Pollution

The impact of heavy metal (HM) pollution on Earth's environment became a major worldwide concern. The expansion of urbanization and industrialization during the past few decades has caused increasing damage to human health and

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natural ecosystems (Farmaki and Thomaidis 2008; Faiz et al. 2009; Zhang et al. 2012; Galal and Shehata 2015). This fact has prompted many countries to impose more severe environmental regulations on HM emissions to the environment (Gücel et al. 2009; Assirey and El-Shahawi 2015). Anthropogenic discharges of HMs usually lead to air, water, and soil pollution, producing serious impacts on terrestrial and aquatic ecosystems (Saeedi et al. 2009). In contrast with organic contaminants, metallic pollutants cannot be degraded to inoffensive carbon-based sub-products (Coupe et al. 2013). Hence, they persist indefinitely in the environment, rising both ecological and human health risks mainly due to the bioaccumulation process along the entire food chain (Coupe et al. 2013; Assirey and El-Shahawi 2015).

Countless places around the globe are currently polluted with dangerous levels of HMs, requiring the urgent application of effective remediation techniques (Coupe et al. 2013). In this scenario, urban areas are the major sources and sinks for HM contamination, due to intensive human activities responsible for high concentration of pollutant emissions (Wiseman et al. 2013; Yang et al. 2015; Galal and Shehata 2015). The main hazardous anthropogenic sources comprise industrial and mining activities, waste disposal, coal and fuel combustion, domestic heating, smelters, construction and infrastructure maintenance, and road traffic (Bai et al. 2009; Coupe et al. 2013; Assirey and El-Shahawi 2015). Road traffic has recently gained wider interest by the scientific community, because together with the industrial activity, it constitutes the most important source of HMs pollution in roadside soils often used for agriculture or grazing (Zereini et al. 2007; Sharma and Prasad 2010; Zhang et al. 2013). Several reports in the pertaining literature have addressed the elevated levels of HMs in roadside soils (Bai et al. 2009; Guney et al. 2010; Khan et al. 2011; Zhang et al. 2012; Radziemska and Fronczyk 2015) and street dusts (Sezgin et al. 2004; Leśniewska et al. 2004; Faiz et al. 2009; Nazzal et al. 2013) associated to high-density traffic of automotive vehicles.

Roadside Soils and Dusts

Soil can be defined as a mixture of mineral constituents, organic matter, living organisms, HMs, water, and air. Roadside soil can also contain materials of anthropogenic origin like vehicle exhaust particles and lubrication oil residues and natural biogenic materials such as vegetal matter pulverized by traffic (Howari et al. 2004; Shi et al. 2008; Faiz et al. 2009). On the other hand, dust can be described as a combination of solid matter (composed of soil), anthropogenic metallic components, and natural biogenic materials (Ferreira-Baptista and De Miguel 2005; Faiz et al. 2009). Road dusts are mainly constituted by deposited atmospheric particles and displaced soil (Faiz et al. 2009). Considering that the deposition of suspended particles is critical for the formation of road dusts, their metal load is therefore highly influenced by airborne particles emitted by traffic,

heating systems, construction, industrial activity, and mineral exploration. In addition, metal levels in road dusts also depend on the displaced soil characteristics and meteorological conditions (Viard et al. 2004). It is worth to remember that road dusts are in constant movement, being easily re-suspended and deposited on the topsoil. Consequently, the metals in suspension may be washed away, contaminating the soil and leaching into groundwater (Ferreira-Baptista and De Miguel 2005; Faiz et al. 2009).

Sources of Metals in Roadside Soils and Dusts

The most abundant HMs found in soils and dusts afflicted by high-density traffic are cadmium (Cd), copper (Cu), nickel (Ni), zinc (Zn), lead (Pb), chromium (Cr), mercury (Hg), cobalt (Co), and vanadium (V) (Viard et al. 2004; Kluge and Wessolek 2012; Zhang et al. 2013; Radziemska and Fronczyk 2015), and platinum group metals (hereinafter designated as PGMs), such as platinum (Pt), rhodium (Rh), and palladium (Pd) (Whiteley and Murray 2003; Leśniewska et al. 2004; Riga-Karandinos et al. 2006) (Table 1).

Table 1 Main sources of heavy metals and platinum group metals on roadside soils and dusts

Heavy metal	Source	Reference
Cadmium (Cd)	Fuel, tires, engine oil, galvanized structures	Sezgin et al. (2004) Nabulo et al. (2006), Nordberg et al. (2007), Faiz et al. (2009), Kluge and Wessolek (2012), Nazzal et al. (2013)
Copper (Cu)	Fuel, engine oil, tires, brake pads, radiators, asphalt, galvanized structures	Sezgin et al. (2004), Denier van der Gon et al. (2007), Faiz et al. (2009), Zhang et al. (2012), Kluge and Wessolek (2012)
Nickel (Ni)	Batteries	Nordberg et al. (2007), Faiz et al. (2009)
Zinc (Zn)	Mechanical abrasion, oil, tires, brakes, asphalt, galvanized structures	Nabulo et al. (2006), Faiz et al. (2009), Khan et al. (2011), Zhang et al. (2012), Kluge and Wessolek (2012), Nazzal et al. (2013)
Lead (Pb)	Fuel, brakes, batteries, asphalt	Turer et al. (2001), Nabulo et al. (2006), Nordberg et al. (2007), Khan et al. (2011), Zhang et al. (2012), Walraven et al. (2014)
Mercury (Hg)	Fuel, batteries, light and headlights	Liang et al. (1996), Khan et al. (2011)
Vanadium (V)	Crude oil and asphalt	Kabata-Pendias (2011)
Platinum (Pt)	Automobile catalytic converters	Leśniewska et al. (2004), Dubiella-Jackowska et al. (2007), Pawlak et al. (2014)
Rhodium (Rh)	Automobile catalytic converters	Dubiella-Jackowska et al. (2007), Pawlak et al. (2014)
Palladium (Pd)	Automobile catalytic converters	Dubiella-Jackowska et al. (2007), Hooda et al. (2008), Pawlak et al. (2014)

Heavy Metals

Roadside environmental media are intensely affected by metal-enriched traffic emissions, due to tailpipe gases related to fuel combustion, as well as releases associated to corrosion of metals and the wear and tear of vehicles parts including brake linings, tires, and automobile catalytic converters (Zereini et al. 2007; Galal and Shehata 2015). Environmental damages caused by the deposition of HMs on roadside soils and dusts are also related to the corrosion of radiators and batteries, fluid leakage, and mechanical abrasion (Nazzal et al. 2013). In heavy traffic roads, dust re-suspension caused by automotive flow can also be a primary contaminant source of HMs for adjacent soils. In such cases, roadside environmental pollution is proportional to the volume of traffic and circulation of heavyweight vehicles (Lough et al. 2005; Galal and Shehata 2015). Other sources of pollution by HMs involve the road infrastructure, including corrosion of galvanized steel, crash barriers, and pavement wear (Assirey and El-Shahawi 2015).

Regarding traffic-related emissions, engine oil consumption is responsible for most releases containing Cd and Cu; tire wear is associated to the discharges of Zn and Cd, while brake wear is the principal source of Cu and Pb (Denier van der Gon et al. 2007; Faiz et al. 2009; Nazzal et al. 2013). Batteries contribute to Ni, Cd, Pb, and Hg pollution (Nordberg et al. 2007), although the latter may also occur due to fuel and fluorescent lamps utilized inside vehicles and in high-intensity headlights. Within the road infrastructure, mineral fillers and bitumen in asphalt floors are sources for different HMs, particularly Cu, Zn, Cd, Pb, and V (Nordberg et al. 2007; Kabata-Pendias 2011; Zhang et al. 2012). Galvanized structures used in some roads represent another contamination source by Zn, Cd, and Cu, due to metal corrosion by rainfall (Kluge and Wessolek 2012).

A considerable number of studies have been dedicated to the assessment of metal distribution according to road proximity and traffic intensity, quantification of HM concentration, as well as the identification of pollution sources in roadside soils and dusts. Turer et al. (2001) investigated HM contamination in roadside soils of a urban highway in Cincinnati, USA. It was observed that metal concentration increased with greater amounts of organic matter, and it decreased as the sampling depth from the topsoil increased. Moreover, the authors attributed the Pb amount found in the roadside soil to exhaust gases from vehicles employing leaded gasoline. Fakayode and Olu-Owolabi (2003) evaluated the effect of traffic intensity on HM levels in roadside soils in Oshogbo, Nigeria. This study found high concentrations of Pb, Cd, Cu, Ni, Cr, and Zn in the topsoil adjacent to the highway and suggested their relationship with emissions related to leaded gasoline. Viard et al. (2004) assessed the pollution by HMs in roadside soils in France. High levels of HMs—especially Pb, Zn, and Cd—were found in soils bordering a heavy traffic highway, whose concentrations decreased with greater distance from the road. The authors credited the pronounced HM contamination to traffic emissions associated to the use of catalytic converters and unleaded petrol additives. Sezgin et al. (2004) conducted a study to identify the metal pollution in road dusts in Istanbul, Turkey. The authors found

high concentrations of HMs (including Pb, Cu, Mn, Zn, Cd, and Ni) and emphasized that the presence of Pb, Cu, Cd, and Zn in road dust is a pollution indicator, due to their use in vehicle components, combustible and oil lubricants (Sezgin et al. 2004). In Iran, Saeedi et al. (2009) determined elevated metal contents (Pb, V, Zn, Ni, Cd) in soils contiguous to highways, which were credited to anthropogenic activities such as traffic, use of leaded gasoline, and tire wear. This result was corroborated by the negative correlation between HM concentration and distance from the road edge. Guney et al. (2010) investigated the influence of traffic on the spatial dispersion of HMs in soils next to urban roads in Istanbul, Turkey. For this purpose, the authors analyzed samples of the topsoil, deep soil, and street dusts. The significant correlations obtained for the concentrations of Pb, Zn, and Cu in all soil sections suggest that traffic operated as the common pollutant source.

Khan et al. (2011) evaluated the levels of HMs on soils adjacent to an important highway in Pakistan. The strong correlations obtained between Pb, Cu, and Zn concentrations in soil indicated that traffic was the main anthropogenic pollution source. Moreover, it was also suggested that HM pollution in sampling points where the relationship among these metals was not significant could be due to illegal dumpsites located along the highway. In Germany, Kluge and Wessolek (2012) investigated HM concentration in roadside soils in function of the depth from the topsoil, as well as of the distance from the highway edge. The authors found greater metal contents (Cd, Cu, Pb, and Zn) as the proximity to the road increased and the depth from the topsoil decreased, suggesting the strong influence from the traffic emissions and road materials on metal pollution. Nazzal et al. (2013) evaluated the HM levels in roadside dust of several highways on Ontario, Canada. Among the elements analyzed (Cd, Cr, Cu, Fe, K, Mg, Ca, Mn, Pb, Ni, and Zn), those that presented higher concentrations were Cu, Zn, Ni, Pb, Fe, and Mg. According to the geo-accumulation index, the results indicated that roadside soils were strongly to extremely polluted with Ni and Pb, and extremely polluted with Cu and Zn, hence representing a serious risk to the environment and human health. Radziemska and Fronczyk (2015) have also used the geo-accumulation index to classify the HM contamination levels along an expressway in Poland. Their assessment showed that roadside topsoils were moderately contaminated by Pb, Cd, Ni, and Cu and moderately to strongly contaminated by Zn. Correlations between HM concentrations suggest the strong effect of the road infrastructure and traffic on metal pollution. Walraven et al. (2014) investigated the source of Pb pollution in roadside soils in the Netherlands. The authors analyzed the Pb isotope composition and concentration in different depths from the topsoil. The results obtained showed that traffic is the major pollution source for this metal, due to the high contents of Pb isotopes from leaded gasoline. Yang et al. (2015) studied the influence of traffic over the spatial concentration of HMs in an urban parking lot located in Chengdu, China. The authors found elevated levels of Pb, Zn, Cu, Mn, Sr, and Fe with an analogous spatial distribution pattern, which is related to traffic density and distance from the road. The yellow road paint, wear and tear of brakes and tires, and tailpipe emissions were identified as the main sources of HMs (Table 2).

Table 2 Heavy metal pollution in roadside soils and dusts in several worldwide locations

Location	Heavy metals (mg kg^{-1})						Reference
	Pb	Zn	Cd	Cu	Ni	Mn	
Istanbul, Turkey	212	521	2	208	32	398	Sezgin et al. (2004)
Athens, Greece	131–1004	173–997	–	101–424	–	–	Riga-Karandinos et al. (2006)
Tehran–Karaj, Iran	924	585	41	–	79	1004	Saeedi et al. (2009)
Okayama, Japan	22–152	30–1475	1.3–3.4	33–261	8–46	–	Suzuki et al. (2009)
Istanbul, Turkey	1573	522	–	136	–	–	Guney et al. (2010)
Beijing, China	35	92	0.2	30	27		Chen et al. (2010)
Pakistan (N-5 Highway)	36	57	0.8	13	9	2	Khan et al. (2011)
Toronto, Canada	32–378	81–367	0.46–0.95	113–392	32–327	–	Nazzal et al. (2013)
Warsaw, Poland	23–56	178–266	2	43–53	17–124	–	Radziemska and Fronczyk (2015)
Zagazig–Banha, Egypt	10–30	49–372	<0.2	23–77	11–53	–	Galal and Shehata (2015)

Platinum Group Metals

Platinum group metals (PGMs) comprise elements such as platinum (Pt), palladium (Pd), rhodium (Rh), ruthenium (Ru), iridium (Ir), and osmium (Os) (Dubiella-Jackowska et al. 2007). PGMs have numerous industrial uses, including pharmaceutical products and vehicle exhaust catalysts, due to their particular chemical and physical properties (Ravindra et al. 2004; Leśniewska et al. 2004). Although the natural occurrence of PGMs generates low concentrations of such metals in the environment, their increasing anthropogenic applications are raising their incidence to toxic levels (Ravindra et al. 2004; Sobrova et al. 2012).

Nowadays, catalytic converters are widely used in automobile exhaust systems to reduce pollutant emissions. Thus, with the introduction of catalytic converters in the automotive market, the use of unleaded combustibles contributed to a significant reduction of Pb discharges on roads (Schäfer and Puchelt 1998). However, catalytic converters also entail environmental impacts due to the emissions of PGMs, caused by the deterioration and abrasion of the surface of the converter as a result of the passage of hot exhaust gases (Riga-Karandinos et al. 2006; Zereini et al. 2007; Hooda et al. 2008).

Several works in the relevant literature have been dedicated to identify the anthropogenic sources of PGMs, as well as to quantify their concentrations in roadside soils and dusts, and determine their environmental impact. Whiteley and Murray (2003) assessed the PGM concentrations in roadside soils and dusts in Perth, Australia. Their results showed elevated concentrations of Pd, Pt, and Rh, consistent with the composition of catalytic converters and, consequently, related to traffic emissions. Leśniewska et al. (2004) evaluated the concentrations of PGMs in

Table 3 Contamination by platinum group metals on several worldwide roadside soils and dust

Location	Platinum group metals (μg kg^{-1})			Reference
	Pt	Rh	Pd	
Perth, Australia	31–108	3–27	14–108	Whiteley and Murray (2003)
Bialystok, Poland	34–111	6–20	–	Leśniewska et al. (2004)
São Paulo, Brazil	6–17	2–8	18–58	Morcelli et al. (2005)
Athens, Greece	141	–	126	Riga-Karandinos et al. (2006)
Oxfordshire, England	16–2	22–4	84–121	Hooda et al. (2008)

road and tunnel dusts in Bialystok, Poland. The authors found high levels of Pd, Pt, and Rh and a clear correlation between the PGM concentrations and the volume of traffic. Riga-Karandinos et al. (2006) investigated the concentrations of Pt and Pd in roadside soils in distinct regions of Athens, Greece. The authors obtained soil concentration ratios (Pt:Pd) proportional to the metal content ratio used in common catalytic converters. Hooda et al. (2008) studied PGM contents in roadside soils of several highways around Oxfordshire, England. Again, the authors attributed the high PGM levels found in soil (particularly Pd), to the use of automobile catalytic converters. Moreover, their work shows that PGM concentrations decrease with increasing distance from the road, indicating that the metal pollution sources were near the road perimeters (Table 3).

Plant Metal Uptake and Phytoremediation

The accumulation of HMs in plants largely depends on the bioavailability of these elements in their growth media. Thus, factors such as pH, oxygen content, and nutrient balance, and the coexistence of other inorganic and organic compounds, play an important role in HM availability (Babula et al. 2008; Sheoran et al. 2009). Some HMs are partially or completely unavailable due to low solubility and strong interactions with the organic or silicate matrix. A number of root mechanisms that include cation exchange, exudation of low-molecular weight organic acids, chelating compounds and enzymes, and the acidification of the rhizosphere through H+ secretion, also influence HMs. These processes contribute significantly to the increment of HM bioavailability and their entrance into root cells over passive or active absorption (Babula et al. 2008; Sheoran et al. 2009; Kabata-Pendias 2011). Once in the root, HMs may be stored or translocated into the shoot, generally through protein-mediated xylem transport, to be detoxified and sequestered in the vacuoles (Rascio and Navari-Izzo 2011). Although to a minor extent, HM absorption may also occur via foliar uptake (Kabata-Pendias 2011).

Some plants have the rare ability of hyperaccumulating HMs. These species, known as hyperaccumulators, are capable of accumulating metals to levels 100-fold greater than those typically measured in aerial parts of common non-accumulator plants living in the same environment (Rascio and Navari-Izzo 2011; Ali et al. 2013).

Hence, hyperaccumulators should present concentrations of a given metal in their shoots above a defined threshold. Classically, the onset levels of HMs in dry shoot matter for hyperaccumulation are at least 100 mg kg^{-1} for Cd; 1000 mg kg^{-1} for Co, Cu, Ni, As, and Se; and 10,000 mg kg^{-1} for Zn and Mn (Baker and Brooks 1989; Sun et al. 2008). However, van der Ent et al. (2013) proposed that the criteria for hyperaccumulation should be revamped and suggested that the thresholds for Cu, Co, and Cr should be lowered to 300 mg kg^{-1} dried plant leaf, and the criterion for hyperaccumulation of Zn should be lowered to 3000 mg kg^{-1} dried plant leaf. In addition, hyperaccumulators should also meet the criteria of presenting a bioconcentration factor (BF) and translocation factor (TF) greater than 1.0. The BF indicates the efficiency of a plant to absorb HMs from the surrounding environment and accumulate them in their tissues. BF is calculated as follows (Ali et al. 2013):

$$BF = \frac{C_{Root}}{C_{Soil}} \tag{1}$$

where C_{Root} is the HM concentration in the plant (mg kg^{-1}) and C_{Soil} is the HM concentration in the soil (mg kg^{-1}). The *TF* denotes the ability of the plant to translocate HMs from the root to the shoot. A TF higher than 1.0 shows that the plant is efficient transport HMs from roots to its aboveground parts. The *TF* is calculated according to the following equation (Novo et al. 2013):

$$TF = \frac{C_{Shoot}}{C_{Root}} \tag{2}$$

where C_{Shoot} is the concentration of HM in the shoot (mg kg^{-1}) and C_{Root} is the concentration of HM in the root of the plant (mg kg^{-1}).

Hyperaccumulators have been described in a large number of studies and may occur in more than 450 species of vascular plants from 45 families of angiosperms, including species belonging to Asteraceae, Brassicaceae, Caryophyllaceae, Cyperaceae, Cunoniaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Poaceae, Violaceae, and Euphorbiaceae (Padmavathiamma and Li 2007; Vamerali et al. 2009; Jaffré et al. 2013). Hyperaccumulators, BF and TF, are key ingredients to phytoremediation, a plant-based technique that can be used to mitigate HM pollution in soils. Phytoremediation is a low-cost and environment-friendly alternative to conventional solutions that are frequently unfeasible due to their high costs, impracticability, and harmful side effects (Jadia and Fulekar 2009; Vamerali et al. 2009; Ali et al. 2013). Two subcategories of phytoremediation are particularly interesting for the recovery of soils afflicted by HM pollution: phytostabilization and phytoextraction. Phytostabilization is a management strategy to immobilize HMs in the rhizosphere of metal-tolerant plants (metallophytes), preventing their migration downward the soil and into water bodies (Mendez and Maier 2008; Marques et al. 2009). The main mechanisms of HM immobilization comprise adsorption and sequestration by the root, in addition to precipitation by bacterial and root exudates.

Besides, the associated plant canopy impedes the eolian dispersion of particles. Plants apt for phytostabilization should exhibit a BF higher than 1.0, but a TF smaller than 1.0 (Mendez and Maier 2008; Ali et al. 2013). Phytoextraction entails the absorption of substantial amounts of HMs by the roots and their translocation into the plant aerial parts. This technique allows the lessening of the soil HM concentrations and their safe disposal after harvest (Marques et al. 2009; Ali et al. 2013). Phytoextraction requires metallophyte species featuring fast growth rate, high biomass yield, hyperaccumulation (or at least high concentrations of HMs in the shoots coupled with elevated biomass production), and BF and TF above 1.0 (Marques et al. 2009; Novo et al. 2015).

Bioaccumulation of Metals by Plants in Roadside Soils

Heavy Metals

Several studies have reported HM accumulation by plants in roadside soils around the world. Galal and Shehata (2015) carried out a study at a heavy traffic highway in Sharqia Governorate, Egypt, to determine the concentrations of HMs in *Plantago major*. A significant decrease of HM concentration in roots and shoots was found with increasing distances from the road. The BF for the assessed HMs, excluding Cd and Sr, were smaller than 1 at most distances. Contrarily, the TF for Cd, Co, Cu, Pb, and Zn were greater with increasing distances from the highway, whereas that of Fe, Cr, and Sr were higher closer to the highway. The soil concentrations of Fe, Al, Cr, Ni, Sr, V, and Zn positively correlated with all HMs analyzed in the roots of *P. major*. While the results suggest the aptness of this species for phytostabilization of Cd, Cu, and Sr, the authors also point its capacity to operate as a bioindicator and biomonitor for traffic-related HM pollution.

A study conducted in the highway connecting Nicosia to Famagusta in Cyprus (Gücel et al. 2009) examined the concentrations of HMs in barley (*Hordeum vulgare*). A positive correlation was found between the levels of HMs in soil and plant tissue. Moreover, HM concentrations in soil and plants diminished with increasing distance from the highway. Considering that plant foliar concentrations of 0.05–3 mg kg^{-1} Pb are a critical sign of Pb pollution, the suitability of barley for bioindication of Pb pollution in roadside soils is suggested. Nabulo et al. (2006) studied 11 farming sites along highways around Kampala City in Uganda, to determine the link between traffic density and HM concentrations in *Amaranthus dubius*. Their results demonstrated that Pb levels in the leaves of *A. dubius* decreased with increasing distance from the highway edge, and that the main pathway for Pb pollution was atmospheric deposition, due to its relationship with Pb concentrations in surface films. The authors have also assessed other leafy vegetables, fruit, and root crops growing on the roadside, in terms of HM content in their roots, leaves, and fruits. The concentrations of Pb in leaves of roadside crops were greater than those in their correspondent roots, with the highest TF observed in *Brassica oleracea* L. The levels of Pb and Zn in the

fruits were significantly lower than those in the leaves of the respective crops. Hence, the authors advise that leaves of roadside vegetables can represent sources of HM contamination to farmers and consumers in high-traffic urban areas. The uptake of HMs by *Spinacia oleracea* L., *Lepidium sativum* L., *Urtica dioica* L., and *Phacelia tanacetifolia*, grown in heavily contaminated highway soil near Vaihingen, Germany, was investigated (Schäfer et al. 1998). The results showed that *L. sativum* presented a BF above 1.0 for Cd, suggesting the ability of this species to immobilize this HM in its root area.

A study about the content of Pb and Cd in *B. oleracea*, *Solanum melongena* L., *Raphanus sativus* L., *S. oleracea*, and *Abelmoschus esculentus* L. was carried out along the Mathura-Kanpur highway, in Agra, India (Sharma and Prasad 2010). It was observed that the reduction of Pb and Cd concentrations in the tissues of the vegetable crops with increasing distances from the highway indicates the relationship between HM uptake and traffic. Furthermore, aerial deposition of metal particulates from motor vehicles is proposed as the main source of HMs at closer distances to the highway. This conclusion is also supported by a recent experiment conducted in Warsaw, Poland, in which particulate matter levels on leaves of *Tilia cordata Mill.* growing at different distances from a heavy traffic urban road were compared (Popek et al. 2015). The results showed that the amount of particulate matter was significantly greater on trees located closer to the emission source and that vegetation represented a barrier that lowered the quantity of PM accumulated on trees growing further away.

Viard et al. (2004) observed that concentrations of Cd, Pb, and Zn in *Festuca arundinacea*, *Phalaris* sp., and *Dactylis glomerata*, growing next to French highway A31 between Nancy and the border with Luxembourg, increased with decreasing distances from the road. Atmospheric deposition of particulate matter from the highway was once again pointed as the main source of HM pollution. Besides, climatic factors such as rainfall and direction of dominant wind are likely to drive the distribution of HMs, as suggested by the correlation between the downwind and the concentrations on the east and west sides of the highway. The species *Lolium perenne* L., *Festuca rubra* L., and *Poa pratensis* L., grown in soil adjacent to highway 17 in Delta, British Columbia (Canada), were assessed for their capacity to accumulate Cu, Mn, Pb, and Zn (Padmavathiamma and Li 2012). All the plants exhibited BF and TF higher than 1.0 for Mn, suggesting their aptitude for phytoextraction of this metal. Additionally, *F. rubra* presented BF higher than 1.0 for Cu, making this plant a potential candidate for Cu phytostabilization.

An experiment using soil contiguous to a heavy traffic road in Tripoli, Libya, investigated the levels of HMs in *Eucalyptus camaldulensis*, *Medicago sativum*, and *Brassica juncea* (Coupe et al. 2013). Given that *E. camaldulensis* and *M. sativum* displayed a BF higher than 1 for Cr, Cu, Pb, and Zn, both species are good prospects for phytostabilization of these HMs, whereas the BF of *B. juncea* only surpassed 1.0 for Cr and Cu, making it suitable however for their phytostabilization. Table 4 compares the concentrations of HMs in different plant species grown in roadside soils around the world.

Table 4 Concentrations of selected heavy metals in different plant species grown in roadside soils around the world

Plant species	Location	Traffic (vehicles day^{-1})	Heavy metals (mg kg^{-1})				Reference
			Pb	Zn	Cd	Cu	
Amaranthus dubius	Najjanankumbi, Uganda	23,819	8.7	–	0.8	–	Nabulo et al. (2006)
Lycopersicon esculenta	Greenhill, Uganda	7853	15	–	0.6	–	
Lepidium sativum	Vaihingen, Germany	–	3	929	7	42	Schäfer et al. (1998)
Solanum melongena	Agra, India	100,000	31.20	–	1.30	–	Sharma and Prasad (2010)
Dactylis glomerata	Nancy, France	40,000	2.1	62	0.06	–	Viard et al. (2004)
Festuca rubra	Delta (BC), Canada	43,316	25	79	–	89	Padmavathiamma and Li (2012)
Lolium perenne			31	71	–	66	
Eucalyptus camaldeulensis	Tripoli, Libya	–	548	47	–	18	Coupe et al. (2013)
Hordeum vulgare	Istanbul, Turkey	–	1.08	40.30	0.84	7.2	Gücel et al. (2009)
Plantago major	Zagazig–Banha, Egypt	–	5.6	68.7	0.5	74.9	Galal and Shehata (2015)
Pleurozium schreberi	Oulu, Finland	43,000	8.9	–	–	–	Niemelä et al. (2004)

Platinum Group Metals

The accumulation of PGMs in plants has also been the object of study by various authors around the world. Djingova et al. (2003) examined the bioaccumulation of Pt, Pd, Rh, Ru, and Ir in *Taraxacum officinale*, *Plantago lanceolata*, *Lolium multiflorum*, *Rhytidiadelphus squarrosus*, and *Vascellum pratense*, growing in different roadside locations of Germany. The concentrations of PGMs in plants growing along the streets of Saarbrucken were higher according to the following order *R. squarrosus* > *T. officinale* > *P. lanceolata* > *V. pratense*, whereas in plant samples collected in highway A1, the order was *T. officinale* > *P. lanceolata* > *L. multiflorum*. Moreover, due to their wide distribution and strong correlation with the street dust levels, *T. officinale* and *P. lanceolata* are pointed as suitable bioindicators of PGM pollution. Although the bioavailability of Pd is often greater than the remaining PGMs (Schäfer et al. 1998; Kalavrouziotis and Koukoulakis 2009; Sobrova et al. 2012), this study and the one developed by Hooda et al. (2008) reported that the soil–plant relationship was higher for Pt. The latter assessed the levels of PGMs in *D. glomerata* found in soils bordering five British road networks in Oxfordshire and West London. The contents of Pd, Pt, and Rh in plant tissue increased, as the sampling distance from the road edge was smaller. Additionally, the BF for Pt was close to 1.0 for plants located at 0, 1.0, and 2.0 m

from the road, and above 1.0 at 5.0 m, highlighting the potential of *D. glomerata* for phytoremediation of roadside soils presenting this PGM. Tankari Dan-Badjo et al. (2007) studied the potential of *L. perenne* to uptake PGMs after exposure to the traffic of highway A33 in the east of France during 90 days. The results showed that the accumulation of Pd and Rh increased with the period of exposure to highway pollution, as well as greater uptake of Pd, followed by Rh and lastly by Pt. In addition, it was also concluded that fodder crops grown near roads might constitute a risk to dairy ruminants.

Samples of *Poa trivialis* and *Pinus sylvestris* were collected from soils neighboring road E-67 and road 19, respectively, in Bialystok, Poland (Leśniewska et al. 2004). The analyses revealed that concentrations of PGMs in *P. trivialis* followed the same order than those in soil, i.e., Pt > Pd > Rh. The results for *P. sylvestris* were below detection limits, which might be explained by the height at which samples were taken (2.0–3.0 m) and the fact that emission of unvolatile particles by car catalytic converters occurs at ground level. Niemelä et al. (2004) determined the concentrations of Pt and Rh in *Pleurozium schreberi*, *Taraxacum vulgare*, *Calamagrostis* sp., and *P. sylvestris*, growing along the E4 motorway in Oulu, Finland. The results depicted greater concentrations of both PGMs in *P. schreberi*, matching previous studies suggesting the advantage of mosses in relation to Pt and Rh uptake. Additionally, the Pt:Rh ratios in *P. schreberi* and *T. vulgare* were very similar to the characteristic Pt:Rh ratio reported for catalytic converters utilized in the European Union, emphasizing their potential as biomonitors of PGM pollution (Table 5).

Table 5 Levels of selected PGMs in plants growing in roadside soils from various locations

Plant species	Location	PGMs (μg kg^{-1})			Reference
		Pd	Pt	Rh	
Taraxacum officinale	Highway A1, Germany	3.1	30	7	Djingova et al. (2003)
Rhytidiadelphus squarrosus	Saarbruecken, Germany	2.4	30	5.4	
Dactylis glomerata	Oxfordshire, England	14.28	12.04	2.34	Hooda et al. (2008)
Lolium perenne	Highway A33, France	6.8	1.4	3	Tankari Dan-Badjo et al. (2007)
Poa trivialis	Bialystok, Poland	3.20	8.98	0.68	Leśniewska et al. (2004)
Urtica dioica	Vaihingen, Germany	1.9	8.6	1	Schäfer et al. (1998)
Taraxacum vulgare	Oulu, Finland	–	1.2	<0.3	Niemelä et al. (2004)
Pleurozium schreberi		–	27.4	4.6	

Conclusions and Perspectives

In general, the literature reveals an unambiguous negative correlation between the levels of metals in soils and plants and the distance of the sampling site to the road, i.e., the concentration of metals in soil and plant tissue decreases as the distance from the road edge increases. Accordingly, a positive correlation was also found by most studies between the concentrations of metals in soil and the levels of metals in plant tissue. Both relationships are unequivocal proof of traffic-related metal pollution. The reports of metal bioaccumulation in plants growing in roadside soils suggest the potential of a wide variety of species for bioindication and phytostabilization of soils afflicted by traffic-related metal pollution, including platinum group metals. The results clearly demonstrate the ability of numerous roadside plants from different climatic regions to stabilize metals in their rhizosphere, as well as to hamper the aerial dispersion of metal-containing road dusts in their canopy. Nevertheless, further studies would be helpful to better comprehend how phytostabilization of roadside soils could be enhanced, particularly through the concurrent application of organic amendments to increment metal immobilization and plant growth, and the use of the harvested biomass for energy generation.

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Soil Quality Protection at Heavy Metal-Contaminated Manufactured Gas Plant Sites: Role of Biological Remediation

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Abstract Soil contamination by metals has been a serious problem since the very beginning of the industrial revolution and nowadays causes widespread environmental concern. Most frequently, the results of soil pollution are due to phenomena of co-contamination (multi-metals or organic and inorganic pollutants) that have negative consequences for soil quality and require appropriate solutions for their reclamation. Remediation technologies have often used invasive processes that impact the characteristics of the soil substantially, causing the degradation of this important resource. The simultaneous presence of different metals is very common in MGP sites, and the remediation of these areas is technically demanding and requires suitable interventions at a reasonable cost. A case study is reported to evaluate both biological and nonbiological approaches used at a former MGP site. Two technologies have been compared in order to evaluate the best possible cost-effective strategies for the maintenance of high soil quality. Results suggest the applicability of biological strategies, in this case phytoremediation, thanks to the efficiency achieved with minimal disturbance of surrounding areas.

Keywords Soil quality • Remediation technologies • Metals • Phytoremediation • Soil washing • MGP site

Introduction

Technical and scientific tools for the study of soil pollution are constantly being developed to find innovative solutions in soil remediation, a topic of increasing interest for international institutions and organizations. Due to unsuitable and inefficient management and hazardous waste disposal techniques in the past, along with the lack of strict environmental policies, today widespread contamination dramatically influences the quality of soil and water. For these reasons, the problem

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of soil contamination is a significant environmental priority that needs to be tackled in all industrialized countries. In this article the problem of remediation is addressed from a European perspective, taking into account that progressive urbanization has led to many industrial sites being located nowadays within urban areas.

Unlike other countries, in Europe there is no common policy for soil remediation, so although soil pollution is recognized as one of the most hazardous soil threats, a regulatory framework to support remediation is lacking, and only a few EU member states have specific legislation regarding this matter. The first Community regulatory references on soil protection were established in 2006 with the Soil Thematic Strategy (EC 2006a) and the proposal of the Soil Framework Directive (EC 2006b), which regarding contaminated sites provide an inventory of them and a soil status report, besides the definition of national remediation strategies. Other environmental directives (EC 2000, 2008) are aimed, although indirectly, at protecting soil through constraints to control its contamination. Although currently any new potentially contaminating activities are restricted by regulations, many historically polluted sites can still pose environmental risks and therefore require urgent management. To date, an estimate of about 2.5 million potentially contaminated sites and 0.5 million sites really contaminated that needing remediation was extrapolated to 33 European countries (EC 2006c; Panagos et al. 2013). However, the differences between member states regarding defining and assessing the pollution of soil do not produce homogeneous data from each country, and consequently it is impossible to obtain a clear evaluation of soil contamination in Europe with the mapping of sites to clean up (Panagos et al. 2013). To minimize the latter problem, the European Environment Agency (EEA) has distinguished the contaminated sites from potentially contaminated sites. The former are any site in which the presence of soil contamination is confirmed, and there is a potential risk to humans, water, ecosystems, or other receptors, while the latter include any site where the suspicion of contaminated soil is not verified and detailed investigations are necessary to verify the risk for receptors (Van Liedekerke et al. 2014).

Although only rough data are available, the presence of pollutants in contaminated sites in Europe shows a trend similar to that of other industrialized countries. Heavy metals, mineral oils, and polycyclic aromatic hydrocarbons (PAHs) are the most relevant contaminants in soil. Industrial activities and treatment and waste disposal plants are the main types of contaminated sites. The lack of a common approach to the problem of contaminated sites has a considerable influence on technological and managerial aspects. In fact there are notable differences in the remediation costs and time between one country and another, closely linked to bureaucratic constraints. The reclamation of a site occurs much more rapidly in the most developed countries (England, France, and Germany) compared to those with a poor environmental culture, such as Italy, where the indiscriminate landfilling of a significant amount of soil remains the most common technological choice. The assessment of risks to human health and environment varies considerably depending on site-specific conditions that dramatically affect the chemical characteristics of contaminants such as solubility, mobility, volatility, persistence, etc. Consequently,

the receptors vary depending on the type of soil, and their exposure can only be evaluated on a case-by-case basis and therefore not at a European level.

While in the past remediation techniques were aimed at eliminating the contamination, often considering the soil itself as hazardous waste, protection of soil functions is becoming a key objective of the new remediation strategies. This new approach, expressed by the practice of "Green Remediation," enables greater long-term environmental sustainability and the conservation of soil quality. In fact, all environmental effects are considered, minimizing environmental footprints and maximizing the benefits resulting from environmental remediation activities (EPA 2008).

"Green and Sustainable Remediation" is the latest innovative approach, which tries to integrate green technologies with the sustainable recovery of contaminated sites. A fundamental step in this kind of remediation is the shared decision-making process with stakeholders to identify the best solution, from environmental, economic, and social points of view (ITRC 2011). Therefore, all three aspects of sustainability (environmental, social, and economic) are involved in every step of the remediation project, optimizing the possible benefits. Thus, green and sustainable reclamation becomes an economic and environmental resource for the surrounding territory. Nevertheless, the risk posed to both human health and environment should remain the main aspects to consider. Today, several remediation technologies are available that differ as to operating principles, time, and cost of realization and in the consequent environmental impact. The new European Community provisions on environmental matters (EC 2010) promote the technologies considered most suitable for reaching a goal without excessive costs, i.e., BATNEEC (best available technology not entailing excessive costs). Thus, identifying the best available technology becomes a tool for the decision-making framework of the remediation project. Considering the project, the implementation, the control of costs, and the desirable level of environmental protection established ensure the correct management of a contaminated site.

Whatever the approach, remediation becomes especially difficult and costly in the presence of co-contamination of the different pollutant classes. This situation is very common in many former industrial sites, especially manufactured gas plant (MGP) sites. The nature and complexity of activities carried out in the past have left us a legacy of multiple contaminations in these areas, and they are among the contaminated sites that prompt the greatest concern.

Approaches for Remediation of MGP Sites

Since the nineteenth century, MGPs have been the main source for lighting and heating homes and industries in the major cities. The first industrial facilities that produced gas from coal, coal and oil mixtures, or petroleum began operating in the United States in the early 1800s and then spread almost everywhere. Peak production occurred around 1920–1930 with approximately 10,000 operative plants spread

throughout Europe and the United States (EPA 1999). During the second half of the twentieth century, after the discovery of more widely available and cheaper natural gas, many MGPs began to close. Most of these buildings have been demolished, but in some countries, they remain as imposing structures that are abandoned or reused as electric substations, storage yards, etc. Although the number of former MGP sites located throughout the world is difficult to estimate, recent studies reported the presence of about 3000 sites in the United States, and about 4000 scattered throughout Europe, the United Kingdom, Canada, and Japan (EPA 1999; Wehrer et al. 2011). The past activity of MGPs led to widespread contamination that still remains, mainly caused by the great amounts of waste materials, especially coal tars, produced during all stages of their operation. The volatile and semi-volatile organic compounds, mono- and polycyclic aromatic hydrocarbons (PAHs), phenolics, inorganic compounds of sulfur and nitrogen, and metals (arsenic, chromium, copper, lead, nickel, and zinc) are the main chemical constituents of by-products and wastes resulting from coal gas manufacture (Luthy et al. 1994; Hatheway 2012; Tarr and McMicheal 2015). These compounds are the cause of soil contamination at MGP sites (Lundstedt 2003; Reddy et al. 2006) and pose a possible threat to human health and the environment. Generally, surface and subsurface soils and surface water are the primary media to be directly contaminated. The groundwater and sediment are consequently polluted, due to the formation of a tar/water emulsion that still migrates slowly in the subsurface.

Although any remediation must be considered site-specific, each site being characterized by a specific geology, the previous gas manufacture activities and the chemical/physical nature of the contaminants have led to similar patterns of pollution, for most former MGP sites. Thus, the remediation of these sites can be tackled with a similar approach to characterization and technology selection, taking into account the possible interactions between contaminants and different surrounding environments. The main problem in remediation technology selection for MGP sites is the presence of mixed contaminants, in addition to the recalcitrant nature of coal tar products, and very often the most common strategy has been soil excavation and transport to landfill. This solution means the destruction of enormous quantities of soil, which could be managed in more environmentally friendly alternative ways.

Table 1 reports a summary of the most frequently employed remedial actions for MGP sites. Many traditional technologies with negative impact on the environment (including atmospheric emissions, dust production, and dispersion) are still used. Thus, aside from canceling any advantages for economic and social growth, there are additional concerns and risks. However, the most recent green remedial action has been able to overcome these effects through the use of in situ techniques for the degradation of organic pollutants (bioremediation) and the removal of inorganic compounds (phytoremediation). Green remediation technologies are much cheaper than conventional ones, thanks to the low cost of realization and management. Besides, they have achieved a great success in scientific and public acceptance because they restore soil quality and minimize the disturbance in the site and in the surrounding area.

Table 1 Examples of some in situ and ex situ remediation technologies for manufactured gas plant (MGP) soil

Type	Technology	
	In situ	Ex situ
Chemical/physical	Soil venting Chemical oxidation Soil flushing Electrokinetics Stabilization/solidification	Soil washing Chemical extraction Stabilization/solidification
Thermal	Thermal enhanced vapor extraction	Thermal desorption
Biological	Biodegradation Bioaugmentation Bioventing Biosparging Phytoremediation	Landfarming Composting Bioreactors Biopiles

Bioremediation has often been chosen as a very successful option for the remediation of organic compounds in MGP soils (Thomas and Lester 1993; Gemoets et al. 2000; Saponaro et al. 2002; Gong et al. 2015). However, the coexistence of heavy metals with organic contaminants raises the need to find appropriate remediation technologies for metals as well. Heavy metal concentration in gasworks site soils is highly variable (Thavamani et al. 2012). Of the toxic metals, Pb was found to be the main contaminant, with a concentration varying from hundreds to thousands of mg kg^{-1}. Lower concentrations (from tens to hundreds of mg kg^{-1}) have been reported for Zn, Cu, As, Cd, and Cr (Mielke et al. 2004; Thavamani et al. 2011). For all these inorganic contaminants, the only green remediation technology that can be used is phytoremediation. Heavy metal mobility and bioavailability largely determine the possible remediation alternatives. The metal speciation should be the basis of feasibility tests, to suggest and compare possible remediation technologies for MGP sites.

The mixed contaminant problem at MGP sites can be approached using both biological and nonbiological strategies. Of the best available technologies (GWRTAC 1997), soil washing and phytoremediation currently appear to be the most frequently used and promising approaches for soil remediation.

Soil Washing

Soil washing is an ex situ remediation treatment of polluted soils based on the concentration of contaminants in a small volume by particle size separation using a scrubbing process in aqueous solution. The technique is commonly used to treat soil contaminated by some of the following compounds: metals, PAHs, polychlorinated biphenyls (PCBs), semi-volatile organic compounds (SVOCs), petroleum and fuel residuals, pesticides, and radionuclides (ITRC 1997).

Although soil washing has not been totally effective in the reclamation of old landfill soils containing mixed wastes, to date it is considered one of the few advantageous and permanent remediation techniques in the case of metal contamination (Dermont et al. 2008).

The term "soil washing" refers primarily to the physical separation process, but is often described as a chemical extraction method, which is a different and much more expensive technology (FRTR 2007; CL:AIRE 2007; Dermont et al. 2008). Contaminated soils generally have a notably variable grain size, and the separation process is based on this characteristic. The fundamental of soil washing is to physically separate the finer particles (<2.0 mm), generally more polluted, from the coarser constituents (>2.0 mm) of the soil, generally less or non-contaminated. In fact, in most cases, the contaminants are preferably adsorbed on finer grain size materials, such as clays or humic substances, which have a greater specific surface than the coarser particles (sand and gravel) (EPA 1993). Thus, physical separation is mostly employed in sandy soil with low content of humus and clay fractions, because if the proportion of fine fraction is higher, the volume of material to be subjected to subsequent treatment may be excessive (EPA 1991; ITRC 1997). The resulting division of soil into different levels of contamination allows considerable reduction of the volume of the effectively polluted soil and the possibility of immediate reuse of a large part of the decontaminated soil.

The ability of the washing process to remove contaminants depends on the properties of each class of substances, for example, polarity, volatility, solubility in water, and characteristics of the contaminated soil such as pH, organic matter, cation exchange capacity, and surface area, in addition to the characteristics of the scrubbing medium and the duration of the process. The best results are obtained in the case of recently contaminated soil, since the phases of adhesion and compaction, which bind the most polluted particles to the other soil constituents, increase with time. Under optimal conditions the physical separation of pollutants can provide very high yields. However, several factors affect this type of process, including the composition of the contaminated soil and the chemical form of pollutants. For example, a metal in mineral form can be successfully separated from soil particles according to different densities, whereas metals in ionic form tend to form bonds with the soil surfaces, becoming more difficult to separate (Petruzzelli et al. 2004).

Many soil washing plants are active in Europe, and despite the specific system configurations, the separation systems are designed so as to separate precisely the material with higher granulometry, generally to 63 μm, from that with lower particle size. The washing process is generally based on a sequence of steps. The first stage is the excavation of the soil and preliminary sifting to remove the coarser material for introduction into the treatment plant. In fact, most of the systems are not able to treat material up to 50 mm. In the second step, the smallest particles of soil are separated and concentrated mainly through intense mixing of the soil with the washing liquid, usually water. Sometimes additives, such as surfactants, can also be added to promote these processes.

The separation step is realized by different physical mechanisms:

(a) Crushing of the soil, when the contaminants are in the aggregates of larger size.
(b) Mechanical disintegration with high-pressure water jets to break the aggregates.
(c) Vigorous rubbing of soil to remove contaminants adhering to the surface.

At the end of these phases, the contaminants bonded to the coarsest particles by adhesion forces and compaction are released during abrasion processes and wet rubbing, moreover obtaining a separation of the solid from the liquid phase. Successively, the recovery of two distinct soil fractions is provided. The more abundant bulk, containing particle sizes greater than 63 μm, is decontaminated and can potentially be reused without further treatment on site or elsewhere without particular problems. Sometimes, a further cycle to remove any contaminants that were not completely separated may be necessary. Otherwise, the lower amount remaining (<63 μm) includes all the contaminants and requires further treatments or the landfill disposal (Anderson 1993; CL:AIRE 2007).

The used washing solutions containing possible additives are also collected, depurated, and reused for subsequent washings. The washing methods can be considered effective when finally the contaminated particles are distributed in a very limited range within the various soil grain sizes. The concentration of contaminants into a reduced soil mass, typically 5–40% of the original soil volume constituted by silty and clayey fractions, demonstrates the applicability of this technology. To conclude, soil washing with pure water or with surfactant solutions produces a cleaned fraction >60–70% of the original mass of the soil, with a consequent reduction of the pollutant concentration volume in the soil fine fraction. Physical soil washing is reliable and cost-effective when the clay and silt content is less than 30–35% of the total soil and when only water is used as washing liquid, because the higher cost of using chemical reagents and implementing further treatments of the contaminated fine fraction and solutions are reduced. However, this technology destroys the physical–chemical properties of the soil, which becomes only material to be reused for filling.

Phytoextraction

Phytoremediation is an in situ technique based on the natural ability of some plants to accumulate, immobilize, or convert many persistent contaminants present in the soil. Phyto-technologies include phytoextraction, phytotransformation, phytovolatilization, rhizofiltration, phytodegradation, rhizodegradation, and phytostabilisation, which differ according to pollutants targeted, decontamination aim, and the biochemical and physiological processes involved (EPA 2000; Pilon-Smits 2005; Parmar and Singh 2015). Like bioremediation, phytoremediation acts on chlorinated solvents, petroleum hydrocarbons, PCBs, PAHs, organophosphate insecticides, surfactants, and radionuclides (Kumar et al. 1995; Salt et al. 1998; Cofield et al. 2007;

Eapen et al. 2007; Sharma et al. 2015). In presence of these contaminants, plants can modify the chemical and physical conditions of soil to make it a suitable substrate for a possible microbial attack able to degrade the organic pollutants. When soil contamination is due to inorganic pollutants such as heavy metals, phytoremediation may be an effective solution for cleanup (Pedron et al. 2009) without destroying the soil structure and fertility. Potentially, phytoextraction seems to be among the most promising methods for metal-contaminated soil remediation (Garbisu and Alkorta 2001). This technology exploits the metal uptake capabilities of plant root systems and their ability to translocate and bioconcentrate the metal into harvestable portions of the plants. Phytoextraction efficiency depends on several factors, including plant species, metal bioavailability, and depth of contamination.

Plants able to grow on contaminated soils deal with the elevated concentrations of toxic metals via different metabolic and physiological strategies. Of these, internal tolerance mechanisms through production of metal-binding compounds and the cellular and subcellular compartmentation in nonsensitive parts are the most important (Küpper et al. 1999; Krämer 2003; Ghosh and Singh 2005; do Nascimento and Xing 2006). Plants with such mechanisms can be employed for phytoextraction purposes, taking advantages of the natural metal-accumulating capacity (hyperaccumulators) or the high biomass crop production. In this latter case, suitable additives in soil can promote the metal uptake.

Normally, plants can accumulate the essential micronutrients (Fe, Mn, Zn, Cu, Mg, Mo) in amounts necessary for their growth and metabolic activities, from 10 to 15 mg kg^{-1}. The hyperaccumulator species are also able to accumulate high amounts of nonessential elements (such as Cd, Pb, Hg), up to concentrations greatly exceeding those in soil, without showing toxic effects (Lasat 2000; Baker and Brooks 1989). A metal concentration ranging from 1000 to 10,000 mg kg^{-1}, according to the type of metal, was recorded in the aerial parts of hyperaccumulator species (Lasat 2002; Krämer 2010; Pollard et al. 2014). Approximately 400 hyperaccumulator species were identified, and in particular the Brassicaceae, Euphorbiaceae, Asteraceae, Lamiaceae, Fabaceae, and Poaceae families are able to concentrate heavy metals at levels 100-fold greater than those typically measured in non-accumulator plants (McGrath et al. 2001; Lasat 2002; Ghosh and Singh 2005; Wuana and Okieimen 2011). The main disadvantages of hyperaccumulators are their slow growth rates and small size and consequently low biomass production, of about one or two orders of magnitude lower than those of species cultivated. However, other crop plants and also woody species with high tolerance for heavy metals can be employed for their potential in phytoremediation (Pulford and Watson 2003; Zhuang et al. 2005; Unterbrunner et al. 2007; de Souza et al. 2012, 2013). The best choice of the species also depends on type of contamination, soil properties, and climatic conditions of the site to be reclaimed.

Two phytoextraction strategies can be distinguished: continuous (or natural) phytoextraction and assisted phytoextraction (Salt et al. 1998; Lombi et al. 2001; Ghosh and Singh 2005). Continuous phytoextraction uses the hyperaccumulator and the other crop species able to naturally accumulate and transfer high amounts of metals, As, Cd, Cr, Se, Pb, and Zn, in the easily harvestable portions of the plant

(Salt et al. 1998). Metal absorption continues throughout the plant's life cycle. As an example, *Brassica juncea* is a fast-growing high biomass plant and one of the best-known species selected for metal remediation, Cd, Pb, and Zn in particular (Kumar et al. 1995; Gisbert et al. 2006). In some studies Cd uptake values up to about 1450 mg kg^{-1} dry weight in shoots of *B. juncea* were found, three times greater than that recorded in *Brassica napus* (Nouairi et al. 2006) and a high removal efficiency of other metals, specifically Pb, up to 6.27 kg m^{-2} (Henry 2000).

Assisted phytoextraction is based on the modification of the soil chemical environment by the addition of specific substances that are able to increase metal bioavailability (Lombi et al. 2001; Ali et al. 2013). In fact, the knowledge of heavy metal bioavailability is essential for phytoextraction because plants are able to uptake these elements only if present in available forms in the soil. Bioavailability is the fraction of the total amount of heavy metals in soil available for plant uptake in a given time period (Peijnenburg and Jager 2003; van Gestel 2008). Metal bioavailability strictly depends on soil characteristics that should be accurately considered to evaluate the potential utilization of phytoextraction, since the ability of the same plants to uptake metals differs widely in soils with different properties. Thus, the total heavy metal concentration in soil has a relatively low importance since only metals in soil solution are readily bioavailable and must be primarily considered in order to evaluate the potential use of phytoextraction. The chemical, physical, and biological conditions of soil determine the distribution of metals among its solid and solution phases, through reactions of adsorption–desorption and precipitation–dissolution, which regulate the amount of heavy metals in the soil solution.

Phyotextraction efficiency depends both on a chemically driven process (i.e., the release of the metal from solid phase) and a physiologically driven process (i.e., the uptake by plants). Thus, the efficiency of phytoextraction is strictly related to bioavailability, which is determined by the chemical characteristics of each heavy metal and above all by the soil characteristics such as pH, organic matter, clay content, cation exchange capacity, and redox potential (Petruzzelli et al. 2015). For remediation with phytoextraction, the bioavailability of metals in contaminated soil should be evaluated using chemical extraction and a bioassay test. The amount of metals in the soil solution and/or that can be easily released from the solid phase can be determined by chemical extraction according to the specific site conditions. Since a chemical extractant cannot provide a good estimate of metal uptake for different plant species, it is necessary to carry out a biological test, with different plant species that are grown in the specific polluted soil. After harvesting, the metal content in plants will provide information on the bioavailable fraction. Chemical and biological tests alone are not able to determine the bioavailability, but both provide information on the heavy metal bioavailable fractions (Petruzzelli and Pedron 2006; Petruzzelli et al. 2015).

As previously stated, to enhance the efficiency of phytoextraction, it is essential to increase metal bioavailability. This is commonly performed by the use of additives such as chelating agents, which increase the metal concentration in the soil solution in forms available for plant uptake. This procedure is the basis of assisted phytoextraction. Many amendments have been used, and several promising results

have been obtained due to the increase in metal solubility, particularly in the lab or greenhouse tests. The low molecular weight (LMW) organic acids, such as ethylenediaminetetraacetic acid (EDTA), hydroxyethyl ethylenediaminetriacetic acid (HEDTA), and diethylenetriaminepentaacetic acid (DTPA), have been among the most frequently used additives. EDTA is the most common chelating agent employed in assisted phytoextraction due to its ability to form stable complexes with many heavy metals (Wu et al. 2004; Gupta et al. 2008; Seth et al. 2011). However, the use of chelating agents, not easily biodegradable, must be closely controlled since the amount of the metal released in the soil solution may exceed the bioavailable quantity that the plants are able to uptake (Luo et al. 2005; Santos et al. 2006; Cao et al. 2007; Bolan et al. 2014). In fact, chemical amendments pose problems of toxicity and persistence in the environment, in addition to potential metal leaching during the phytoextraction process (Wu et al. 2004; Grčman et al. 2001). Currently studies research environmentally friendly mobilizing agents. To contrast these possible negative side effects, the highly biodegradable LMW organic acids such as EDDS, oxalic, and tartaric acids can be used (Wu et al. 2004; Evangelou et al. 2007; Doumett et al. 2008; Pedron et al. 2015). Also, fertilizers have been used as mobilizing agents. Phosphate and thiosulfate have been positively used in soils contaminated by As and Hg (Tassi et al. 2004; Moreno et al. 2005; Pedron et al. 2013, 2011; Petruzzelli et al. 2014). Phytoextraction efficiency can be promoted also by the use of phytohormones, such as cytokinines, in foliar treatment of crop plants (Barbafieri and Tassi 2011; Cassina et al. 2012). In addition, the plant's microbial consortia, which change the rhizosphere environment, have proven to have good efficiency in the increase of bioavailability and uptake of heavy metals (Vetterlein et al. 2007; Wenzel 2009; Lin et al. 2010; Franchi et al. 2016).

Case Study

Experiments were undertaken to investigate the feasibility of two different remediation methods to clean up a soil contaminated by heavy metals: soil washing and phytoremediation. The decontamination efficiency of both techniques was evaluated by the degree of metal removal and the posttreatment soil quality.

Materials and Methods

Description of Site and Soil Characterization

Co-contaminated soil samples were taken from a former manufactured gas plant located in northern Italy. The site worked throughout the twentieth century, for gas production by coal gasification and later by catalytic reforming processes of light petroleum hydrocarbons for neighboring cities. A high pollution of soil, due to As,

Pb, and Zn, was discovered in the area. From three different zones (A, B, C), selected as representative of these MGP site, soil samples were collected (0–1 m depth) to evaluate soil washing and phytoremediation techniques. The soil samples were air-dried, ground to pass through a 2-mm sieve, and thoroughly homogenized before laboratory analysis. Soil physical properties were determined according to procedures reported in *Methods of Soil Analysis* (Sparks et al. 1996). The total As and heavy metal (Cr, Cu, Ni, Pb, Zn) concentrations were determined via acid digestion (EPA 1995a).

Solubility Test and Soil Sequential Extraction

The metals interact with the soil components through many different reactions, with formation of chemical bonds characterized by different energies. The evaluation of metal solubility and bioavailability was needed to achieve the goal of this study. Heavy metal solubility and potential bioavailability were determined following a sequential extraction procedure with H_2O, 1.0 M KNO_3, and 1% EDTA (Petruzzelli et al. 1989; Pedron et al. 2009), with a soil/extractant ratio of 1:5 and a shaking time of 3 h for all extractions. As solubility and potential bioavailability were determined by the first two steps of modified Wenzel's sequential extraction (Wenzel et al. 2001) using 0.05 M $(NH_4)_2SO_4$ and 0.05 M KH_2PO_4, in the first step, 1.0 g of soil was treated with 25 mL of $(NH_4)_2SO_4$ in 50-mL polypropylene centrifuge tubes and shaken for 16 h. The soil residue was treated with 25 mL of KH_2PO_4 and stirred for 4 h. Pb, Zn, and As in the extracts from each sequential extraction were determined after centrifugation and filtration.

Experimental Setup

Soil Washing Test

Soil sample (1.0 kg each) was subdivided into subsamples (no separation of coarser particles and various residues was carried out), which underwent a treatability test according to the following steps:

1. Water was added to contaminated soil samples in a ratio liquid to solid (L/S) 3:1 to make slurry in 2 L plastic containers. The L/S ratio was selected after preliminary experiments in which the increase of L/S from 3 to 10 did not change the results significantly. Very low amounts of floating materials were observed and skimmed off.
2. The slurry was shaken at 20 °C overnight in a high-speed agitator, which facilitated an intense scrubbing of the soil particles. In this step the finer were separated from coarser size particles.
3. The slurry was fed to the screening process through different sieves in order to fractionate soil particles of various sizes. The following fractions were obtained: >5 mm, 5–2 mm, 2–0.2 mm, 0.2–0.1 mm, 0.1–0.05 mm, and <0.05 mm.

The soil material remaining on each sieve was repeatedly washed with water until finer particles passing under the sieve were not found visually. The washing waters were recovered and passed once on to the sieves. The residual material on every sieve was collected and oven-dried at 105 °C. The weight was recorded, and heavy metal analyses were carried out after acid digestion (EPA 1995a). All process waters were also analyzed for Pb, As, and Zn. Before and after the washing process, the soil pH was determined and no significant variation was recorded.

Phytoextraction Test

The phytoextraction experiment was carried out at mesocosm scale in a greenhouse. Only the A soil sampled in the MGP site was used and only Pb and As were investigated. The samples were homogenized, and the coarser materials were removed in order to simulate a natural water flow through the soil and recreate a situation most representative of field conditions. Three plant species, *Lupinus albus* (lupine), *Brassica juncea* (Indian mustard), and *Holcus lanatus* (velvet grass), were selected. Plant growth and contaminant accumulation, translocation, and uptake in vegetal tissues were examined and compared.

Mesocosm pots were polypropylene containers, 32 cm × 50 cm, arranged to collect leachates by a hole in the bottom connected to a plastic tank with a PVC tube. Mesocosms were filled with 30 kg of contaminated soil, and NPK 8.24.24 (3.5 g pot^{-1}), ammonium nitrate (NH_4NO_3) (2.5 g pot^{-1}), and urea (2.1 g pot^{-1}) were mixed uniformly and added to soil in liquid form as base fertilizers. After 1 week of equilibrium, the mesocosms were sown with 1.5 g of *B. juncea* seeds and 10 seeds of *H. lanatus* and *L. albus* per pot. At the floral stage, after about 2 weeks from sowing, two different soil treatments were started. A solution of EDTA 2 mMol kg^{-1} and one of DAP (biammonium hydrogen phosphate) 33.3 mMol kg^{-1} were used as mobilizing agents for Pb and As, respectively, and were added separately to the soil. The concentration of both chemical agents was chosen according to specific characteristics of the soil characterized by a basic pH.

The experimental design provided a total of 18 pots, where one half was devoted to Pb analysis and the other half for As investigation. Three mesocosm groups for species were set up: untreated control, pots with EDTA addition (+EDTA), and mesocosms treated with DAP (+DAP). All experiments were performed in triplicate, and simultaneously three nonvegetated control mesocosms were prepared for leachate studies. The greenhouse was maintained at 18–25 °C with natural day/night cycles, and the plants were watered daily with tap water through a system of automatic irrigation. The experiment lasted about 30 days, after which the plants were harvested and their biomass production was evaluated. Aerial parts and roots were separated by cutting with scissors and washed with deionized water. The roots were further washed in an ultrasound bath (Branson Sonifier 250 ultrasonic processor; Branson, Danbury, CT, USA) for 10 min to eliminate soil particles that could have remained on radical surfaces. Subsequently, samples were dried in a ventilated oven at 60 °C until constant weight, and the dry weights of tissues were determined gravimetrically. Shoots and

roots were analyzed for Pb and As content, after acid digestion (EPA 1995b). The leachates of each pot were collected before and after treatments.

Metal Analysis

Soil and plant samples were digested with HNO_3 (65%, v/v) and H_2O_2 (30%, v/v) mixture into a PTEF-TMF (polytetrafluoroethylene-tetra-fluoromethoxil) pressure digestion vessel using a microwave oven (FKV-ETHOS 900) by US-EPA method 3051-A (EPA 1995a) and US-EPA method 3052 (EPA 1995b), respectively. Determination of metals in soil, plant samples, washing water, leachates, and extracts was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) Liberty AX, Varian. The concentrations of metals were expressed in milligrams per kilogram dry weight (mg kg^{-1}), and their total accumulation in vegetable samples, expressed in milligrams (mg), was calculated as the product of metal concentration in plants tissues for the dry biomass produced (Jarrell and Beverly 1981). All data reported are the average of three replicate mesocosms.

Statistical Analysis

Statistical analysis was performed by Statistica version 6.0 (Statsoft, Inc.). The data were analyzed using a one-way analysis of (ANOVA). Tukey honestly significant difference test was used for pairwise comparison of means at 0.05 significance levels ($p < 0.05$).

Results and Discussion

Soil Characterization

The soil samples were characterized by similar mean values: pH about 7.0, 11.5 $Cmol_{(+)}$ kg^{-1} of CEC, and 2.1% of organic matter. The textures were composed of gravel 13% (skeleton), sand 52%, silt 29%, and clay 6%. As, Pb, and Zn were the only pollutants found at high concentrations (Table 2). The other heavy metals were present (data not shown) below the acceptable concentration limits for soil in

Table 2 Total concentration of Pb, Zn, and As (mg kg^{-1} dry weight) in three different soil samples

Soil	Pb	Zn	As
A	535 ± 32	259 ± 27	352 ± 62
B	767 ± 53	302 ± 69	58 ± 17
C	288 ± 31	172 ± 15	740 ± 84

Data are the mean values ($n = 3$) with standard deviation

conformity with Italian legislation (Italian Legislative Decree 152/06 2006; Annex 5, Title V, Part IV).

Solubility Test and Soil Sequential Extraction

The evaluation of metal solubility is an essential step in the phytoextraction feasibility test since plants can take up only bioavailable metals. The same test is essential for evaluating the washing liquid for the soil washing treatment. Sequential extractions were performed to quantify chemical forms of metals in soil (Table 3). The water and KNO_3 extractions allowed assessment of the soluble and exchangeable metal fractions, that is, the immediately available forms for plant uptake. The data suggested that most of the metals in the soil were in a form unavailable for absorption by plants. The negligible amounts of metals solubilized by water and KNO_3 supported the feasibility of soil washing using water as the washing liquid. The greater portion of metals extracted with EDTA indicated the suitable use of this complexing agent in assisted phytoextraction in these soils. In phytoremediation studies, EDTA has often been employed due to its high complexing ability regarding different metals, particularly Pb, facilitating the heavy metal's release from solid to liquid soil phases (Huang et al. 1997; Luo et al. 2005; Saifullah et al. 2009; Pereira et al. 2010).

Arsenic was not immediately bioavailable since the amount extracted with the sulfate, the first amendment used in Wenzel's sequential extraction, was below the detection limit. The addition of phosphate ions to soil by KH_2PO_4, the second step of the extraction, significantly promoted the release of arsenate in the liquid phase for effects of competition and exchange with the same adsorbent surfaces. For this

Table 3 Concentration of metals (mg kg^{-1} dry weight) extracted by the sequential procedures in different soil samples

Soil	H_2O	KNO_3 1 M	EDTA 0.01 M
	Pb		
A	0.01 ± 0.004	12.0 ± 1.1	123 ± 15
B	0.02 ± 0.01	10.3 ± 1.3	136 ± 13
C	0.04 ± 0.02	11.9 ± 1.4	51.3 ± 2.0
	Zn		
A	0.03 ± 0.01	1.12 ± 0.01	36.2 ± 3.4
B	0.01 ± 0.01	1.24 ± 0.02	23.5 ± 1.3
C	0.02 ± 0.01	1.35 ± 0.01	16.5 ± 2.1
	$(NH_4)_2SO_4$ 0.05 M	*KH_2PO_4 0.05 M*	
	As		
A	BDL	19.5 ± 6.2	
B	BDL	3.15 ± 1.2	
C	BDL	40.2 ± 5.3	

Data are the mean values (n = 3) with standard deviation
BDL below detection limit

reason, phosphate is the additive most widely used in assisted As phytoextraction (Cao et al. 2003; Petruzzelli et al. 2014). The results from the sequential extraction procedures reveal that most of the metals are strongly bound to soil, addressing the choice of water as liquid phase in the soil washing and suggesting the need for a suitable mobilizing agent to select phytoremediation as an effective alternative.

Soil Washing Test

The weight distribution of the different soil fractions was fairly similar between the three different samples (Fig. 1). After the washing procedure, about 57% of the total soil weight was composed of the size fraction >5.0 mm and the remaining 43% by the finer fractions, distributed in the following ranges: 8% of 5.0–2.0 mm, 18% of 2.0–0.2 mm, 3% of 0.2–0.1 mm, 2% of 0.1–0.05 mm, and 12% of size fraction <0.05 mm. The distribution of the metal amounts in the different particle size classes (Fig. 2) shows that the washing process concentrated most of the pollutants, over 85% of the total, in the finer fractions (<2.0 mm), accommodating one of the requirements necessary for implementation of soil washing. Furthermore, in accordance with the site-specific risk analysis (Bonomo et al. 2000), the target value achievements of Pb (400 mg kg^{-1}) and As (50 mg kg^{-1}) were evaluated. The Zn value limit was not fixed by the risk analysis since it is an essential trace element not particularly toxic.

In all samples, the Pb concentrations in the coarse soil fraction were found below the target value. In the size particles <0.1 mm, the average metal amounts ranged from 536 mg kg^{-1} to 178 mg kg^{-1} for Pb and from 356 mg kg^{-1} to 114 mg kg^{-1} for Zn. The As concentrations were below the remediation target only in sample B, in which just the <0.05 fraction exceeded the threshold value. In the other two samples,

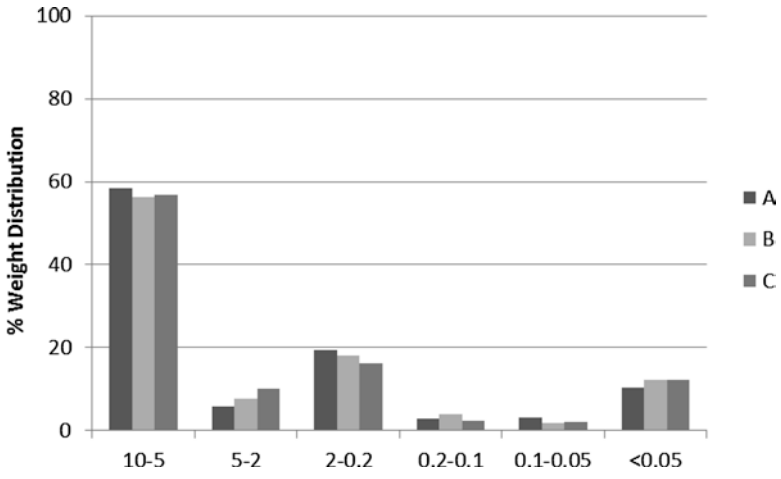

Fig. 1 Weight distribution (%) of the different particle size fractions (mm) of the soil samples

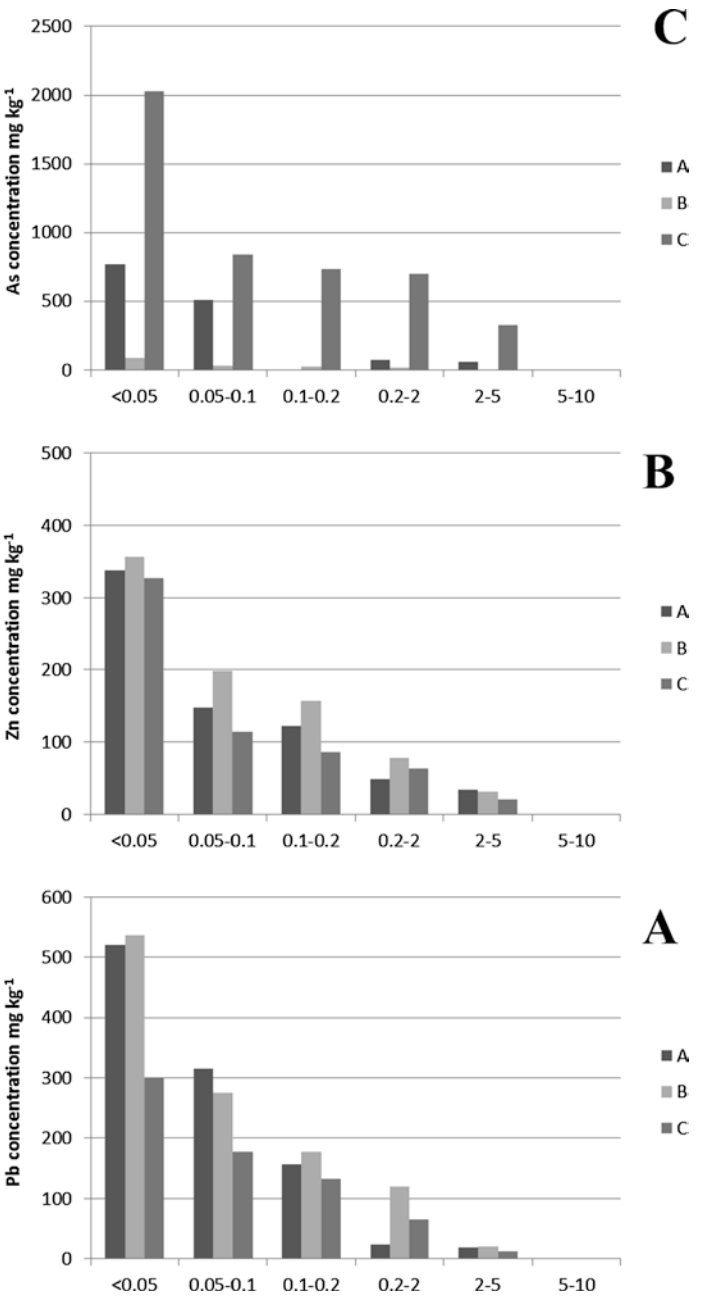

Fig. 2 Pb (**a**), Zn (**b**), and As (**c**) concentration (mg kg^{-1}) in the different soil size fractions (mm). Values are expressed as the means of the three replicates

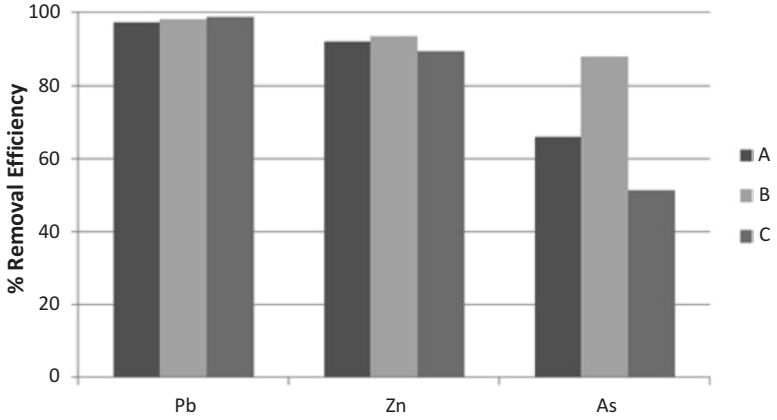

Fig. 3 Efficiency of soil washing in <2.0 mm soil fraction. Data are expressed as percent of metal removal (with respect to the total concentration in the original unwashed soil)

the amount of arsenic in the coarse fraction varied from 65 to 735 mg kg^{-1}. The soil washing efficiency was evaluated by analyzing only the soil fraction below 2.0 mm (Fig. 3). The best performance was achieved in Pb and Zn removal, with an average efficiency of about 98% and 92%, respectively. Arsenic was removed less efficiently, only 88% in the sample B, which had the lowest initial concentration of the metal. In the A and C samples, about 51% and 66% of As was eliminated, respectively. The water washing was also analyzed, and all metals were found below the detection limits (data not shown).

The results obtained suggest the use of physical soil washing as an appropriate technology for Pb and Zn remediation. The goals established by the risk analysis were achieved, and a reduction by over 50% of the initial contamination in the fraction >2.0 mm was obtained. In contrast, the reduction of As concentration in the coarse fraction was not enough to reach the threshold value. If soil washing is selected as the remediation technology, soils from different areas will be mixed, and the efficiency will be estimated by the mean values of the three samples. Also in this case, the inverse relationship between metal concentration and size of particles is obtained for each metal (Fig. 4).

Phytoextraction Test

Biomass Production

Biomass is an important parameter for evaluating phytoextraction efficiency, since the plants absorb and concentrate toxic metals from contaminated soil in their tissues. The biomass yield can be affected by several agronomic practices including application of fertilizers. The aerial part dry masses of the selected species are illustrated in Fig. 5. No visible symptom of metal toxicity during germination and plant

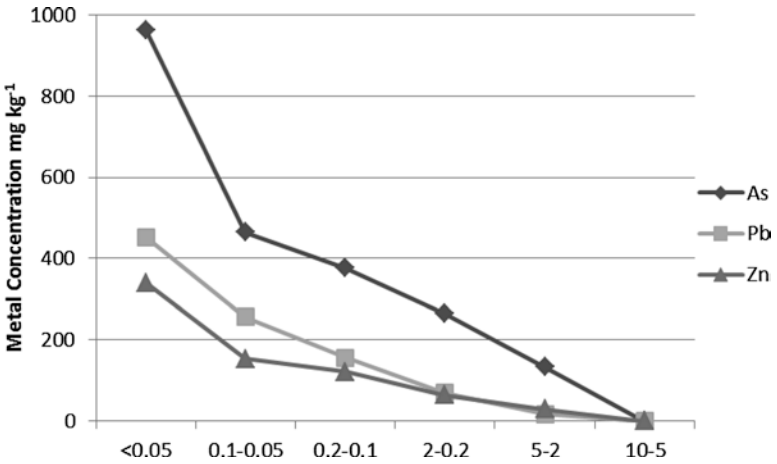

Fig. 4 Inverse relationship between metal concentration (mg kg^{-1}) and different particle size (mm). Values are the means of the *A*, *B*, and *C* soil samples

growth was observed. The highest biomass production was recorded for *B. juncea* and *H. lanatus*. EDTA and DAP addition induced a slight shoot biomass decrease, greater in Indian mustard plants after DAP application, by around 9% of reduction. Only the aerial biomass of lupine increased by about 15% with the phosphate treatment.

Metal Analysis

L. albus, *B. juncea*, and *H. lanatus* species were chosen because they have the ability to tolerate and accumulate high amounts of various heavy metals (Pb, Cu, Cd, and Zn) in their tissues from contaminated soils with suitable yields of biomass (Liu et al. 2000; Ximenez-Embun et al. 2002; Marchiol et al. 2007; Adesodun et al. 2010). All selected crops are considered As-tolerant (Pickering et al. 2000; Esteban et al. 2003; Quaghebeur and Rengel 2003; Gupta et al. 2009; Petruzzelli et al. 2014).

After treatment application, significant differences in metal concentrations, translocation, and accumulation in vegetal tissues were observed. Total concentrations of Pb and As in roots and shoots of plants and their translocation factor (TF) are given in Table 4. The application of EDTA and DAP has allowed the mobilization of Pb and As, respectively, in soil samples, increasing their concentration in vegetal tissues, especially in aerial parts of *L. albus* and *B. juncea*. As expected, after treatment, the metal concentration in the aboveground part of all selected species was higher than in the roots, except for *H. lanatus*, which concentrated most of the Pb and As in the root portion, even after addition of the complexing agent. Moreover, EDTA addition significantly increased Pb concentration by about 28, 13, and 14 times in shoots of lupine, Indian mustard, and velvet grass, respectively. In the presence of DAP, the shoot As concentration for lupine and Indian mustard was

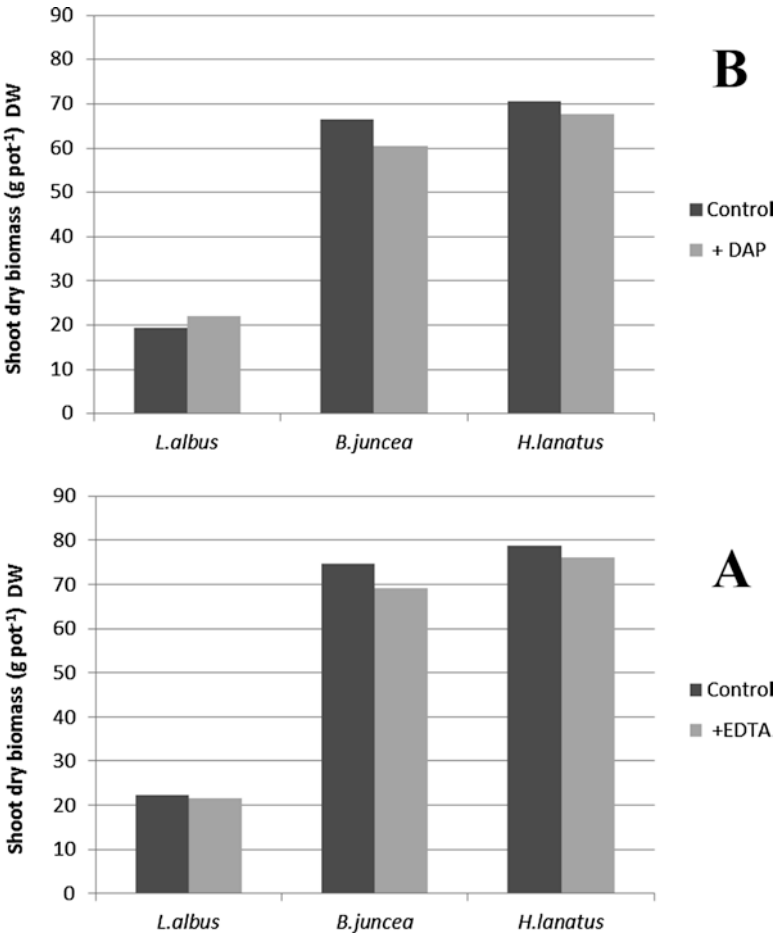

Fig. 5 Effect of EDTA (**a**) and DAP (**b**) treatments on shoot dry biomass (g pot^{-1}) of *Lupinus albus* (lupine), *Brassica juncea* (Indian mustard), and *Holcus lanatus* species grown in soil contaminated by Pb and As. Data are the means of three mesocosms

greatly enhanced by around 28 and 40 times, respectively, relative to control shoot concentration.

The translocation factor, calculated as the ratio of metal content in the shoots to the roots, reveals the plant's ability to translocate metals from radical tissues to aboveground biomass. Usually, the plants suitable for phytoextraction have TF values ≥1.0 (Tu et al. 2002; Yoon et al. 2006). Data shows a significant effect of different treatments on the translocation factor of Pb and As. In all untreated plants, the TF values were lower than 1.0, except for Pb translocation in Indian mustard that showed a value of 1.8, similar to that reported in other phytoextraction tests (Kumar et al. 1995; Ghosh and Singh 2005). Pb and As transfer factors, reaching values up to 17.6 and to 7.83, respectively, indicate that *B. juncea* effectively translocated the

Table 4 Pb and As concentration (mg kg^{-1} dry weight) in plant tissues and translocation factor (TF) of *Lupinus albus* (lupine), *Brassica juncea* (Indian mustard), and *Holcus lanatus* species grown in control and EDTA- or DAP-treated pots

Metal	*L. albus*			*B. juncea*			*H. lanatus*		
	Root	Shoot	TF	Root	Shoot	TF	Root	Shoot	TF
Pb									
Control	22.6 ± 3.2	8.5 ± 1.9	0.38	10.8 ± 1.5	19.8 ± 4.8	1.83	25.2 ± 3.2	2.1 ± 0.6	0.08
+ EDTA	150 ± 25	240 ± 16.9	1.60	15.0 ± 4.2	265 ± 34.6	17.6	36.1 ± 4.3	30.2 ± 6.2	0.84
As									
Control	4.23 ± 2.1	0.76 ± 0.1	0.18	2.23 ± 1.8	0.42 ± 0.2	0.19	2.78 ± 1.4	0.01 ± 0.002	0.004
+ DAP	8.54 ± 2.8	15.8 ± 3.6	1.85	2.49 ± 1.4	19.5 ± 2.8	7.83	2.23 ± 1.1	0.01 ± 0.004	0.004

Data are the mean values ($n = 3$) with standard deviation

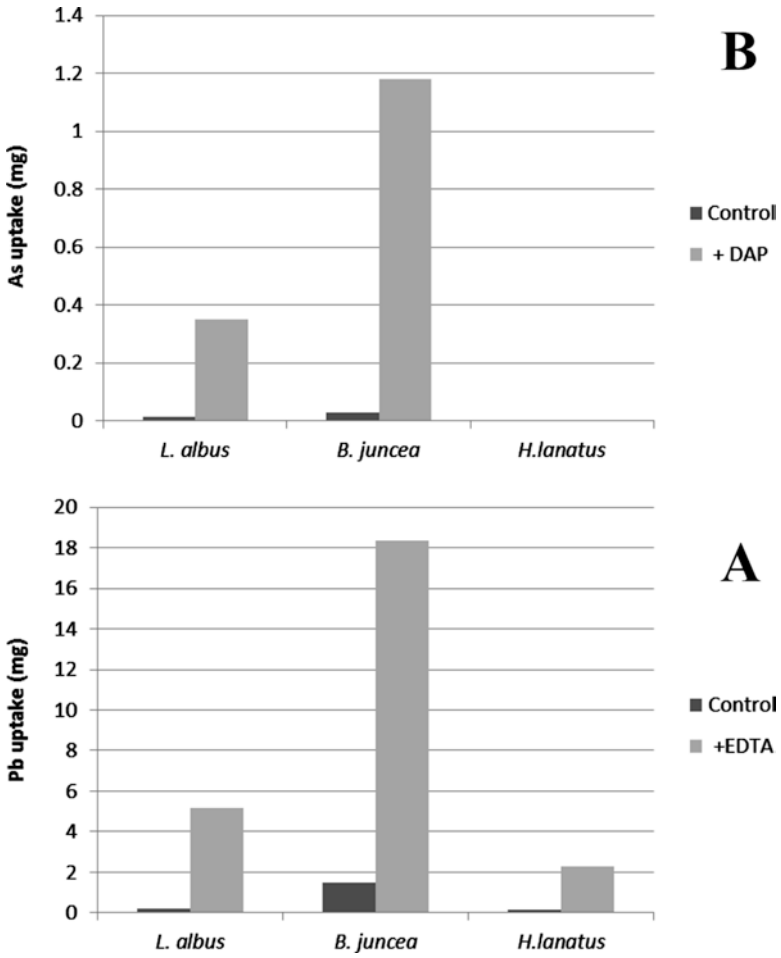

Fig. 6 Effects of EDTA (**a**) and DAP (**b**) on Pb and As uptake (mg), respectively, in the aerial parts of *Lupinus albus* (lupine), *Brassica juncea* (Indian mustard), and *Holcus lanatus* species. Data are the means of three replicates

metals in the presence of EDTA and DAP. Even in *L. albus*, the addition of two mobilizing agents raised the TF to over 1.0. The treatments performed increased the removal efficiency of Pb and As, which is expressed as total accumulation. The data are reported in Fig. 6. The amount of metals found in plants differs considerably according to the species selected, indicating different abilities to absorb metals from soil. The best results for Pb accumulation after treatment were obtained for *B.juncea*, which reached up to 18.3 mg, while *L. albus* accumulated about 5 mg of lead in their aerial parts. By EDTA treatment, the Pb uptake of *L. albus* and *B. juncea* was, respectively, of about 27- and 12-fold greater than those of the control plants. Similarly, DAP application led to an increase in As uptake in the shoots of Indian

mustard and lupine. DAP-treated *B. juncea* accumulated up to 1.18 mg of As, about 42 times more than control plants, while in *L. albus* supplemented with phosphate, the accumulation reached 0.35 mg, approximately 23 times greater than the corresponding untreated plants. Inversely, *H. lanatus* was not able to effectively extract either of the two metals, especially As, even after the treatments. The results suggest *B. juncea* and *L. albus* species as possible candidates for phytoextraction.

Evaluation of phytoremediation efficiency is based on the reduction of the bioavailable fraction of heavy metals that are the most hazardous to human health and the environment. The remediation approach is based on the enhanced bioavailable contaminant stripping (EBCS) procedure so that evaluation of the residual hazard involves a specific risk assessment considering bioavailable amounts instead of the total content of metals in the contaminated soil (Pedron et al. 2013; Petruzzelli et al. 2013, 2014). Data from soil sequential extractions carried out after harvest allow observation of the variation in metal amounts in the EDTA extractable fraction (potential bioavailable fraction) due to presence of the plants. After only one growing cycle, Pb extractable by EDTA was considerably lower than the original amount, suggesting that the plants took up the Pb mainly from this fraction. After four regrowing cycles, the EDTA extractable quantity was substantially negligible (Fig. 7). The decrease in the extractable amount in EDTA was maximum after the first growth, and subsequently this bioavailable quantity tended to decrease to zero after four cycles of growth. The DAP effect on As also had a pattern similar to that of EDTA on Pb. In fact, the As bioavailable form in soil, extracted by KH_2PO_4 0.05 M, became negligible after four regrowing cycles.

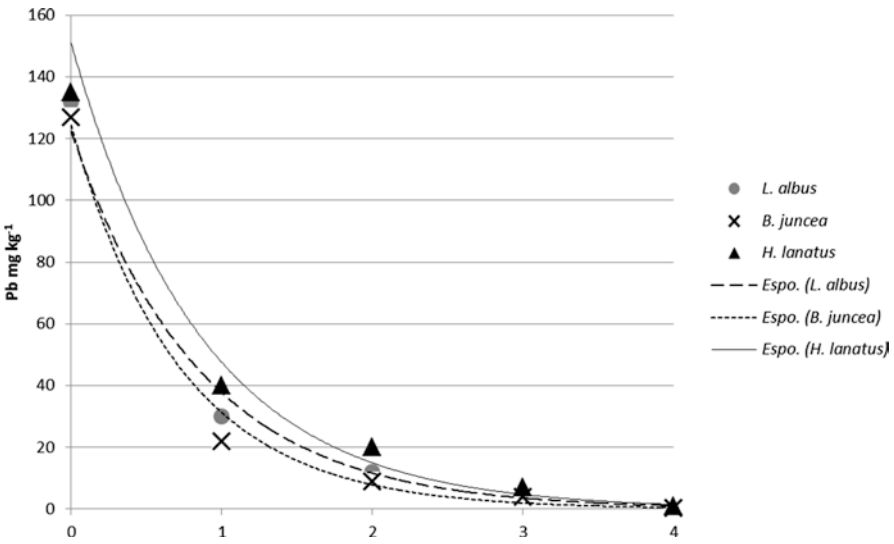

Fig. 7 Pb concentration (mg kg^{-1}) extracted by EDTA after four plant regrowths. Data are means of three mesocosms ±SD

Leachate

The mesocosm leachates were collected to investigate the presence of metals in leaching water due to the effects of addition of chemical agents. To obtain the same amount of leachate from mesocosms vegetated with *B. juncea*, more water additions were needed, probably due to the greater transpiration of this species. The Pb and As concentrations in the leachate solutions are presented in Table 5.

The EDTA addition significantly raised the Pb concentration in the leachates of lupine, Indian mustard, and velvet grass, reaching values of up to 10.4, 55.7, and 117 μg L^{-1}, respectively. After DAP application the As concentrations in leachates of vegetated mesocosms increased about 35-fold compared to those in untreated soil, amounting to average values of 10 μg L^{-1} for all species. As expected, the greater amount of metals found in the leachates of *H. lanatus* mesocosms was caused by the low extraction efficiency of the species. The obtained data show that after treatment with the mobilizing agents, metals were present in the leachate; indeed, the plants were unable to absorb the total quantity of bioavailable metals produced by the addition of EDTA or DAP (Pedron et al. 2014). Other bench-scale studies of assisted phytoextraction have shown a substantial rise in metal leaching after addition of chemical chelates (Grčman et al. 2001; Lombi et al. 2001; Wenzel et al. 2003). The increased mobility of the metals in soil, due to addition of agents such as EDTA and DAP, can result in a certain leaching of toxic metals into groundwater. This environmental hazard can be prevented by an accurate application of additives and by suitable irrigation management (Römkens et al. 2002; Huang and Cunningham 1996), as well as by repeated phytoextraction cycles. Also in this case, after three growing cycles, the amount of Pb and As in leachates was negligible. The data are in agreement with several studies that have shown the effectiveness of repeated harvests with suitable species, for metal content reduction in soil leachate (Tlustoš et al. 2006; Jiang et al. 2010; Li et al. 2014).

Table 5 Pb and As leachability (μg L^{-1}) before and after the treatments in the pots of soils vegetated with *Lupinus albus* (lupine), *Brassica juncea* (Indian mustard), and *Holcus lanatus*. Data are expressed as mean values and standard deviation of three mesocosms

	Pb		As	
Species	Before	After	Before	After
L. albus	0.33 ± 0.05	10.4 ± 2.1	0.31 ± 0.04	9.5 ± 3.1
B. juncea	0.28 ± 0.06	55.7 ± 13.6	0.29 ± 0.05	10.3 ± 3.5
H. lanatus	0.27 ± 0.04	117 ± 22.1	0.27 ± 0.05	11.5 ± 2.9
No vegetation	0.31 ± 0.08	147 ± 12.4	0.32 ± 0.04	16.9 ± 1.8

Data are the mean values ($n = 3$) with standard deviation

Case Study Conclusion

In the present study, soil washing and phytoremediation techniques were evaluated for the reclamation of MGP site soil contaminated by metals. The results obtained suggest the use of a physical separation process as remediation technique for Pb and Zn but less for As. In particular, Pb concentrations in the >2 mm fraction achieved the threshold value established by the risk analysis with a reduction by over 50% of its initial concentration. The phytoextraction findings demonstrated the effectiveness of the application of selected additives to increase metal mobility in soil and consequently their uptake by plants. Specifically, EDTA greatly enhanced the Pb uptake and DAP of the As accumulation by plants. Of the selected species, only *B. juncea* and *L. albus* were found to be possible candidates for phytoextraction. The feasibility tests performed offer decision-makers an overview of the possible technological options. On one hand, a physical technology can resolve the problem of contamination in a relatively short time but completely destroys the soil. In this case, the material resulting from soil washing can be only reused even on site for fills. On the other hand, a green technology takes longer, but improves the quality of the soil, avoiding any problems for neighboring areas. Phytoextraction is able to maintain green areas within the site, which is one of the stakeholder requests.

Conclusion

In recent years, there has been increasing attention to soil conservation and soil quality, which can be still considered evolving concepts. This concern stems from an awareness of the enormous amounts of soil lost every day to erosion and sealing. Nowadays, the concept of soil quality is taken into account as an important issue in the remediation of contaminated sites. After years, the erroneous idea of defining soil quality by contaminant concentration values has finally been abandoned. The univocal assessment of soil quality is basically impossible due to the natural differences between soils in different geographical locations, the specific evolution that accompanied the formation of the soil, and the subsequent pollution processes. The quality estimation of soil at a contaminated site after remediation can be based on the ability of the soil to perform its essential functions, mainly as a living filter for the protection of water and the food chain. In addition to consolidated parameters such as time, cost, and efficiency of contaminant removal by means of appropriate physical, chemical, and biological parameters, critical soil functions can be used to evaluate the effects of alternative technologies on the environment. Thanks to several technologies available for the remediation of contaminated sites, decision-makers and stakeholders have an opportunity to evaluate innovative remediation strategies, considering the soil as a valuable resource rather than a waste to dispose of. There is an imperative need to consider all environmental effects of remediation in the selection of technologies optimizing the maximum environmental benefits according to strategies of “Green Remediation.” In this way, the reuse of reclaimed

sites will be promoted without destroying the soil while reducing the production of waste, energy consumption, and pollutant emissions into the atmosphere.

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Plant Physiology Processes Associated with "Plant-Plant Growth-Promoting Rhizobacteria" Bioassays for the Enhanced Heavy Metal Removal

Angélica Rodríguez-Dorantes and Leonor Angélica Guerrero-Zúñiga

Abstract Rhizoremediation considers the phytoextraction and soil bioaugmentation strategies and optimize the synergistic effect between plants and microorganisms with a physiological basis related only to plants. It is known that plant growth-promoting rhizobacteria (PGPR) affect the plants growth facilitating the uptake of nutrients and protecting them; this interaction has been attractive because the biotechnological potential of microorganisms for metal removal from soils and transport of them to the plants. In the following sections of this chapter, the authors give some analysis of the importance about the establishment of "plant-PGPR bioassays" as tools to compare the relationships between in vitro physiological characteristics of rhizobacteria isolated from plant metal accumulators and the plant's physiology response, as follows: importance of siderophores as plant growth-promoting trait, evaluation of the in vitro production of siderophores by rhizobacteria, utility of the measurement of antioxidant activity in plants as indicator of heavy metal stress, and, finally, the description of cadmium effect by the antioxidant activity in two bioassays, with plant cell cultures and plantlets inoculated with a siderophore-producing bacteria (SPB).

Keywords Plant growth-promoting rhizobacteria • Plant bioassays • Siderophores • FRAP

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Siderophores and Important Plant Growth-Promoting Trait for the Heavy Metal Removal

There has been known that genera like *Agrobacterium*, *Ralstonia*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Serratia*, *Pseudomonas*, and *Rhizobium* reported by O'Sullivan and O'Gara (1992), Höflich et al. (1994), Carlot et al. (2002), and Glick (2003) are recommended for the metal removal associated with plants. Gadd (2004) mention that the participation of these microorganisms increase solubility and change speciation of metals/metalloids through the production of microbial siderophores that can complex cationic metals or desorb anionic species (like arsenate) by ligand exchange and may also immobilize cationic metals such as cadmium, copper, or zinc by the differential surface charge of metal-siderophore complexes. Generally, the siderophores are low-molecular-mass iron chelators with high association constants for complexing iron Neilands (1983) and Miethke and Marahiel (2007) produced by a diverse group of microorganisms. Siderophores production is most common among plant growth-promoting rhizosphere bacteria, which exhibit their optimum growth and siderophores production activity at extreme environmental conditions, including the presence of elevated concentrations of heavy metals (Winkelmann 2007; Rajkumar et al. 2010).

Dimkpa (2008) have proposed two different ways to analyze the stimulating effect that heavy metals involved in siderophores production; firstly, that the heavy metal might be directly involved in siderophores biosynthesis pathways or their regulation, and secondly as alternative, the free siderophores concentration in the medium might be reduced by complex formation with heavy metal ions. This process interferes with the complexation of siderophores with iron and thus decreases the soluble iron concentration. As iron deficiency stimulates siderophores production, more siderophores would then be produced. It is known that diverse plant species, including *Cucurbita pepo*, *Brassica juncea*, *Helianthus annuus*, *Medicago sativa*, and *Vigna unguiculata*, acquire iron from Fe-siderophore complexes, and their growth be stimulated in metal-contaminated soils, increasing it with the presence of potential siderophore-producing bacteria (SPB) (Rajkumar et al. 2010).

Importance of the In Vitro Siderophores Production by SPB

Alexander and Zuberer (1991) mention that it is important to analyze the siderophores production by rhizobacteria, not only to evaluate the bioavailable Fe to plants and to measure populations of SPB but also important to determine the amounts and if it is possible, the types of siderophores that are produced by the isolated bacteria, to understand the dynamics of siderophores. The authors recommended the CAS (chrome azurol S) medium (Neilands 1981; Sung et al. 2001; Pérez-Miranda et al. 2007) to study the ecology of this important trait in PGPR. The in vitro siderophores production can be quantified with the CAS assay solution of Schwyn and Neilands

(1987) modified by Alexander and Zuberer (1991), which provides certain advantages over CAS agar medium to detect siderophores producing rhizobacteria. These authors recommended that the employ of low-Fe liquid medium and bacteria filtrates could be tested for the presence of siderophores with the assay solution and also mention that CAS assay solution has been employed to analyze the kinetics of siderophore accumulation in vitro, where in simple experiments with batch cultures of bacteria isolates, they obtained data which confirmed the expected relationship between growth and siderophores production. The sensitivity of the assay with CAS solution enabled them to measure concentrations of siderophores directly in culture filtrates without the need to concentrate or purify the compounds.

Avilés (2016) and Laguna (2013) analyzed this important trait in *Serratia* sp. strain 6, grown in liquid cultures with the presence of heavy metals added to the medium. The rhizobacteria employed in their study was isolated by Melo et al. (2011), from the rhizosphere of *Sphaeralcea angustifolia*, a plant species that grows in a metal-contaminated soil located in Villa de la Paz in the state of San Luis Potosí, México. These authors determined the minimal inhibitory concentration (MIC) to As and Cd for 1.0, 5.0, 10, 15, and 20 mM concentrations. The authors at first sign (Fig. 1) reported that this rhizobacteria presented an increase of plant growth at lower concentrations of both metals and inhibition of it at higher concentrations, with more effect on plants by the presence of arsenic. The authors suggest that this plant growth promotion at the low concentrations of the heavy metals could be a hormesis response proposed by Calabrase and Blain (2009). Guo et al. (2010) demonstrated this effect in *Bacillus* sp. L14, grown at 10mM Cd concentration; Leedjärv et al. (2008) founded the same hormesis response in the growth of *Pseudomonas putida* KT2440 with the addition of Zn, Cd, and Pb to the medium culture at concentrations of 10, 10, and 100 mM of each metal, respectively. Avilés (2016) proposed the employ of immobilized rhizobacteria to analyze the siderophores in vitro production, with the same rhizobacteria: *Serratia* sp. strain 6.

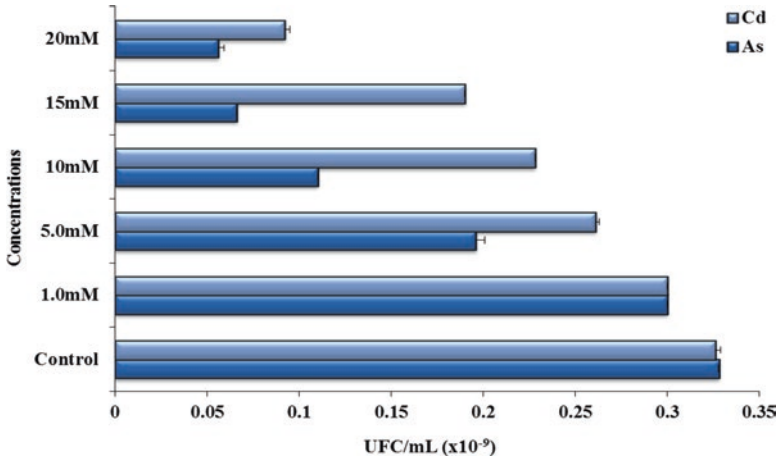

Fig. 1 Minimum inhibitory concentration (MIC) of *Serratia* sp. strain 6 to arsenic and cadmium

Entrapment of cells is a useful and effective strategy for application of bioinoculants in soil, which provides protective niche together with the provision of nutrient source (Minaxi and Saxena 2011), where the carrier is the delivery vehicle of live microorganisms to the environment, like the soil (Trevors et al. 1992), and it is the major portion (by volume or weight) of the inoculant. The materials of which the carrier is composed and the type of formulation vary. The carrier can be slurry or a powder. Bashan (1986, 1991), Smith (1992), and Trevors et al. (1992) mentioned that a good carrier should have one essential characteristic: the capacity to deliver the right number of viable cells in good physiological condition at the right time. The encapsulation of microorganisms into a polymer matrix is still experimental in the field of bacterial inoculation technology, keeping the concept to immobilized microbial cells of beneficial microorganisms into a matrix. Bashan (1986) and Bashan and Carrillo (1996) established that encapsulated bacterial formulations in agriculture have at least two important aspects: temporarily protect the encapsulated microorganisms from the soil environment and microbial competition and release them gradually for the colonization of plant roots. Alginate is the material most commonly used for encapsulation of microorganisms, and as some authors reported, the resulting inoculum was used for the immobilization of cell. Bashan (1986) noted that the preparation of beads containing bacteria is fairly easy and involves a multistep procedure. Following these recommendations, Avilés (2016) analyzed the in vitro siderophores production by no immobilized and immobilized bacterial cells of *Serratia* sp. strain 6 in sodium alginate, exposed to As and Cd, employing the methodology proposed by Sánchez (2013), and evaluated the siderophores production according to the method of Alexander and Zuberer (1991).

For the quantity of siderophore accumulation in vitro, a standard curve for the modified CAS assay was prepared according to Alexander and Zuberer (1991) and analyzing the absorbance at 630 nm. A standard solution curve was also prepared, and this relationship was linear at siderophore-selected concentrations. The author reported that in most of the treatments, the measurement of siderophores in liquid medium supplemented with each metal by the CAS concentration quantified were higher in the culture medium from the immobilized bacteria than no immobilized (Fig. 2; above); the rhizobacteria maintained siderophores production in almost all the Cd concentrations tested (Fig. 2b); even the concentration quantified in cultures from both rhizobacteria conditions tested decrease in 15 mM Cd. It is important to mention and analyze the response of this rhizobacteria exposed to As in both conditions (Fig. 2a); even the siderophores production with 1.0 mM and 5.0 mM Cd concentrations for both conditions of bacteria growth was less compared to the control treatment and higher in 5.0 mM As. The color of the complex formed by the combination of the rhizobacteria culture filtrates and CAS solution gives a particularly colored appearance related to the As concentration tested as Fig. 3 shows, where control filtrates had yellow-green color with and without the immobilization of the rhizobacteria; filtrates from no immobilized rhizobacteria had orange color in treatment with 1.0 mM As and purple color in filtrates with 5.0 and 15 mM As. Filtrates from immobilized bacteria had yellow-green color in treatment with 1.0 mM As and also purple in treatments with 5.0 and 15 mM As. These results could

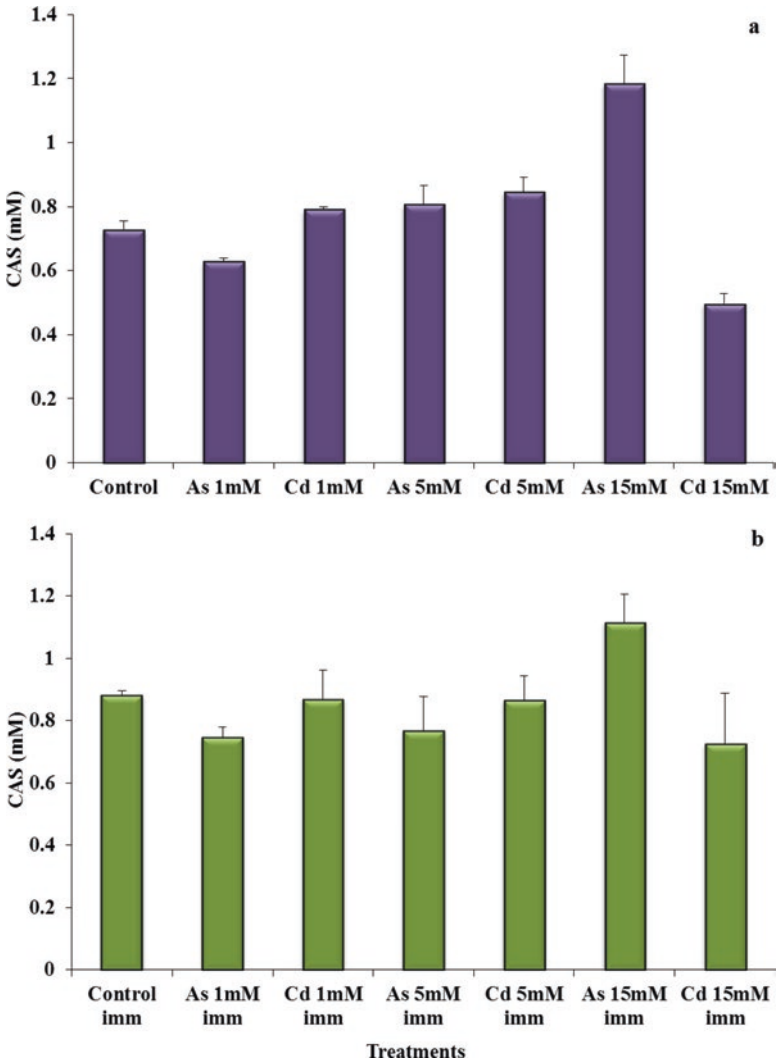

Fig. 2 Siderophores production of *Serratia* sp. strain 6 exposed to arsenic and cadmium and quantified by CAS assay

correspond to the diverse types of siderophores excreted by *Serratia* sp. strain 6 to the medium and also induced by the increase of metal concentration added to the culture medium. Color of the filtrates from culture medium of no immobilized and immobilized rhizobacteria exposed to all Cd concentrations had in all treatments a yellow-green color. Pérez-Miranda et al. (2007) reported a color change of CAS medium by siderophores excretion from blue to purple, particularly in the case of *Bacillus cereus* characterized as hydroxamate siderophores producer, with a change of color in the culture medium from greenish blue to orange and *Rhizopus oligosporus*

Fig. 3 Developed colors in culture filtrates of *Serratia* sp. strain 6 with the CAS assay by siderophores excretion

as carboxylate siderophores producer that changed the medium to a light yellow color. The authors also reported that the changed color of the medium culture by the bacteria strains to purple corresponds to catechol-type siderophores. Avilés (2016) results agree with the report by Pérez-Miranda et al. (2007) regarding to the fact that it is possible that some rhizobacteria produce more than one type of siderophores containing both catecholate and hydroxamate groups. Alexander and Zuberer (1991) reported the accumulation of siderophores in cultures of *Pseudomonas putida* B10 and *Pseudomonas fluorescens* Q6 isolates from Bermuda grass, with substantial quantities of siderophores in vitro; the highest concentrations measured with the modified CAS assay solution ranged from 130 to 230 μM. The authors mention that these quantities agree with the results obtained by Buyer et al. (1989) and Teintze and Leong (1981), from cultures of *Pseudomonas putida* B10. Many pseudomonads were reported to produce a diffusible, yellow-green fluorescent compound when grown in modified M9 medium without the addition of Fe.

Antioxidant Response in Plants as Indicator of Heavy Metal Effect

It is known that metals, essential or not, are toxic for plants when they are present in high concentrations and produce some effects like redox-active (Cu, Fe) and non-redox-active (Cd, Ni, As) by metals, and also these may catalyze, directly or indirectly, the formation of reactive oxygen species (ROS), which generate oxidative stress and cell damage (Clijsters et al. 1999; Briat 2002; Sharma and Dietz 2009). Also, ROS signaling is involved in many plant metabolic processes, including regulation and development of plant growth, responses to biotic and abiotic stresses, and cell death (Marquez and Córdoba 2009). To prevent and counteract the increase and effects of ROS, Inze and Van Montagu (1995), Arrigoni and DeTullio (2002), Tung et al. (2007), and Kelman et al. (2009) noted that tolerant plants to heavy metals possess different antioxidative mechanisms involving enzymes, such

as superoxide dismutase, catalase, ascorbate peroxidase, or small metabolites, such as ascorbic acid, phenolics, and carotenoids.

A biological antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate." This definition is clear and covers every member of the antioxidant defense team. However, unless an antioxidant prevents the generation of ROS by metal chelation or enzyme-catalyzed removal of a potential oxidant, a redox reaction still occurs and the difference is that the oxidizing species reacts with the antioxidant instead of the "substrate" (Benzie and Strain 1996). One important function of antioxidants toward free radicals, such as $^{\cdot}OH$, $O^{2\cdot}$, and $ROO^{\cdot}$, is to suppress free radical-mediated oxidation by inhibiting the formation of free radicals and/or by scavenging radicals. The formation of free radicals may be inhibited by reducing hydroperoxides and hydrogen peroxide and by sequestering metal ions (Niki 2002) through complexation/chelation reactions. Radical scavenging action is dependent on both reactivity and concentration of the antioxidant. In a multiphase medium (such as an emulsion), the localization of the antioxidant at the interphases may be important (Apak et al. 2007). The antioxidant capacity assays are classified as electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays (Huang et al. 2005; Prior et al. 2005), where most of nonenzymatic antioxidant activity (e.g., scavenging of free radicals, inhibition of lipid peroxidation, etc.) is mediated by redox reactions (Pulido et al. 2000). In addition to these two basic classes considering mechanism, reactive oxygen species (ROS) scavenging assays could also be considered. In most ET-based assays, the antioxidant action is simulated with a suitable redox-potential probe, and the antioxidants react with a fluorescent or colored probe (oxidizing agent) instead of peroxyl radicals. Spectrophotometric ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change (either an increase or decrease of absorbance at a given wavelength) is correlated to the concentration of antioxidants in the sample. In the ferric-reducing antioxidant power (FRAP), there is an increase in absorbance at a prespecified wavelength as the antioxidant reacts with the chromogenic reagent, where the lower valencies of iron (Fe(II)) form charge-transfer complexes with the ligands. Although the reducing capacity of a sample is not directly related to its radical scavenging capability, it is a very important parameter of antioxidants (Apak et al. 2007). Thus, it has been employed a simple and quite method to determinate the total antioxidant capacity of homogenates, called "ferric-reducing ability of plasma" (FRAP) by Benzie and Strain (1996), and it is known as "FRAP assay," it depends upon the reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH. (Fe(II)-TPTZ) has an intensive blue color and can be monitored at 593 nm (Benzie and Strain 1996). Although this method was elaborated for human plasma, this assay is applicable for investigation of fresh plant samples and herbs (Szöllösi and Varga 2002; Szöllösi et al. 2009; Gohari et al. 2011). The next two sections describe the use of this assay applied to two particularly "plant-PGPR bioassays."

Plant Antioxidant Response by FRAP Assay: Two Important Studies on Plants Inoculated with SPB

FRAP assay applied to "plant cell culture-SPB bioassay" exposed to cadmium.

Frommel et al. (1991), Yang et al. (1991), and Nowak (1998) established that the associations between beneficial microbes and cultured plant cells, tissues, or organs represent a unique method of enhancing induction of regenerants and the adaptation of tissue-cultured propagules to environmental stresses. It is known that PGPR stimulate plant growth by mechanisms that include the production of siderophores (Glick 1995; Tang et al. 1995), but little is known about the interactions between bacteria and the plant cells or tissues within the microenvironment of a tissue culture system (Murthy et al. 1999). Herman (1996) named the in vitro responses as "biotization" defined as a metabolic response of in vitro grown plant material to a microbial inoculant(s), leading to the developmental and physiological changes enhancing biotic and abiotic stress resistance. Thus, in vitro plant bioassays between callus and cell cultures inoculated with PGPR are considered in this category. Pillay and Nowak (1997) mention that tissue cultures are simple model systems designed to test single factors and their combinations and this kind of plant cultures promotes the establishment of stable associations between plants and beneficial organisms in vitro and ex vitro that help to understand mechanisms of signal recognition and transduction in plant-microbial associations under different environments, with the establishment and management of plant cell cultures as bioassays (Nowak 1998). Toledo (2012) analyzed the antioxidant response in cell cultures of *Epithelantha micromeris* inoculated with rhizobacteria *Serratia* sp. strain 6 exposed to Cd, by the FRAP method. This bioassay analyzed the effect of the reported SPB that has been known to produce high concentration of siderophores in in vitro conditions and exposed to this metal. This author reported that the response of *E. micromeris* callus culture inoculated with the rhizobacteria showed the highest antioxidant activity (Fig. 4; above), compared to all the treatments of the bioassay, and there was an evident effect of damage in inoculated callus cultures with a diminished cell growth and cell viability also with necrosed callus (Fig. 5; above). Comparing only the callus cultures exposed to cadmium, they showed almost the same higher antioxidant response than control cultures, particularly response of callus cultures inoculated and exposed to the heavy metal with the highest activity at 0.5 mM of Cd concentration and the lowest antioxidant activity with 1.0 mM Cd + rhizobacteria.

FRAP assay applied to analyze the response of plants inoculated with PGPR exposed to cadmium.

Sánchez (2013) analyzed the antioxidant response of *Axonopus affinis* plantlets exposed to Cd concentrations: 0.5, 1.0, and 1.5 mM, inoculated with *Serratia* sp. strain 6, no immobilized and immobilized in alginate beds, also by FRAP analysis. Sánchez et al. (2014) founded that the presence of Cd *A. affinis* caused an inhibitory effect on plantlets growth, but the gain of plant biomass with the no immobilized

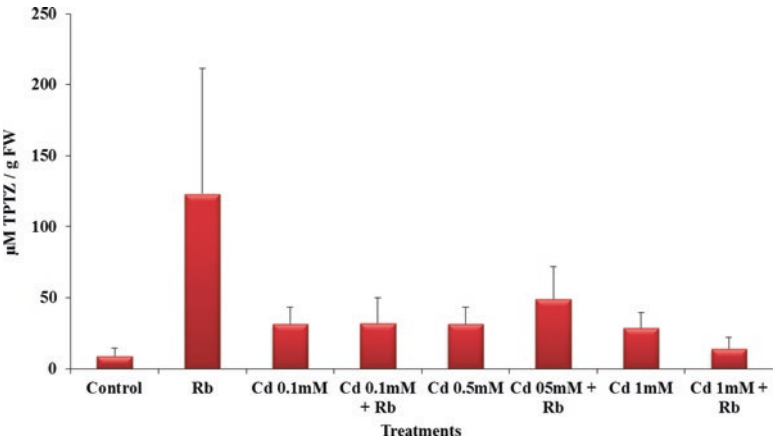

Fig. 4 Antioxidant activity of *Epithelantha micromeris* callus culture inoculated with *Serratia* sp. strain 6, exposed to cadmium

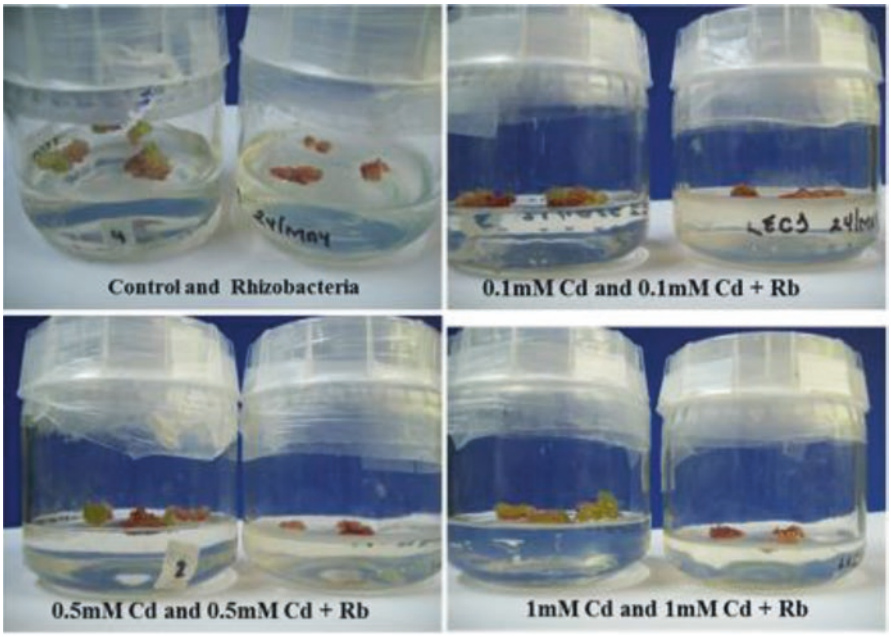

Fig. 5 Appearance of *Epithelantha micromeris* callus culture inoculated with *Serratia* sp. strain 6, exposed to cadmium

rhizobacteria, *Serratia* sp. strain 6, were mainly in the experiments of plantlets exposed to Cd, with a tolerance index of 1.29, 1.23, and 1.23, against 0.5, 1.0, and 1.5 mM Cd concentrations, respectively, and the fresh biomass increased from 23% to 29% than plantlets without the immobilized inoculum and only with the heavy metal.

The authors reported that the rhizobacteria tested not only maintained its survival immobilized in alginate beds; this condition also proved microenvironmental conditions that allowed the bacterial growth. Even the results reported by Sánchez et al. (2014) regarding to the effect on the maintenance of the *Axonopus affinis* plantlets against to Cd exposition where the growth of them were short than the control plantlets; the plant's protection was maintained by the presence of the immobilized rhizobacteria. The measurement of the antioxidant activity support this evidence, because the activity quantified by FRAP assay in root extracts of *A. affinis* plantlets was the highest obtained in plantlets inoculated with immobilized SPB, mainly in control plants, followed by 0.5mM and 1.0mM of Cd, finally with a less antioxidant activity at the highest Cd concentration (1.5 mM) (Fig. 6; above). Toledo (2012) suggests that the induction of antioxidant response was an evidence of some protecting mechanism that plants generate against the rhizobacteria and also by the presence of the heavy metal. Söllözy et al. (2009) reported that Cd is known as nonessential heavy metal and cause oxidative stress in plants; the authors evaluated the antioxidative responses in early stages of ontogenesis in *Brassica juncea* seeds exposed to Cd stress (0, 50,100 and 200 mg/L Cd), employing the FRAP assay, with respect to both time and concentration dependence. They demonstrated that *B. juncea* seeds react differently to Cd in the early stage of ontogenesis than in older plants as shown in former studies and particularly, the FRAP assay showed that concentration dependent increase during 24 h, but it decreased later. Authors finally founded that this assay is a recommendable parameter to assess the antioxidant capacity of heavy metal stressed in plants, as Toledo (2012) and Sánchez (2013) agree with these authors.

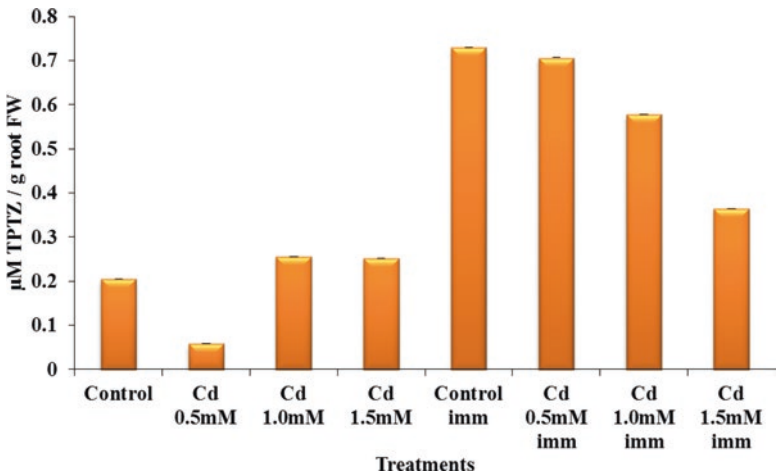

Fig. 6 Antioxidant activity of *Axonopus affinis* roots inoculated with no immobilized and immobilized *Serratia* sp. strain 6, exposed to cadmium

Conclusions

Finally, this chapter tries to give an introduction to the management of "plant- PGPR bioassays" that involves not only the characterization of rhizobacteria isolated from soils contaminated with heavy metals, through conventional methods employed in microbiology; it is also important to analyze their plant growth-promoting attributes like siderophores production and the plant's response when there are bioassays that established a relationship between plants and bioinoculants, given this systems and opportunity to applied some techniques regarding the physiology of plant response. One of those analyses consider the effect of heavy metals in plant's growth by the measurement of the antioxidant activity by FRAP assay, considering the celerity and facility of this assay; it could be an adequate parameter for the study of heavy metal-stressed plants.

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Exploiting Nitrogen-Fixing Rhizobial Symbionts Genetic Resources for Improving Phytoremediation of Contaminated Soils

Alice Checcucci, Marco Bazzicalupo, and Alessio Mengoni

Abstract Rhizobia are one of the most relevant components of the plant-associated microbiota. They are found both in soil and associated as commensals or symbionts with several plant taxa. In particular, with leguminous plants, they establish a symbiotic association, which allow the bacteria to express the enzyme nitrogenase responsible for the reduction of atmospheric dinitrogen. Consequently, rhizobia allow host plants to colonize marginal lands and nitrogen-deficient soils, for instance, contaminated soils. The use of legume-rhizobial symbiosis for phytoremediation would allow increasing plant coverage (then phytostabilization) of contaminated areas, without the need of expensive nitrogen fertilization of the soil. Moreover, among host legumes, both pioneer plants (of for instance degraded lands) and crops (as alfalfa) are present, which allow an easy implementation of agronomical practices. Finally, the large genomic and phenotypic diversity of rhizobia allows the selection of elite strains resistant to harsh soil conditions and the creation of potentially new strains with the desired features for assisting legume-based phytoremediation.

Keywords Rhizobia • Legumes • *Sinorhizobium meliloti* • *Mesorhizobium metallidurans* • Pangenome

The Diversity of Rhizobia

Rhizobia constitute a fraction of bacteria inhabiting on the root of plants (rhizobacteria), which have effect in promoting growth and alleviating the stress of the plant (plant growth-promoting rhizobacteria, PGPB) (Dimkpa et al. 2009; Lugtenberg and Kamilova 2009). The PGPB may supply the host with a higher amount of nitrogen, synthesize several different phytohormones which can

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enhance various stages of plant growth, synthesize siderophores which can solubilize and sequester iron from the soil providing it to plant cells, and have mechanisms for the solubilization of phosphates, which then become more readily available (Lugtenberg and Kamilova 2009). Rhizobia include all those rhizobacteria which form endosymbiotic nitrogen-fixing association with legumes. Rhizobia are a paraphyletic group of soil-inhabiting bacteria that fall into two classes of the *Proteobacteria*—the alpha- and beta-proteobacteria with the potential for establishing symbiotic relationships with many legume plants. The first known species of rhizobia was *Rhizobium leguminosarum* and, with some subsequent known species, was initially placed in *Rhizobium* genus (van Rhijn and Vanderleyden 1995). Then, more advanced methods allow a reclassification into distinct genera (*Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium/Ensifer*) (Table 1).

The symbiotic relationship allows rhizobia to live inside the roots of the legume plant, consuming carbohydrates from the host and providing the legume with nitrogen that the bacteria convert into plant-usable form. Most of the symbiotic associations that involve legumes and rhizobia are characterized by species specificity, mainly due to the specific signal molecules present in the root exudates of legume plants that allow partner recognition and symbiosis development. In particular, rhizobia are able to sense specific secondary metabolites called flavonoids secreted by the roots of their host legume plant. The perception of the flavonoid signal modifies the rhizobial behavior (Spini et al. 2015) and initiates a cascade of events which then lead to the entry of rhizobia inside the plant root tissue and establish the symbiotic interaction (Gibson et al. 2008). In fact, flavonoids trigger the secretion of Nod factors by the rhizobia that are recognized by transmembrane receptors on root hair cells of specific legume: different strains of rhizobia produce different Nod factors, and different legumes produce receptors of different specificity. Following the root bacterial colonization, the root cortical cells begin to divide generating the nodules where the rhizobia differentiate into nitrogen-fixing bacteroids. In effective nodules, the bacteria convert atmospheric nitrogen (N_2) into ammonia (NH_3), which then enters into plant biosynthetic pathways via glutamine. This allows plants to grow better and thrive especially in nitrogen-deficient soils. This biological process is possible, thanks to a multi-enzymatic complex called *nitrogenase*, active inside bacteroids. In return, the rhizobia can benefit of the nutrients that the plant provides and are protected inside the nodule structure. In ineffective nodules, no or low nitrogen level is fixed but the rhizobia are still supplied with nutrients, and in this situation, the bacteria could be considered parasitic (Denison and Kiers 2004). To control the efficiency of the symbiotic partnership allowing the improvement of their growth, the plants have evolved mechanisms which favor nodules colonized by beneficial rhizobia (Denison 2000).

Table 1 List of representative genera and species of the most known nitrogen-fixing PGPB and respective host legumes

Root-nodule bacteria		
Genus	Species	Host legume (genus)
Azorhizobium	*caulinodans*	*Sesbania*
Bradyrhizobium	*elkanii*	*Glycine*
	japonicum	*Glycine*
	liaoningense	*Glycine*
	yuanmingense	*Lespedeza*
Mesorhizobium	*amorphae*	*Amorpha*
	chacoense	*Prosopis*
	ciceri	*Cicer*
	huakuii	*Astragalus*
	loti	*Lotus*
	mediterraneum	*Cicer*
	plurifarium	*Acacia*, *Leucaena*
	tianshanense	*Glycyrrhiza*, *Sophora*
Rhizobium	*etli*	*Phaseolus*
	galegae	*Galega*, *Leucaena*
	gallicum	*Phaseolus*, *Dalea*, *Onobrychis*, *Leucaena*
	giardinii	*Phaseolus*
	hainanense	*Stylosanthes*, *Centrosema*
	huautlense	*Sesbania*
	indigoferae	*Indigofera*
	leguminosarum bv trifolii	*Trifolium*
	leguminosarum bv viciae	*Pisum*, *Vicia*, *Lathyrus*, *Lens*
	leguminosarum bv phaseoli	*Phaseolus*
	loessense	*Astragalus*
	mongolense	*Medicago*, *Phaseolus*
	sullae	*Hedysarum*
	tropici	*Phaseolus*, *Leucaena*, *Dalea*, *Macroptilium*
	undicola	*Neptunia*
Sinorhizobium	*abri*	*Abrus*
	americanus	*Acacia*
	fredii	*Glycine*
	indiaense	*Sesbania*
	kummerowiae	*Kummerowia*
	medicae	*Medicago*
	meliloti	*Melilotus*, *Medicago*, *Trigonella*
	morelense	*Leucaena*
	saheli	*Sesbania*
	sahalense	*Sesbania*
	terangae	*Sesbania*, *Acacia*
	xinjiangense	*Glycine*

Host Legumes Relevant for Phytoremediation

Plant-associated bacteria have been recognized as one of the most relevant issues in improving phytoremediation yields (Glick 2003; Afzal et al. 2014). In the last years, the association between leguminous plants and rhizobia has stirred the attention of researchers involved in the restoration of degraded lands and phytoremediation (Zahran 1999; Sheaffer and Seguin 2003; Wang et al. 2005; Hao et al. 2014; Teng et al. 2015). In particular, the possibility offered to cultivate legumes on marginal and nutrient-poor soils, thanks to the intimate association with PGPB and in particular with nitrogen-fixing symbiotic partners, has been seen as an opportunity to increase phytoremediation yields, while reducing its costs (Safronova et al. 2011; Hao et al. 2014) (Table 2). Among the most relevant legumes tried for phytoremediation are those belonging to genera *Lupinus* and *Sesbania*, which have been used in the remediation of mine deposits. Interestingly, for the forage legume *M. sativa*, application trials for decontamination from PCB have been carried out, in symbiosis with *S. meliloti* strains, possibly able to degrade such recalcitrant compounds. In some cases, interesting results were obtained when the symbiotic partnership between the host legume and rhizobial symbiont included also arbuscular mycorrhizal fungi, which allowed to reduce the toxic effect of the contaminant (Garg and Bhandari 2012).

Use of Rhizobia in Phytoremediation

Tough potentially tolerant to pollutants, legumes relevant for phytoremediation can be limited in their growth. Symbiotic rhizobia, providing fixed nitrogen, can allow to promote host legume growth in marginal and degraded soils, as usually are those claimed for restoration. Several efforts have been carried out both on improvement of plant traits to cope with unfavorable conditions (Dwivedi et al. 2010, 2015). Since the efficiency of the process of N_2 fixation is related to both the physiological state of the host plant and to rhizobial partner (Zahran 1999), efforts have been made to find symbiotic rhizobia which can tolerate harsh conditions. Rhizobial strains in natural environments showing tolerance to various stressors (e.g., salinity, heavy metals, pH, etc.) have been found and tested as inocula on target plant (Hungria et al. 1993; Zahran 2001; Provorov and Tikhonovich 2003; Roumiantseva 2009; Elboutahiri et al. 2010; Trabelsi et al. 2010; Boukhatem et al. 2012).

Concerning salt stress, this inhibits the initial steps of symbiosis (Coba de la Peña et al. 2003). Several studies have been performed looking at salt-tolerant rhizobia, especially in subarid regions, where conditions may likely have contributed in selecting rhizobial strains with the ability to cope with osmotic stress (Mnasri et al. 2007; Trabelsi et al. 2010) (Fig. 1). These studies showed the presence of rhizobia tolerant to high NaCl concentrations (up to 1.0 M), which can be used as inocula for crop production in salinized soils. Often linked to salt stress are also

Table 2 List of representative host legumes with their rhizobial symbionts extensively used in phytoremediation of major metals/metalloids

Host legume	Use in phytoremediation	Rhizobial symbiont	Reference
Anthyllis vulneraria	Heavy metals	*Mesorhizobium metallidurans*	Vidal et al. (2009)
Cajanus cajan	Cd	*Rhizobium sp.*	Garg and Bhandari (2012)
Cicer arietinum	Cr	*Mesorhizobium* sp.	Wani et al. (2008a)
Cicer arietinum	Zn, Cu, Cr, Cd, Fe	*Rhizobium* sp.	Gupta et al. (2004)
Glycine max	Cd	*Pseudomonas putida*, *Pseudomonas monteilli*	Rani et al. (2009)
Lablab purpureus	Spent engine oil, Cu	*Rhizobium sp*. *Bradyrhizobium lablabi*	Younis (2007), Ismail et al. (2014)
Lens culinaris	Zn	*Rhizobium leguminosarum*	Wani et al. (2008c)
Leucaena	Cd, Zn	*Rhizobium* sp.	Saraswat and Rai (2011)
Lolium multiflorum	Cd, Zn, and Ni	*Bradyrhizobium* sp.	Wani et al. (2007), Guo and Chi (2014)
Lotus edulis	Cd, Pb, and Zn	*Mesorhizobium loti*	Safronova et al. (2010)
Lotus ornithopodioides	Cd, Pb, and Zn	*Mesorhizobium loti*	Safronova et al. (2010)
Lupinus albus	Cd, Cu, Pb, and Zn	*Bradyrhizobium* sp., *Ochrobactrum* sp.	Pajuelo et al. (2008)
Lupinus luteus	Cd, Cu, Pb, and Zn	*Bradyrhizobium* sp., *Ochrobactrum* sp.	Pajuelo et al. (2008)
Medicago ciliaris	Cd, Pb, and Zn	*Sinorhizobium* sp.	Safronova et al. 2010
Medicago sativa	As	*Sinorhizobium* sp.	Pajuelo et al. (2008)
Medicago sativa	Polychlorinated biphenyls (PCB)	*S. meliloti*	Mehmannavaza et al. (2002)
Mimosa pudica	Pb, Cu, and Cd	*Cupraividus taiwanensis*	Chen et al. (2008)
Pisum sativum	Ni and Zn	*Rhizobium* sp.	Wani et al. (2008b)
Pisum sativum	Cd	*Rhizobium leguminosarum*	Engqvist et al. (2006)
Prosopis juliflora	Fe, Mn, Cu, Zn, Cr, and Pb	*Rhizobium* sp.	Rai et al. (2004), Sinha et al. (2005)
Robinia pseudoacacia	Zn	*Agrobacterium tumefaciens*	Smith and Giller (1992)
Robinia pseudoacacia	Cu	*Mesorhizobium amorphae*	Hao et al. (2015)
Sesbania cannabina	Pb and Zn	*Azorhizobium caulinodans*	Chan et al. (2003)
Sesbania grandiflora	Pb and Zn	*Azorhizobium caulinodans*	Chan et al. (2003)

(continued)

Table 2 (continued)

Host legume	Use in phytoremediation	Rhizobial symbiont	Reference
Sesbania rostrata	Pb and Zn	*Azorhizobium caulinodans*	Chan et al. (2003), Shuguang et al. (2009)
Sesbania sesban	Pb and Zn	*Azorhizobium caulinodans*	Chan et al. (2003)
Vigna mungo	Cd	*Pseudomonas aeruginosa strains*	Ganesan (2008)
Vigna radiata	Cd, Zn, and Ni	*Bradyrhizobium* sp.	Wani et al. (2007), Guo and Chi (2014)
Vigna radiata	Cr	*Ochrobactrum intermedium*	Faisal and Hasnain (2006)

Host legume, rhizobial symbiont and the related use in phytoremediation are reported

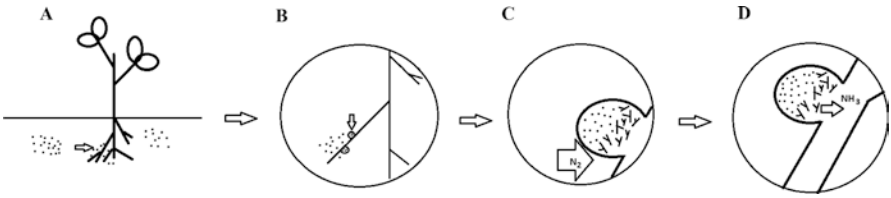

Fig. 1 Steps in the symbiotic interaction. (**a**) The symbiotic process starts with the approaching of the rhizobia present in the soil to the leguminous plant roots. (**b**) The rhizobia are attracted by specific chemoattractants, the flavonoids, released by legume roots. Then, the first step in developing the symbiosis is their consequent attachment to the surface of the plant root hair and the formation of the nodules. The nodules hold the bacterial symbiont providing specialized conditions necessary for nitrogen fixation. (**c**) Inside the nodules, part of the rhizobial population differentiate into nitrogen-fixing bacteroids, (**d**) the only form capable to transform molecular nitrogen in ammonia, thanks to the activity of the bacterial enzyme nitrogenase. The produced ammonia is then released within the plant

drought and temperature stresses (Alexandre and Oliveira 2013). In particular, desiccation is a critical step if proper inocula have to be prepared for spraying as biofertilizers as well as for long-term survival of inoculated rhizobia in arid soils. Adaptation to desiccation has been shown for strains which experienced salt stress before desiccation, but the molecular mechanisms of desiccation survival, which may involve between genes and exopolysaccharide production, are still not fully understood (Vriezen et al. 2007).

Related to the phytoremediation, highly relevant is the contamination by heavy metals. In the last few years, there has been an increasing interest in rhizobial symbionts from wild legumes growing in soil rich in trace metals (as nickel, copper, etc.). In particular, the flora of serpentine soils has been deeply investigated. Serpentine soils are distributed all over the world and originated from an array of

ultramafic rock types characterized by high levels of nickel, cobalt, and chromium; low levels of N, P, K, and Ca; and a high Mg/Ca ratio (Brooks 1987). The flora of serpentine soil contain several endemics, including many legume species (Brady et al. 2005). The bacteria-inhabiting serpentine soil and endophytes of serpentine plants have attracted the attention of many investigators ((Mengoni et al. 2010) and references therein). In particular, several bradyrhizobial strains with tolerance up to 15 mM Ni(II) have been isolated from the endemic legume *Serianthes calycina* growing in New Caledonia serpentine soils (Chaintreuil et al. 2007). Moreover, metal-tolerant rhizobia have been isolated also from legume species growing on mine deposits. A new highly tolerant to Zn rhizobial species (*Mesorhizobium metallidurans*) has been identified as symbiont of *Anthyllis vulneraria*, a legume species growing in zinc mine deposits (Vidal et al. 2009). Interestingly, the association between *A. vulneraria* and *M. metallidurans* has been demonstrated effective for the growth of the host plant in soil contaminated by Zn, Pb, and Cd (Mahieu et al. 2011). *A. vulneraria*/*M. metallidurans* association was able to promote *A. vulneraria* growth on strongly contaminated soil (16% Zn, 9.2% Pb, and 1.3% Cd) and to obtain fix up to 80% of its total nitrogen from atmospheric N_2. In conclusion, there are several rhizobial strains which have been isolated and characterized for tolerance to many environmental stresses and proved to be effective in improving legume growth under unfavorable conditions, for instance, as pioneer species for restoration ecology in marginal lands (Wang et al. 2005).

Genomics as a Way for Improving Rhizobial Performances

Rhizobial tolerance to pollutants is related to the presence of specific genes or metabolic pathways able to degrade the pollutant (e.g., organic pollutants) or tolerate the toxic effects (e.g., trace metals). Consequently, to improve phytoremediation yields of rhizobial-legume partnerships, selection of tolerant elite rhizobial strains (Dwivedi et al. 2015) is as crucial as that of tolerant plant germplasm. In the last years, thanks to the efforts of the scientific communities in the sequencing of the genomes of a plethora rhizobacteria and rhizobia (Reeve et al. 2015), a lot of information is available which can be used to identify, by genome analysis, useful strains or gene traits. In particular, comparative genome analysis has helped to identify additional genetic determinants for plant-bacteria interaction relevant phenotypes (Mengoni et al. 2014; Galardini et al. 2015). In fact, bacterial genomes are composed by two main parts, a common gene set (shared by all members of a given taxon) named core genome and a dispensable gene set (dispensable genome) which is specific of some members only (Tettelin et al. 2005, 2008). Core genome includes genes, which confer the taxon identity (e.g., basic cellular machinery, housekeeping metabolism, etc.), while the dispensable genome fraction is related to genes, which confer strain-specific features, such as environmental adaptation. The modular feature of the bacterial pangenome (as sum of core and dispensable genome fractions) allows bacterial strains to easily adapt to changing environmental conditions by

integrating genes (and replicons, such as accessory plasmids) into the core genome machinery (Young et al. 2006). It is consequently plausible that genes related to improving plant-bacteria relationships in terms of plant productivity and especially plant tolerance to abiotic stress can be found in the dispensable genome fraction. The analysis of rhizobial pangenomes (viz., the comparative genome analysis of conspecific strains) can then disclose the genetic basis of their different tolerance to pollutants allowing to potentially designing new strains, which may combine the genetic relevant features of different individual strains (e.g., for both high nitrogen fixation rate and heavy metal tolerance). The modular nature (di Cenzo et al. 2014) and redundant feature (González et al. 2006; di Cenzo and Finan 2015) of many rhizobial genomes help in defining nearly independent genetic cassettes (even containing several genes), which can be delivered through the large plasmids typical of many rhizobial strains in a sort of "pangenome-assisted strain improvement" or in analogy with plant breeders.

For the alfalfa symbiont *Sinorhizobium meliloti*, the pangenome analysis approach has allowed to identify genes related to the different cooperative behavior of strains (Galardini et al. 2011, 2013), which could be used for improving the symbiotic performance of strains. Moreover, the same analysis has helped to identify and characterize a core genome determinant (Sma1641), encoding an ortholog of *nreB* gene involved in Ni^+ efflux (Grass et al. 2001). This gene has been shown to have pleiotropic effects also toward the tolerance to Cu^+ (Pini et al. 2013) and in particular, its deletion, induced a higher plant growth under in vitro culture conditions (Pini et al. 2013), indicating that metal homeostasis, symbiosis, and plant growth promotion can be tightly linked. A copper-tolerant *S. meliloti* strain (CCNWSX0020) has been isolated from mine tailing, and its genome has been completely sequenced (Li et al. 2012) and shown to positively influence the growth of the host legume *Medicago lupulina* in copper-contaminated soils (Li et al. 2014). Functional genomics analyses of *S. meliloti* CCNWSX0020 revealed that genes involved in copper homeostasis (copper chelation and export to the bacterial periplasm) were responsible for the copper tolerance (Li et al. 2014). Interestingly, those genes are widespread in *Rhizobiaceae* and several genomes of strains of *S. meliloti*, as well as *R. leguminosarum* contain such genes (Fig. 2), indicating that common cellular mechanisms (then being part of the core genome) can be used also, in adjunct to dispensable genes, for biotechnological improvement of elite strains. A similar investigation carried out on a copper-tolerant strain of *Mesorhizobium amorphae* nodulating *Robinia pseudoacacia* indicated that a P-type ATPase, possibly involved in the efflux of cytoplasmic copper, can be one of the determinants of the copper-tolerant phenotype (Hao et al. 2015). However, the same authors found that other genes, with unknown function, are related to copper tolerance in such strain. This latter evidence indicates that our understanding of heavy metal tolerance in bacteria is still far from being complete.

The genomic features of rhizobia in terms of modular and functionally redundant genomes and the large pangenomes they harbor allow the search for both elite strains and elite genes to be used in phytoremediation. In particular, elite genes (for instance, the abovementioned Sma1641 from *S. meliloti*) could be transferred from tolerant to sensitive strains to possibly combine tolerant features with strain

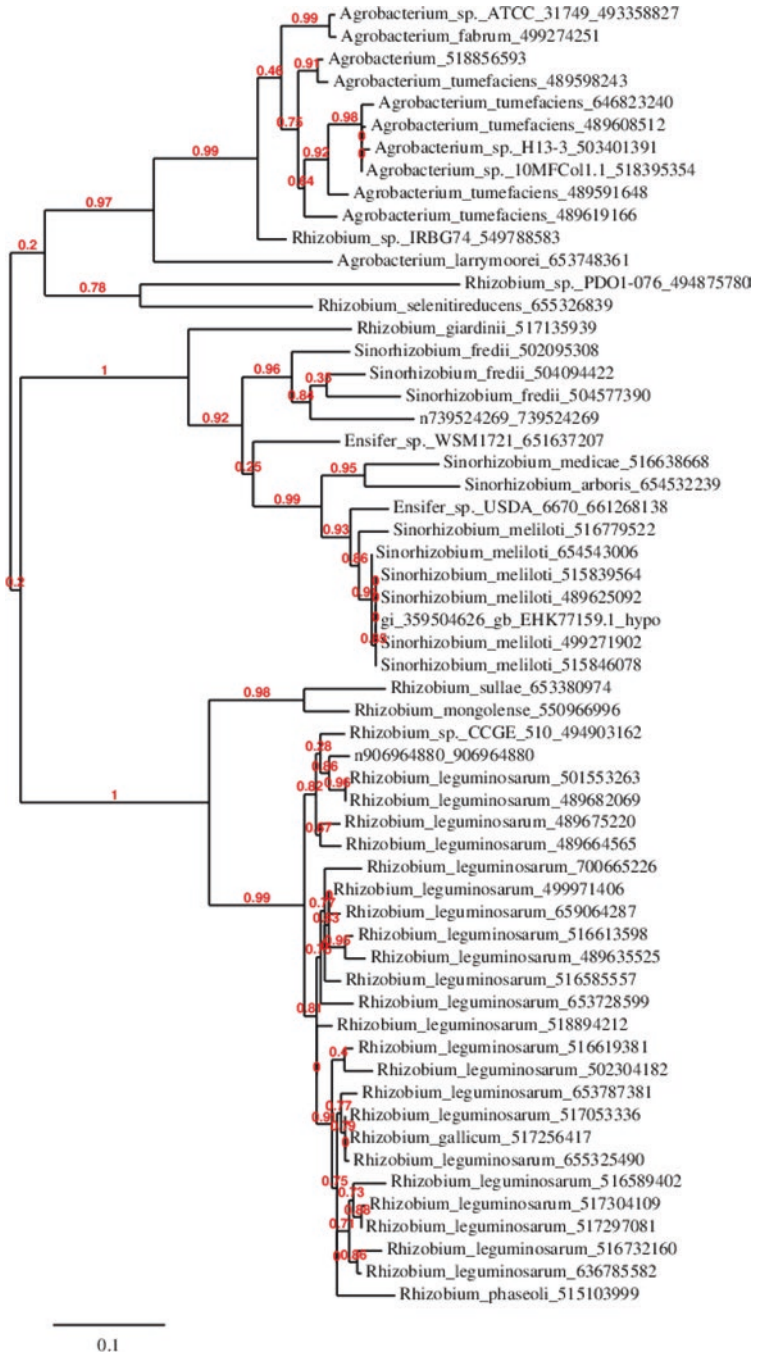

Fig. 2 Widespread occurrence of genes for metal homeostasis in rhizobia. Maximum likelihood phylogenetic tree of the orthologs of the protein SM0020_14779 from *S. meliloti* CCNWSX0020. A number at nodes indicate bootstrap values. Arrow indicates the protein SM0020_14779 (gil359504626|gb|EHK77159.1|)

competitiveness and other important features for the symbiotic interaction (Dwivedi et al. 2015). The perspective of rhizobial synthetic biology has granted credits and genome sequencing of relevant strains that have been promoted by the US Department of Energy Joint Genome Institute (Biondi et al. 2009).

Conclusions

This chapter presented here a brief summary of the use of the legume-rhizobium symbiosis for increasing phytoremediation yields and application. There exist a number of investigations, mainly done under laboratory and controlled conditions, which indicate that rhizobia with the ability to tolerate toxic compound and/or to degrade contaminants are present in nature. These bacteria can be found as symbionts of legumes thriving on contaminated soils, by either geological features (e.g., serpentine outcrops) or anthropic causes (e.g., mine deposits). The study of their physiology and of their genome may enable to use them (or their genes) as partner of the same host legumes or of legumes for which better growth and agronomic potential are present. In this respect, the use of legume crops in phytoremediation could allow the use of already settled agronomic practices and then reducing the cost and time of the translation of lab-based evidences to the field.

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Environmental Bioremediation by Biosorption and Bioaccumulation: Principles and Applications

Raluca-Maria Hlihor, Laura-Carmen Apostol, and Maria Gavrilescu

Abstract The historical and everyday environmental pollution generates numerous impacts on the environmental quality and human health. Anthropogenic activities, in particular the industrial and agricultural systems, release in the environment large quantities of pollutants of inorganic and organic nature, which can be transported, immobilized, degraded, or bioaccumulated in the environmental compartments (water, air, soil) and in the ecological components (plants, animals). From there, they are easily available to humans through the food chain. This is why numerous efforts have been invested for the reduction and/or removal of pollution from the environment, together with preventive actions. Diverse physico-chemical and biological options and processes were applied to remove and/or transform different kind of pollutants (heavy metals, dyes, persistent organic pollutants) from the environment. Physico-chemical processes including chemical precipitation, ion exchange, adsorption, membrane separation, coagulation, flocculation, flotation, electrochemical technologies, etc. were applied for the mobilization, immobilization, or degradation of various pollutants. However, some of these processes, although with fast results in some cases, proved to be less efficient and more expensive than bioremediation-based processes. The biological applications, considered

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as low-cost alternatives, gained more and more the interest of scientists and stakeholders for ensuring a sustainable environmental remediation. This work discusses some current aspects and perspectives in the environmental bioremediation by biosorption and bioaccumulation, which exploit the potential of non-living and living biomass to immobilize and biodegrade persistent contaminants. A focus on past and present studies addressing the bioremediation of both inorganic and organic pollutants, their bioavailability in the environment, mechanisms, and impacts of environmental factors on the removal efficiency by biosorption and bioaccumulation was considered. Various biosorbents for the removal of these contaminants, such as agricultural or industrial wastes, microbial-based biomass (bacteria, fungi), algae, and plant-based biomass, are considered from impact, tolerance to persistent pollutants, effectiveness, and cost perspectives. This approach contributes to a better understanding of biological processes, so as to overcome the technical barriers in the application of biosorption and bioaccumulation processes that delay the commercialization and to increase their scale-up potential for practical applications.

Keywords Inorganic pollutants • Organic pollutants • Biosorbents • Bioaccumulators • Environmental impacts

Introduction

The increase of environmental contamination is a major consequence of industrial development, shifting in people lifestyle, and consumer demands. Furthermore, it constitutes a continuous challenge for policy and decision-making processes as well as for society as a whole. In particular, heavy metal pollution is usually the result of the activity of a number of industries such as pigments, mining, electroplating, plastics manufacturing, and metal plating facilities. Xenobiotic organic compounds originating from industries in the form of chemicals, personal care products, pharmaceutical, or food products represent a complex mixture of pollutants found in effluents that must be submitted to sewage treatment. In this context, attention has to be paid to the health hazards generated by the existence of heavy metals in the environment, because of their accumulation in living tissue through the food chain. On the other side, xenobiotic organic compounds strive to oppose to biodegradation (the natural attenuation that results in degradation of the molecule, implicitly the mass or concentration reduction), leading to persistence and also to bioaccumulation for extended periods. Environmental challenges nowadays have encouraged scientists to search for new approaches for wastewater treatment since the established physico-chemical methods for these two groups of pollutants removal do not show the best results. One of the most promising options is the bioremediation technology emphasized in many studies through biosorption and bioaccumulation. These two methods are attractive due to their low cost, biomass availability, and high efficiency (Apostol et al. 2015a, b; Gavrilescu 2004, 2010, 2014; Hlihor et al. 2013, 2014, 2015).

Considering the above mentioned concerns, it is opportune the focus on different aspects of biosorption and bioaccumulation processes in which different categories of pollutants are involved, for a better understanding of their behavior in living and/or inactive biomasses. This information, based on knowledge gained from literature, laboratory, and pilot experimental data, is useful in generating the scientific and technical support for large-scale application in environmental bioremediation by biosorption and bioaccumulation. This chapter focuses on inorganic and organic pollutants, their bioavailability in the environmental compartments and biosorbents and bioaccumulators of different categories; it also evaluates factors affecting environmental bioremediation by biosorption and bioaccumulation and kinetics and equilibrium, highlights major mechanisms underlying previous processes, and lists major applications and future perspectives.

Environmental Pollutants Susceptible for Bioremediation

Inorganic Pollutants: Heavy Metals

Inorganic pollutants such as heavy metals were considered essential in the industrial development due to their critical role in technological advances. Contrary to many organic pollutants, which eventually degrade to carbon dioxide and water, heavy metals are accumulated in the environment, leading to several environmental and health effects (Hlihor et al. 2015, 2016). New environmental requests and their imminent threats to various environmental compartments have focused the attention of many researches on more efficient methods of monitoring and on rethinking the remediation strategies (Zuykov et al. 2013). Since metals are transported easily from one environmental compartment to another, and their discharge is higher than before, the remediation task seems to be more difficult than ever for environmental engineers and researchers focusing on removing heavy metals from the environment (Wang and Chen 2009; Kotrba 2011; Hlihor et al. 2014; Iriel et al. 2015). It is a well-established fact that absorption of metals occurs in the microbial world and in plants. As they progress through the food chain, metals tend to get concentrated in microorganisms and plants, affecting in the end human health while leading to toxicity symptoms, disorders in the cellular functions, long-term debilitating disabilities, and eventually death (Mudhoo and Mohee 2012; Tavares and Figueiredo 2012).

According to IUPAC, heavy metals are defined as "metals with a density larger than 4–5 g cm^{-3}" (Duffus 2002). In their natural state, metals are part of the Earth's crust, being essential elements for maintaining the metabolism of each life form, even if, in higher concentrations, they could pose toxic effects on the entire ecosystem. In general, metals are divided into four major categories, in terms of environmental threat but also on the angle of interest: (i) toxic heavy metals, (ii) strategic metals, (iii) precious metals, and (iv) radionuclides. When considering the environmental impact of metals, the "big three," mercury, lead, and cadmium, are in the limelight (Volesky 1990; Naja and Volesky 2009).

In the entire world, due to the toxic effects of heavy metals, environmental laws focused on increasingly tight limits for their concentration in industrial effluents. Chojnacka (2009) provided comprehensive information including the maximum admissible concentration of heavy metals (mg/L) in domestic and industrial wastewaters according to American and European regulations. To meet these safe discharge standards of heavy metals, essential for maintaining life in all forms, it is critical to remove heavy metals from effluents before their release into the environment. Chemical precipitation, ion exchange, electrochemical methods, reverse osmosis, and adsorption are usually used for heavy metal removal from industrial effluents.

Although these methods are in general simple and effective procedures with successful metal recuperation, there are several disadvantages of their application such as hard separation, generation of secondary residues, commonly inefficiency for low metal concentrations, and high costs or sensitivity under determined conditions, such as the presence of interfering agents (Gavrilescu 2004, 2010). In recent years, environmental and public health engineers focused more on the use of biomaterials in wastewater treatment. These studies are relevant in the context of low costs, relatively short operating time with no secondary products released and reusability of the biomaterials. These biomaterials include agricultural or industrial waste, microorganisms, and plants. The observation of natural-occurring processes in the environment leads to application of bioremediation strategies that rely on the natural capacity of microorganisms (fungi and bacteria), algae, and plants to bind heavy metal ions or, in some cases, to promote their conversion to their less toxic forms, though treatment of polluted effluents (Brinza et al. 2009; Gavrilescu 2014; Gavrilescu et al. 2015; Volf et al. 2015; Todorciuc et al. 2015).

Organic Pollutants: Dyes and Xenobiotics

Modern societies are highly dependent on commodities and processes that involve the extensive production and use of synthetic organic compounds which lead to a high amount of xenobiotic compounds lost in the industrials effluents that, in many cases, are harmful to organisms (including humans) and ecosystems (i.e., they are hazardous substances). The essential properties such as solubility, volatility, and biodegradability of organic xenobiotic compounds are just as varied as their potential sources and uses (Donner et al. 2010). Natural compounds can become xenobiotics if they are taken up by another organism, such as the chemical defenses produced by some organisms as protection against predators. The term xenobiotic is understood as substances foreign to an entire biological system, which did not exist in nature before their synthesis by humans (Csaba and Csaba 2011). The properties of xenobiotic compounds attributed due to their recalcitrant properties include the toxicity in nature, the stability and insolubility to water, large molecular size which determines difficulty to be input in the microbial cell, non-recognizable as substrate by microbes to act upon and degrade it, and the absence of permease essential for its

transport into microbial cell (Ekerue 2014). The recalcitrant xenobiotic compounds can be divided into different groups depending on their chemical composition. Polycyclic aromatic hydrocarbons (PAHs), halogenated aliphatic as well as aromatic hydrocarbons, nitroaromatic compounds, azo compounds, organic sulfonic acids, and synthetic polymers are important classes of pollutants with xenobiotic structural features. Those compounds are raw material for the production of different synthetic compounds (Fetzner 2008). The hazards induced by xenobiotics are huge. These compounds are highly toxic in nature and can influence the existence of all living organisms on the strength of persistence (they remain in the environment for a long period of time leading to bioaccumulation and/or biomagnifications) and the route to enter into the food chain. This properties lead to high concentrations of compounds in organisms that do not come in contact with xenobiotics directly.

Sixteen individual PAH compounds have been classified as priority pollutants due to their toxic, mutagenic, and carcinogenic characteristics, and chronic toxic effects and were proposed as potential persistent organic pollutants (POPs). High PAH concentrations have been reported in sediments on benthic and aquatic organisms (Perelo 2010). PAHs are ubiquitous pollutant by-products of coke production and coal tar. A large number of different PAHs compounds are formed by incomplete combustion of organic substances. Some are of industrial area, such as PAHs used in medicine and in production of dyes, plastics, and pesticides. Because of their low water solubility and hydrophobicity, they tend to adsorb on and accumulate in sediments, where the degradation of PAHs with high molecular weights is particularly slow (Zaki and Hammam 2014). Coal tars are complex and variable mixtures of phenols, PAHs, and heterocyclic compounds and are the main constituent of food dyes. For example, with respect to the food dye Erythrosin B, the tetraiodo analogue of fluorescein is produced by iodination (the reaction of iodine or potassium iodate in an ethanolic solution converted to the sodium salt) of fluorescein, the condensation of resorcinol with phthalic anhydride (Fig. 1).

The presence of xenobiotic compounds from industries has led to environmental research focusing on identifying methods which can effectively be applied to different environmental components in order to minimize their toxic effects. In order to contribute to sustainable development, extensive research should focus on the use of biological remediation processes by using by-products or crops.

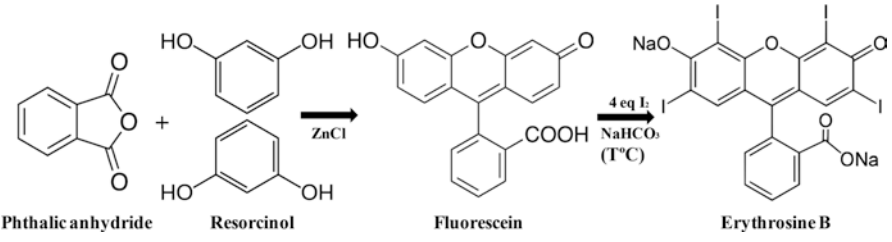

Fig. 1 Schematic representation of the production of the tetraiodo analogue of fluorescein

Bioavailability of Pollutants in the Environment

Due to their potential for human exposure and increased health risk, major pollutants such as heavy metals and xenobiotic organic compounds present a major interest in terms of their bioavailability into the environment. Many factors are associated with the bioavailability of pollutants: climatic conditions, transfer processes, sequestration and speciation, redox states, and response of plants, microorganisms, and animals to seasonal cycles (Chojnacka et al. 2005). Several definitions for the term bioavailability were summarized by Hlihor et al. (2009). As suggested by Peijnenburg et al. (2004), "bioavailability is, to an increasing extent, recognized as the key issue linking the increased levels of toxicants to actually occurring adverse effects in ecosystems, whilst taking the modifying effects of the abiotic components of the environment into account." The bioavailability is actually a function of the environmental processes that act on contaminant's mobility in the ecosystem making them accessible through different routes, such as oral exposure route, dermal absorption, inhalation, and plant uptake. The mobility of metals in the environmental compartments is determined through a variety of processes by microorganisms, therefore influencing metal bioavailability. The bioavailable fraction is usually used in risk assessment purposes (Bondarenko et al. 2008). For ecological evaluations, bioavailability can be assessed by evaluating direct exposures to the available fraction of metals present in the environmental media (i.e., sediment or soil), estimating or measuring bioaccumulation directly from the environmental media, or estimating uptake from ingestion of food, while for human health risk assessment, bioavailability is measured in terms of absolute and relative bioavailability (Battele and Exponent 2000).

Numerous reactions can be engaged in physical, chemical, and biological transformation of heavy metals in soil, influencing metal bioavailability. The mechanisms involved in the transformation of metal(loid) ions in soil are illustrated in Fig. 2 and are related to retention (mediated by sorption, precipitation, and complexation reactions) or loss (plant uptake, leaching).

Xenobiotic organic compounds can be mineralized by the mixed culture of microorganisms. Microbial consortium including fungi or aerobic and anaerobic bacteria is involved in the degradation of xenobiotics. The biodegradative activity of the microbial population depends on external physico-chemical factors (temperature, pH, redox potential, moisture content) and on intrinsic biological parameters (the presence of appropriate enzymes and uptake systems, toxic effect) (Jaspers et al. 1999; Gourlay-Francé and Tusseau-Vuillemin 2013) (Fig. 3).

Bioavailability is controlled by parameters such as the physical state of the xenobiotic compound (solid, liquid, gaseous), its solubility in water, and its tendency to adsorb or bind to particulate matter (Committee on the Design and Evaluation of Safer Chemical Substitutions 2014). In general, the bioavailable fraction of organic compound is present in the aqueous phase resulting from different kinetics: hydrodynamic transport, mass transfer, and biodegradation. Fetzner (2008) argues that the extent of biodegradation and the rate at which it occurs depend on the chemical

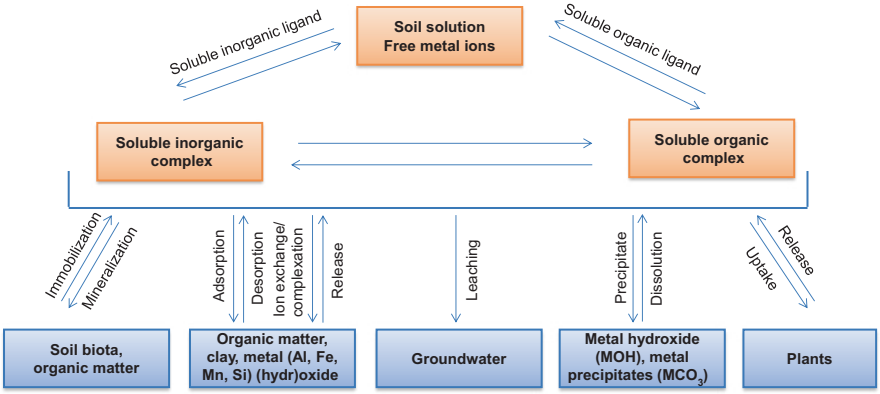

Fig. 2 Reactions involved in the transformation of metal(loid) ions in soil (Adapted after Seshadri et al. 2015)

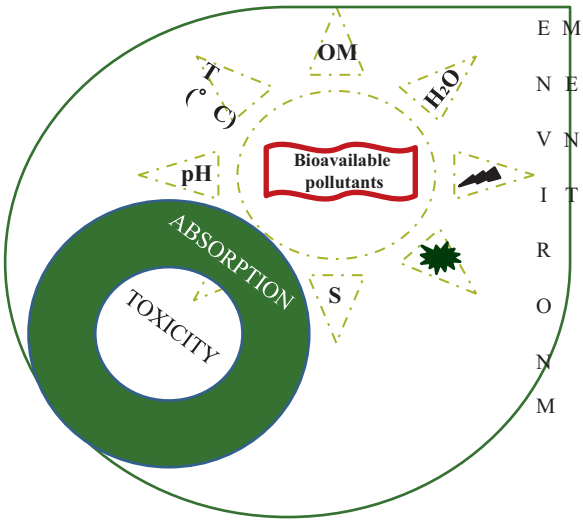

Fig. 3 Factors inducing environmental pollutant's bioavailability (Adapted after Gourlay-Francé and Tusseau-Vuillemin 2013)

structure and concentration of the compound being degraded, the type and number of microorganisms present, and the physicochemical properties of the environment. A significant number of organic xenobiotic compounds manifest hydrophobic interaction with media. After release into the environment, they are immobilized on the solid particles of the matrix by sorption.

Desorption process (the release of contaminant) is the result of collaboration between the physico-chemical factors, reaction or surface properties of the sorbent and biological factors, and the activity of microorganisms, plants, and animals. The released contaminants are transported by diffusion and dispersion, which may lead to the xenobiotic contact with the microorganism's surface. Passing the physiologi-

cal barrier of cellular membranes of microorganisms is the key stage in the process of transformation of xenobiotics, taking place with the participation of more or less specialized enzymes of xenobiotic decomposition pathways (Gren 2012).

A part of those compounds have medium- to long-term stability in soil, air, and water, and their persistence results in significant impact on the ecosystem. Some xenobiotics showed to persist, and they can resist to microorganism's actions on it or can be toxic. The products of partial biodegradation of a xenobiotic may be less harmful as the original compound, or they may be as hazardous or even more poisonous as the original compound (Fetzner 2008). The methods to evaluate the intrinsic bioremediation potential take into account the migration of the contaminants as well as the desorption-limited mass transfer and biodegradation. From the studies conducted by Baveye and Bladon (1999), it can be found that the key determinant of the bioavailability of organic xenobiotics in environment is not the rate (supposedly fixed) of their release by the substrate but the ability of microbial cells and higher organisms to act as immerse for these compounds. The experiments carried out with near-perfect sinks (resin beads) have shown delayed aging of organic xenobiotics and heavy metals in soils. The "sink theory" of the bioavailability of organic xenobiotic compounds has estimated a number of practical repercussions in particular in terms of environmental policy decisions (Baveye and Bladon 1999).

Biosorbents and Bioaccumulators

Bioremediation is a multidisciplinary area of knowledge and expertise which involves the use of microorganisms (or other types of biomaterials of natural origin) by virtue of their bioconcentrating and metabolic properties, to degrade, sequester, or remove environmental contaminants. Seen as a branch of environmental biotechnology, bioremediation thus involves the detoxification of hazardous substances (Gavrilescu 2004; Fingerman and Nagabhushanam 2005).

In nature, biomass plays an enormous role in detoxifying all kinds of waste streams. Soluble chemicals interact with materials of biological origin and are bound to cellular surfaces in the process called biosorption or become accumulated inside the cells via bioaccumulation (Kadukova and Vircikova 2005; Chojnacka 2009). Because many mechanisms can be involved in the biosorption and bioaccumulation processes, sometimes these processes can be difficult to be defined or understood (Volesky 1990; Gadd 2009; Gavrilescu 2010). Biosorption is a nondirected physico-chemical mechanism, including surface complexation, ion exchange, electrostatic attraction, and microprecipitation (Fingerman and Nagabhushanam 2005; Robalds et al. 2015). Biosorption is a metabolically passive process (made primarily by nonliving microorganisms or biological materials (e.g., agricultural waste)), while bioaccumulation requires living organisms, being achieved in subsequent stages of biosorption. Therefore, the first stage in bioaccumulation is biosorption; after this stage the pollutant is transported inside cells mainly via energy-consuming active transport systems (Chojnacka 2009). It is very difficult to

assess bioaccumulation quantitatively because the chemical transport may work both ways – into the cell and out of it and across the cell wall and cell membranes, including some organelles (e.g., vacuoles) serving as deposition or storage sites inside the cell. In addition to it, some cells tend to produce extracellular chemicals (Naja and Volesky 2011). Because of their potential application in industry, biosorption and bioaccumulation processes are hot topics in the field of biotechnology. Recent research on biosorption focuses mainly on the removal of toxic metals commonly used in industrial processes, such as Cu, Cd, Pb, Cr, Zn, and Ni. There are few studies devoted to Hg, Al, Co, As, and Th. In bioaccumulation, literature concentrates mainly on Cd, Cu, Hg, Pb, Cr, Zn, As, and Ni (Chojnacka 2009). In the case of xenobiotic organic compounds, the principal classes studied were persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), dyes, and pesticides. The capacity of certain types of microbial biomass to remove and concentrate inorganic or organic pollutants from solutions provides the basis for a cost-effective technology that can be used in detoxifying industrial effluents. Many biological materials can bind pollutants, but only those with sufficiently high-binding capacity and selectivity are suitable for use in a full-scale biosorption process (Wang and Chen 2009; Naja and Volesky 2011). Microorganisms fully conform to these features and are being often proposed as suitable sources of natural-occurring materials used for biosorption purposes.

A wide range of microbial biomass has been investigated during the past decade for biosorption and bioaccumulation studies. Biomaterials interact effectively with heavy metals and represent a new opportunity for pollution control, recovery, and recycling (Gadd 2009). The biological effects associated with microorganism's exposure to heavy metals are diverse and depend upon the metal speciation and the model organism tested (Poljsak et al. 2009). From the viewpoint of organic pollutants, the hydrophobicity/hydrophily or the polar/nonpolar character of these compounds contributed on the success of sorption process (Sayara et al. 2015). While choosing a biomaterial for organic and inorganic pollutants sorption, its origin is a major factor to be taken into account and can come from (a) microorganisms as a by-product of fermentation industry, (b) organisms naturally available in large quantities in nature, and (c) organisms cultivated or propagated for biosorption purposes using inexpensive media. Three easily available groups can be used as biosorbent materials: algae, fungi, and bacteria, the former two perhaps giving broader choices (Ahluwalia and Goyal 2007; Wang and Chen 2009; Zeng et al. 2013).

Algae have gained a lot of attention from scientists due to their biosorption potential. The multifunctional utilization of micro- and macro-algae in food, cosmetics, medicine, energy, etc. demonstrates their large availability for other purposes, such as their use in bioremediation. Algae can be used either in the living form or as dead in a biosorption system, the dead form being more practical because it does not require nutrients and it is not subjected to pollutants toxicity (Anastopoulos and Kyzas 2015; Bulgariu and Gavrilescu 2015; Ungureanu et al. 2015). Brinza et al. (2007) provided a comprehensive review on practical aspects of the application of algal biomass for the biosorption of heavy metals from wastewater. The authors highlighted the possibility of reusing algal biomass in several adsorption/

desorption cycles and the influence of morphology and environmental conditions on the reusability of algal tissue.

Another type of biomaterial used for biosorption studies is represented by agricultural and industrial waste. The main components of agricultural wastes are lignin, cellulose, and hemicelluloses. Biosorption technology applied for the removal of heavy metals and dyes using lignocellulosic materials and by-products is a proper method for detoxifying polluted streams due to material abundance, cost-effectiveness, and efficiency for decontamination (Hlihor and Gavrilescu 2009; Abdolali et al. 2014; Hlihor et al. 2014; Zhou et al. 2015). The functional groups present in this type of waste play an important role in heavy metals and dyes removal from solutions. Many studies have shown the relevancy of enhancing the functional groups in order to increase the active sites by different pretreatment methods (Abdolali et al. 2014; Zhou et al. 2015; Zeng et al. 2013).

The application of hyperaccumulating plants in remediation of metal-contaminated environments has been intensively studied over the past 20 years. These plants play a significant role in phytoremediation because they absorb and concentrate the metals in their roots and shoots, having a direct implication in human health through the food chain (Prasad and de Oliveira Freitas 2003). According to Fasani (2012), metal hyperaccumulation occurs in approximately 500 taxa of angiosperms and is particularly common among the *Brassicaceae*. Hyperaccumulator plants do not only absorb high concentration of micronutrients such as Fe, Zn, Mn, Ni, Cu, and Mo, or macronutrients such as N, P, K, S, Ca, and Mg, but also significant amounts of nonessential metals (Lasat 2000). Table 1 presents a short overview of biosorbents and bioaccumulators used for heavy metal removal from aqueous solutions, while Table 2 considers biosorbents and bioaccumulators used for dyes removal.

Table 1 Application of biosorbents for the uptake and/or removal of heavy metals

Biosorbent	Heavy metal	Uptake (mg/g)	Removal efficiency (%)	Reference
Microorganisms				
Activated sludge biomass (ASB)	Cd(II)	–	59.3	Ahmad et al. (2010)
	Pb(II)	–	68.5	
	Zn(II)	–	86.5	
Mycobacterium sp.	Cr(III)	40.09	94.19	Aryal and Liakopoulou-Kyriakides (2014)
	Cr(VI)	23.06	71.08	
Stenotrophomonas maltophilia PD2	Cu(II)	11.86	83	Gosh et al. (2015)
Saccharomyces cerevisiae	Cr(VI)	–	100	Hlihor et al. (2013)
T. viride (dead)	Cd(II)	8.86	99.99	Hlihor et al. (2015)
T. viride (living)	Cd(II)	3.48	100	

(continued)

Table 1 (continued)

Biosorbent	Heavy metal	Uptake (mg/g)	Removal efficiency (%)	Reference
Rhizopus arrhizus	Cr(VI)	21.72	–	Shroff and Vaidya (2012)
Bacillus thuringiensis strain OSM29	Cd(II)	59.17	87.8	Oves et al. (2013)
	Cr(VI)	71.94	89.4	
	Cu(II)	39.84	91.8	
	Ni(II)	43.13	94.6	
	Pb(II)	30.76	90.6	
P. lilacinus (dead)	Cd(II)	41.13	46.05	Zeng et al. (2013)
P. lilacinus (living)	Cd(II)	36.51	38.98	
Bacillus cereus (dead)	Cd(II)	31.95	90	Huang et al. (2013)
Bacillus cereus (living)	Cd(II)	24.01	65	
P. aeruginosa B237	Zn(II)	17.67	–	Limcharoensuk et al. (2015)
	Cd(II)	16.49	–	
Algae				
Ulva lactuca sp.	Pb(II)	181.82	–	Bulgariu et al. (2013)
	Cd(II)	43.02	85	
Sargassum muticum	Sb(III)	5.5	–	Ungureanu et al. (2015)
Mixture of *Ulva lactuca* sp. and Purolite A100 resin	Pb(II)	–	98.17	Bulgariu and Bulgariu (2013)
Mixture of green and blue-green algae	Pb(II)	77.2	98	Brouers and Al-Musawi (2015)
Cystoseira indica	Cu(II)	94.33	>90	Akbari et al. (2015)
	Co(II)	54.64	>90	
Sargassum asperifolium	Cr(VI)	–	92.7	Hamdy (2000)
	Co(II)	–	67.6	
	Ni(II)	–	87.8	
	Cu(II)	–	91.2	
	Cd(II)	–	89.7	
Cystoseira trinode	Cr(VI)	–	96.3	
	Co(II)	–	60.6	
	Ni(II)	–	82.6	
	Cu(II)	–	97.3	
	Cd(II)	–	87.7	
Turbinaria decurrens	Cr(VI)	–	97.8	
	Co(II)	–	86.1	
	Ni(II)	–	92.6	
	Cu(II)	–	97.1	
	Cd(II)	–	93.9	
Laurencia obtusa	Cr(VI)	–	98.6	
	Co(II)	–	50.0	
	Ni(II)	–	75.8	
	Cu(II)	–	98.2	
	Cd(II)	–	98.0	

(continued)

Table 1 (continued)

Biosorbent	Heavy metal	Uptake (mg/g)	Removal efficiency (%)	Reference
Agricultural and industrial wastes				
Rice straw	Cd(II)	13.9	–	Ding et al. (2012)
Peanut shell	Cu(II)	25.39	99	Witek-Krowiak et al. (2011)
	Cr(III)	27.86	80	
Pistia stratiotes	Pb(II)	–	96	Volf et al. (2015)
Mustard husk	Cd(II)	33.56	80	Bulgariu et al. (2012)
Ficus religiosa	Cr(VI)	26.25	65	Qaiser et al. (2007)
	Pb(II)	37.45	90	
Pomelo peel	Cd(II)	21.83	–	Saikaew et al. (2009)
Nutshells	Cd(II)	7.22	75.38	Hlihor et al. (2014)
Straws	Cd(II)	5.99	62.52	
Bean hulls	Cd(II)	6.76	70.53	
Pumpkin seed hulls	Cd(II)	7.5	69.90	
Plants				
Carthamus tinctorius L.	Cd(II)	–	4.8	Tlustoš et al. (2006)
	Zn(II)	–	1.1	
Different types of dried plants	Cd(II)	–	94	Chiban et al. (2011)
	Cu(II)	–	92	
	Pb(II)	–	99	
	Zn(II)	–	97	

Table 2 Application of biosorbents for the uptake and/or removal of dyes

Biosorbent	Dyes	Uptake (mg/g)	Removal efficiency (%)	Reference
Microorganisms				
Aspergillus niger Trichoderma sp.	Orange G	0.48	83	Sivasamy and Sundarabal (2011)
		0.45	76	
Aspergillus sp.	Dunkelblau		69	Cretescu et al. (2010)
Trichoderma asperellum (free cell)	Crystal violet	12.97	–	Chew and Ting (2016)
	Methyl violet	12.54	–	
	Cotton blue	14.43	–	
	Malachite green	11.44	–	
Activated sulfidogenic sludge	Congo red	239.9	–	Rasool and Lee (2015)
Aspergillus fumigatus	Acid Violet 49	136.98		Vaigan et al. (2010) Gao et al. (2010)
Granular activated carbon	Brill Blue KN-R		23.6	
Nonliving aerobic granular sludge	Acid yellow 17	20	86	

(continued)

Table 2 (continued)

Biosorbent	Dyes	Uptake (mg/g)	Removal efficiency (%)	Reference
Algae				
Turbinaria conoides	Rhodamine B	16.7	–	Hii et al. (2009)
	Acid Blue 9	38.46	–	Rajeshkannan et al. (2010)
Ulothrix sp.	Methylene blue	86.1	–	Doğar et al. (2010)
Chlorella pyrenoidosa	Malachite green	0.015	–	Horník et al. (2013)
Chara sp.			92.75	Khataee et al. (2010)
Agricultural and industrial wastes				
Jujuba seeds	Congo red	34.64	–	Somasekhara et al. (2012)
Pumpkin husk	Reactive red 120	93.7	–	Çelekli et al. (2014)
Pumpkin seed hull	Erythrosine B	16.4	–	Apostol et al. (2015a)
Jatropha curcas shells	Reactive red 120	65.63	–	Prola et al. (2013)
Cashew nut shell	Methylene blue	71.33	–	Kumar et al. (2014)
Cocos nucifera		20.74	–	Mondal et al. (2014)
Tea leaves		180	–	Kumar et al. (2010)
Banana peels		181	–	
Wheat straw		156	–	
Sugarcane bagasse		137	–	
Lemna gibba	Direct Red 89	20	60	Guendouz et al. (2013)
	Reactive green 12	6.13	47	
Plants				
Lepidium sativum L.	Erythrosine B	–	–	Apostol et al. (2015b)
Lemna minor	Methylene blue	10.93	–	Reema et al. (2011)
Alyssum caricum	Reactive green 19	27.6	–	Bayramoglu et al. (2013)
	Reactive red 2	16.5	–	
Aster amellus	Remazol orange 3R	–	42	Kabra et al. (2011)
Glandularia pulchella		–	37.2	
	Rubine GFL	–	100	Kabra et al. (2012)
	Scarlet RR	–	100	
	Brilliant Blue R	–	85	
	Red HE3B	–	55	
	Navy Blue 2R	–	60	

Removal Efficiency by Biosorption and Bioaccumulation: Role of Environmental Factors and Process Parameters

Overall, the biosorption is studied based on general principles of adsorption. The amount of sorbent and sorbate, equilibrium time between the two phases, contact time, pH, and temperature are important parameters that significantly affect the biosorption process. These parameters need to be optimized one by one, keeping all

others constant, for a fast and effective process. Literature shows tremendous work of researchers on optimizing all these parameters, in order to improve biomass uptake efficiencies.

pH

One of the most important influencing factors for biosorption and bioaccumulation of inorganic and organic compounds is the pH of solution. It does not only influence the chemical speciation of metal ions or the charges on the biosorption sites of biomass but also the metallic ion competition for active sites. It is very important to consider the ionic states of the functional groups of the biosorbent, as well as the metal solution chemistry at different pH values. At pH values <3.0 and >9.0 or 10, the microbial growth is highly inhibited by pH values in the system, since it causes changes in the microbial metabolism, nutrient availability, metal bioavailability, and solubility (Vijayaraghavan and Yun 2008; Mudhoo and Mohee 2012; Hlihor et al. 2014). For instance, Hlihor et al. (2015) investigated the biosorption and bioaccumulation of *Trichoderma viride* for Cd(II) bioremoval from aqueous solutions. The process was strongly influenced by the solution pH. The authors found that the biosorption capacity of dead biomass increases with the pH, exhibiting a maximum of 7.31 mg/g at pH 6.0, for 100 mg/L cadmium in solution, while the efficiency of the process was around 60%. On the other side, the living biomass was able to bioaccumulate 100% of 50 mg/L Cd(II) at pH 6.0, while lower pH values showed a decrease in metal uptake. With respect to Cd(II), Pb(II), and Zn(II) removal from wastewater by activated sludge biomass (ASB) in an anaerobic digestion system, as noted by Ahmad et al. (2010), maximum biosorption was observed at pHs 3.5, 4.0, and 4.5, respectively. In this case, the interaction of metal ions with ASB due to microbial biomass and metal cations with the electron-rich functional groups located on the ASB may be strongly sensitive to the pH value of the environment. The removal of Cr(VI) is found to increase when decreasing the pH to acidic values. At low pH values, $HCrO_4^-$ species are dominant and enter in reactions, when Cr(VI) is reduced to Cr(III) (Hlihor et al. 2013). The increase of solution pH from 1.0 to 4.0, according to Silva et al. (2009), increases the negative charge on the cell surface due to the deprotonation of the metal-binding sites hence attracting Cr(III) ions resulting from the reduction of Cr(VI). The experimental data reported in different studies indicated that the pH of the solution exerts a strong influence on the uptake of organic xenobiotic compound (e.g., dyes) molecules due to its influence on speciation such as ionization (protonation/deprotonation) and dissociation of the compound molecules as well as on the surface properties (the activity of the functional groups) of the biomass. In the case of bioremediation studies using living organisms, Gül and Dönmez (2014) studied the decolorization capacity of *Aspergillus versicolor* for Remazol Blue. The maximum removal capacity of 95.75% was found to be achieved at pH 6.0. The effect of initial aqueous phase pH (2.0–10) on biosorption of Orange G by *Aspergillus niger* and *Trichoderma* sp. was carried out by

Sivasamy and Sundarabal (2011). In the range of studied pH, for the biomass *Aspergillus niger*, it was found that the biosorption process efficiency decreased from 61% to 6%, whereas for biomass *Trichoderm*a sp., it decreased from 55% to 3%. The authors observed that the percentage removal of dye was maximum at pH 2.0 for both fungal biomasses. In the case of biosorption process using agricultural waste, Apostol et al. (2015a) recently investigated the pH effect of pumpkin seed hull (PSH) biosorption capacity for Erythrosin B removal from aqueous solution. The studies were carried out at different initial solution pH values, varying from 4.0 to 10. The investigations show that the maximum amount of dye sequestered by PSH (point of zero charge, pHpzc, of PSH was found to be 6.2) was achieved at pH 5.0. For pH less than 5.0, Erythrosin B contact with the sorbent reacts with the buffer solution and the absorbance of the solution decreases very much due to the strong acidity of the medium that conducts to dye precipitation. In another study conducted by Çelekli et al. (2014), a tremendous reduction of RR120 by biosorption on pumpkin husk was observed at high pH value. More positively charged pumpkin husk surface (pH_{pzc} = 6.4) at pH 1.0 could exert strong electrostatic attraction for RR120 molecules, and as a consequence the high amount of dye is adsorbed in this condition. This will be advantageous if organic compounds will be stable in these conditions. A lot of studies do not take into account the change in pH due to material addition and the effect on compound chemistry or metal speciation, leading to inaccurate interpretation of biosorption properties.

Biosorbent Dosage

Biosorbent dosage or biomass concentration in solution affects the specific uptake of inorganic or organic pollutants: for lower values of biomass dosages, there is an increase in the specific metal uptake. In many instances, lower biosorbent dosages yield higher uptakes but lower percentage removal. An increase in the biomass concentration generally increases the amount of solute sorbed, due to the increased surface area of the biosorbent, which in turn increases the number of binding sites (Sahmoune et al. 2011; Hlihor et al. 2014). These findings are in agreement with the work of Mishra et al. (2013). The authors concluded that an increase in biomass dosage at a fixed Zn(II) ion concentration led to a rise in removal efficiency from aqueous solutions, while a very sharp decrease in metal uptake capacity could be observed. In the case of organic pollutants, since they can have large molecule sizes toward heavy metal compounds, the biosorbent properties (e.g., concentration and particle size) exhibit a strong influence on pollutant biosorption.

The biosorbent dosage is established in the phase where the concentrations of xenobiotic compounds on the surface of biosorbent and in solution come to equilibrium with each other. For example, in the studies conducted on pumpkin by-products as biosorbent, the concentration at equilibrium was 20 mg/L for Erythrosin B (Apostol et al. 2015a) and 0.5 g/L for RR120 (Çelekli et al. 2014), respectively. The fungal biomass concentration (*Aspergillus niger* and *Trichoderma* sp.) for biosorp-

tion of Orange G was established by Sivasamy and Sundarabal (2011) at 90 g/L. In the studies conducted by Çelekli et al. (2014) and Sivasamy and Sundarabal (2011), a single parameter variation was taken into account considering the percentage of pollutant removed, while in the study of Apostol et al. (2015a), the authors considered the amount and the percentage of pollutant removed.

Concentration of Pollutant

The initial solute concentration seems to have impact on biosorption process. A higher concentration results in a high solute uptake. This is because at lower initial solute concentrations, the ratio of the initial moles of solute to the available surface area is low; subsequently, the fractional biosorption becomes independent of the initial concentration (Vijayaraghavan and Yun 2008). Ghosh et al. (2015) were able to optimize the uptake of copper by *Stenotrophomonas maltophilia* strain PD2 for the first time. The authors showed that the uptake capacity of the biomass was increased when increasing the initial Cu(II) concentration from 10 to 50 mg/L, while the maximum removal efficiency was found to be around 84% at a biomass dose of 0.5 g/100 mL metal solution. Murugavelh and Mohanty (2014) observed that the bioaccumulation efficiency of active *Phanerochaete chrysosporium* decreased from 81.47% to 23.82%, in solutions where Cr(VI) concentration was increased from 10 to 40 mg/L. Rasool and Lee (2015) reported that the amount of Congo red (CR) sorbed increased from 82.64 to 238.90 mg dye/g activated sludge cell, with increase in the initial CR concentration from 100 to 1000 mg/L. Thus, the equilibrium removal of CR decreased from 79.80% to 23.08%. In the bioremediation assays where the subject is the xenobiotic organic compound degradation, the toxicity is the first factor influencing the results. The results reported by Apostol et al. (2012) confirm the negative impact of Erythrosin B (Ery) and Eosin Y (Eos) on anaerobic granular sludge activity. In terms of kinetics, the first-order rates increased with increasing the dyes concentration followed by a decrease at higher dyes amount, 0.4 mM of Ery and 0.9 mM of Eos. Inhibitory effect at high dye concentration occurred with both dyes, being higher for Ery. These results highlight one of the advantages of biosorption over bioaccumulation: living organisms may not be preferable for effluents treatment since they can be subjected to toxic effects of the contaminants. The data obtained from the investigation of initial pollutant concentrations effects on biosorption process are fundamentally for the application of adsorption isotherm models.

Contact Time

The biosorption capacity and the removal efficiency of metal ions and organic xenobiotics by different kinds of biomass can become higher with prolonging the contact time, but not necessarily. Many studies demonstrated the biosorption process efficiency in a short period of time (Lupea et al. 2012; Bulgariu et al. 2013; Aryal and

Liakopoulou-Kyriakides 2014). The contact time studies can assist in establishing the duration of apparent equilibrium attainment: the time beyond which no significant pollutant is uptaken over the biosorbent surface. However, in practice, it is necessary to assess the contact time, considering the efficiency of desorption and regeneration of the biomass. Similarly, the duration of exposure is also a factor in bioaccumulation. Most exposures to chemicals in the environment vary continually in concentration and duration, sometimes including periods of no exposure. In these cases, equilibrium is never achieved and the accumulation is less than expected (Gavrilescu 2004; Wang and Chen 2006). As an application, the contact time study is used in the kinetic approach and rate-determining/limiting step establishment.

Temperature

The study of thermodynamics of the biosorption process shows, at a global analysis of the literature, that temperature is an important parameter since it influences the removal efficiency of different kind of pollutants. It is directly related to the kinetic energy of the metal ions. Thus, it can account for the diffusion process. An increase or decrease in temperature should cause a change in the amount of metal removed or sorbed by the biomass. Temperature plays a vital role in biosorption and bioremediation processes. In the case of dye removal by biosorption process, the real application is advantageous in terms of temperature because the maximum amount of dye removal is generally reached at temperatures around 50 °C. The value is close to that at which industrial coloring process release the effluents. In the study conducted by Apostol et al. (2015a), the positive value of enthalpy change (ΔH°) shows that the biosorption process is endothermic, increasing temperature leading to a higher Erythrosin B amount uptake at equilibrium by pumpkin seed hulls. The endothermic nature of the biosorption process was also reported by Çelekli et al. (2014) for RR120 removal by pumpkin husk. In the case of an exothermic process, where the dye uptake decreases with the temperature rise, a temperature adjustment of the industrial effluents before adsorption process application is necessary. In the case of bioremediation, the process temperature is the key parameter used for microorganism's growth, reproduction, and enzyme activities. The results obtained to date suggest that two different biosorption mechanisms, energy dependent or energy independent, for different metal-biomaterial systems are encountered (Feng et al. 2012). In the case of living organisms, the temperature is a limiting factor. Temperature is opportune only in the case of thermotolerant culture treatment of xenobiotics or heavy metal effluents.

Kinetics and Equilibrium

Kinetic and equilibrium data are modeled using different approaches in order to explain the biosorption mechanism involved in the removal of inorganic and organic pollutants. Understanding of biosorption mechanism can facilitate the

application of these techniques in wastewater treatment or other associated fields (Gavrilescu 2004). Kinetic models are involved in the identification of the rate and mechanism of reaction associated with the establishment of rate-controlling step. The study of kinetic models also helps in the design of continuous adsorption columns (El-Latif et al. 2010). The rate is an essential factor in adsorption systems and is represented by prediction of at which adsorption takes place in a given system (Ho 2006). In order to analyze the sorption kinetics, various models have been suggested: Lagergren pseudo-first-order model and pseudo-second-order model, Elovich model, etc. Kinetic models exploited for investigating the biosorption mechanisms related to convective mass transfer include intraparticle model and Bangham's model (Febrianto et al. 2009; Mishra 2014). In the case of bioaccumulation assays, the growth kinetics of cells is described with Monod equation when limiting concentration of substrates is used. If the expression did not represent the inhibitory effects of the pollutant tested, noncompetitive inhibition model can be used for process characterization. Competitive inhibition model is conversely the equation that can be applied if the inhibitory effects manage the process (Siva Kiran et al. 2012; Apostol et al. 2012).

Adsorption isotherm is the basic requirement for designing any adsorption system. The most used method of determination of the best fitting isotherm is linear regression, while the method of least squares has been used for determining the isotherm parameters. As a universal phenomenon governing the mobility of substances, the transfer of pollutants from liquid phase to solid phase is described by the "isotherm". The isotherm is a curve that highlights the retention of a certain substance on a solid at various concentrations and is used as a major tool, which predicts the mobility of pollutants in the environment. These retention/release phenomena are sometimes strongly kinetically controlled, so that time dependence of the sorption isotherm must be specified (Limousin et al. 2007). The equilibrium of biosorption of inorganic and organic pollutants follows an adsorption-type isotherm, according to literature. Equilibrium isotherm models are usually classified into the empirical equations and the mechanistic models. The experimental behavior is explained and predicted by mechanistic models (Wang and Chen 2009). The adsorption equilibrium determines (i) the amount of species adsorbed under a given set of conditions (concentration and temperature) or (ii) how selective adsorption takes place when two or more adsorbable components coexist. When an adsorbent is in contact with the surrounding fluid of a certain composition, adsorption takes place, and after a sufficiently long time, the adsorbent and the surrounding fluid reach equilibrium. This means that the equilibrium distribution of metal ions between the sorbent and the solution is important in determining the maximum sorption capacity (Igwe and Abia 2006). Several isotherm models are available in the literature to describe the equilibrium sorption distribution and the possible mechanism implied in the sorption process: Langmuir, Freundlich, Temkin, Dubinin-Radushkevich, etc.

Mechanisms of Biosorption and Bioaccumulation

The distribution of different elements and compound species in water is highly dependent on pH, composition, temperature, and the oxidation-reduction potential of the solution. These variables define their precipitation, dissolution, and complexation reactions. Especially for the metallic ions, phenomena such as chemical and biological transformations, metal mobility, bioavailability, bioaccumulation, toxicity, and persistence in the environment frequently depend on the chemical form or speciation of a given ion (Ibanez et al. 2007). In the biosorption system, the mechanisms that take place in metal bonding need to be well understood, while the metal speciation in aqueous solutions has to be taken into consideration since it plays an important role (Hlihor et al. 2014). The complexity of the microorganism's structure implies that there are many ways for the metal to be captured by the cell. Kotrba et al. (2011) proposed a complex scheme of all possible mechanisms occurring during biosorption-bioaccumulation of heavy metals. According to this scheme, it is possible to meet the action of a single, particular mechanism, or several mechanisms that can act simultaneously or in a train, depending on the organism and physico-chemical properties of cellular environment. Some of these mechanisms for metal species bioremoval are as follows: extracellular immobilization, precipitation, intracellular detoxification, solubilization, and mobilization of metals.

Biosorption mechanisms are therefore various and, in some cases, still not very well understood. Robalds et al. (2015) proposed a new diagram for classifying the sorption mechanism while considering that biosorption is referred to adsorption to biological materials. Dye decolorization can take place during three processes: biosorption, bioaccumulation, and biodegradation. The key factors controlling and characterizing these mechanisms are the type of biological ligands available for pollutant sequestering, the status of the biomass (living/non-living), and the characteristics of the pollutant solution such as pH and the presence of competing co-ions (Dhankhar and Guriyan 2011). Another important issue in understanding the biosorption mechanisms is focused on the physical and chemical characteristics of the biosorbent before and after the process, which is of considerable concern for the development of adsorption and separation processes. Depending on the nature of the biosorbents, a variety of techniques are useful tools for this purpose, e.g., Fourier transform infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), scanning electron microscopy (SEM), X-ray diffraction (XRD), energy dispersive X-ray (EDX) fluorescence spectrophotometry, nitrogen sorption, etc. These methods are commonly applied together to obtain a complete description of the structure, morphology, and composition of the biosorbents (Arief et al. 2008; Apostol et al. 2015a) (Fig. 4). The concept of point of zero charge can also be used to explain the ion exchange interaction capacity and saturation percentage of the biosorbents.

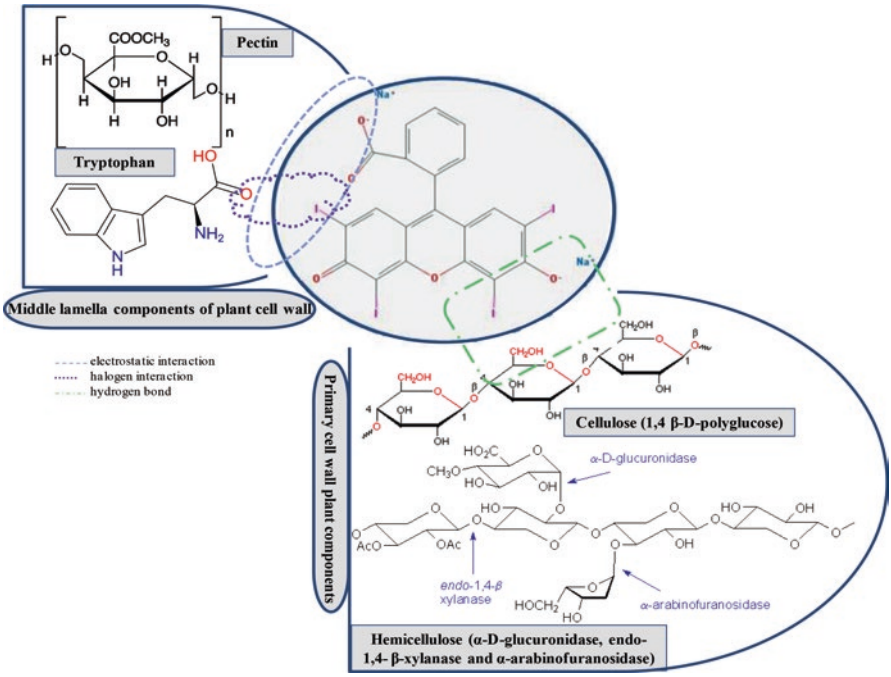

Fig. 4 Possible interaction between Erythrosin B and agro-waste wall components established for the biosorption process of the dye and pumpkin seed hull (Adapted from Apostol et al. 2015a)

Applications, Conclusions, and Future Perspectives

Up to now, many researchers at laboratory scale, in batch or dynamic conditions, have applied biosorption technology. Few attempts have been made considering large-scale conditions. An increase amount of work was done in order to elucidate the mechanism that underlines the process in order to scale-up the laboratory process for practical applications. In laboratory conditions different kind of biomasses were successfully used, from different algae types to agricultural or industrial wastes, plants, and microorganisms. Considering the high number of biosorbents proposed for inorganic and organic pollutant removal from wastewaters, there are some instances where biosorption process has reached commercialization as described by Gavrilescu (2004), Wang and Chen (2009), Vijayaraghavan and Yun (2008), and Oliveira et al. (2011): B.V. SORBEX Inc. (several biosorbents of different biomaterials from biomass such as *Sargassum natans*, *Ascophyllum nodosum*, *Halimeda opuntia*, *Palmaria palmata*, *Chondrus crispus*, and *Chlorella vulgaris*), Advanced Mineral Technologies Inc. (biosorbents based on *Bacillus* sp.), AlgaSORB (Bio-recovery Systems Inc., biomass *Chlorella vulgaris* immobilized in silica and polyacrylamide gels), AMT-BIOCLAIM™ (Visa Tech Ltd., biosorbent from *Bacillus subtilis* immobilized in polyethyleneimine and glutaraldehyde beads), and BIO-FIX (US Bureau

of Mines, biosorbent based on several biomasses, including *Sphagnum* peat moss, yeast, bacteria, and/or aquatic flora immobilized in high density polysulfone).

Release of wastewaters contaminated with inorganic and organic pollutants has been known for decades to have adverse effects to the environment and human health. Up to now, solving this problem has been presented as a challenge, since physicochemical and biological processes proved to be less efficient, especially when small concentrations of pollutants are found in the environmental compartments. Biosorption and bioaccumulation are ubiquitous property of dead or living biomass and derived products and are undoubtedly important processes that naturally occurred in the environment. In this work, biosorption and bioaccumulation have been presented as promising processes for environmental bioremediation. Biosorbents such as algae, agricultural or industrial wastes, plants, and microorganisms proved to be available and efficient biosorbents and/or bioaccumulators for inorganic and organic pollutants from wastewaters. The bioavailability of these pollutants, the impact of environmental factors on the removal efficiency, the kinetic and equilibrium modeling options, and the mechanisms involved have been outlined. When the interaction mechanisms between inorganic/organic pollutants and biomass (in its different forms) are well understood, it opens the possibility of process optimization, which is useful in large-scale applications.

Future studies for practical implementation of the biosorption process should focus on several challenges such as: realistic conditions (e.g., real wastewater which includes all kinds of pollutants); competitive adsorption studies of inorganic pollutants with organic pollutants; new strategies able to elucidate the mechanisms, when these are not known; design of biosorption system; and assessing the environmental impacts and costs of the process (Anastopoulos and Kyzas 2015; Miksch et al. 2015; Zhou et al. 2015). Once performed, the highlighted works can be helpful for environmental scientists to understand the current trends in biosorption and bioaccumulation processes, their practical applications, and future direction and perspectives that should be taken into account. More efforts need to be made for large-scale application of biosorption and bioaccumulation of inorganic and organic pollutants from wastewaters and also in terms of environmental impacts of the processes and their costs.

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