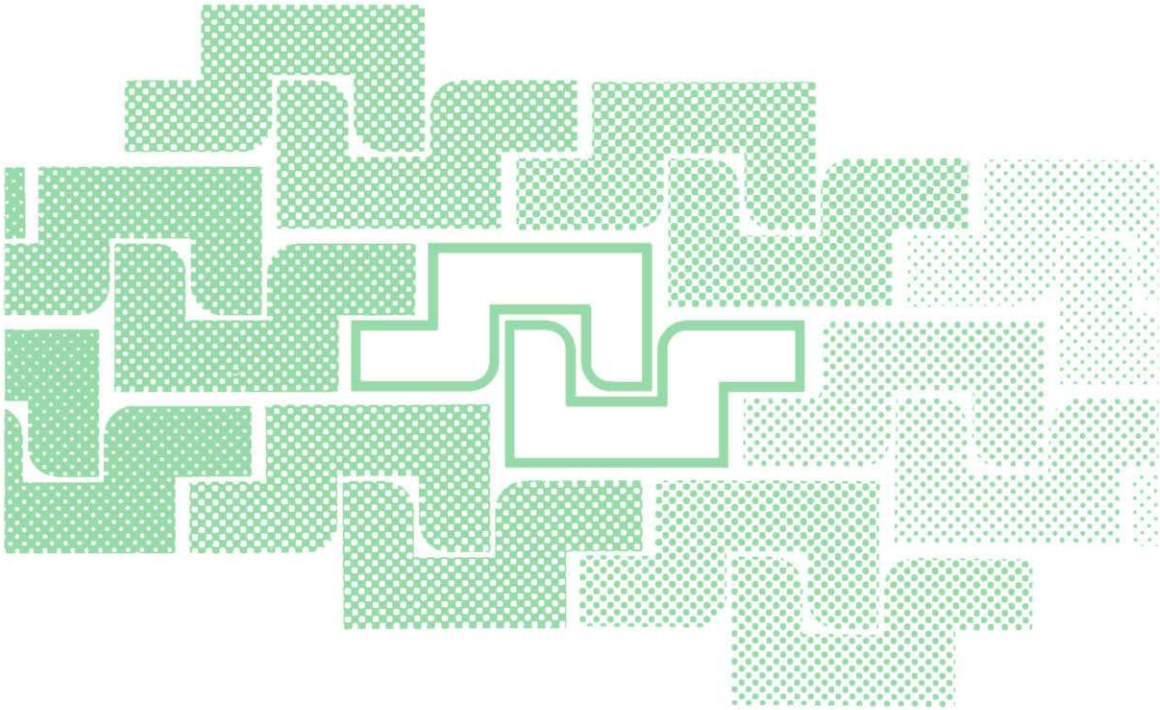

Bioremediation of Soils Contaminated with Aromatic Compounds

Edited by

Hermann J. Heipieper

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edited by

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PREFACE

Environmental biotechnology, which was in its infancy in the early 80's, has evolved thanks to the revolution brought about by molecular biology. Multiple successes in the biological cleanup of civil and industrial wastewater and of hydrocarbon soil pollution, demonstrate the vast power of clean technologies. In addition, the buildup of information on the activities of microorganisms as catalysts in all sorts of natural, industrial and animal environments has flourished. There is a continuing realization of the critical role of microbial processes in biological, industrial and geological systems.

Since environmental biotechnology has matured, it is ready to tackle bigger challenges: the scaling up of many bioremediation systems still in progress, the search for novel biocatalysts for industrial applications, the continuing effort against common human life-threatening processes such as antibiotic resistance, the accumulation of hormone-mimicking substances (endocrine disrupters), the deposition of air-borne pesticides in the environment and, the degradation of recalcitrant contaminants. These endeavors will help prevent the contamination of food chains, protect human life and allow for human activity and economic development that do not compromise environmental sustainabijity.

This volume includes the key lectures and participants' contributions delivered at the NATO-funded Advanced Research Workshop (NATO-ARW No. 980838) *Bioremediation of Soils Contaminated with Aromatic Compounds: Effects of Rhizosphere, Bioavailability, Gene Regulation and Stress Adaptation,* held in Tartu, Estonia, from the 1st to the 3rd of July 2004, and attended by participants from 15 countries.

The purpose of the workshop was to bring together scientists from NATO and Partner countries to establish collaborative research on bioremediation, Bioremediation has become a generally accepted means of cleaning up polluted sites, particularly ones contaminated with various xenobiotic compounds. The main topic of the workshop was bioremediation of soils contaminated with aromatic compounds such as herbicides, BTEX, and phenols.

The programme included 18 oral presentations and a poster session. Each of the 4 major sessions: Rhizosphere; Bioavailability and Transport; Molecular Biology, Gene Regulation and Genomics; Biodiversity and Environmental Genomics, Stress Adaptation was represented by a key scientists who presented their field.

This book is addressed to a wide readership. Specialized workers in the field of environmental biotechnology should find the updated materials on several areas of this topic very useful. University teachers could use the material in this book for introductory or graduate courses, and those who have a general interest in the subject should find the offered overviews particularly interesting. There are extensive literature references for further detailed studies.

Many people have contributed to the success of the ARW on which this volume is based. We wish to thank especially Ene Talpsepp and all her co-workers from the University of Tartu for their outstanding work, availability and kindness in the organization of the meeting. We thank all the participants, mainly the invited key speakers: Alexander Boronin, Ildefonso Cases, Victor de Lorenzo, Hauke Harms, Ain Heinaru, Hermann J. Heipieper, Janet K. Jansson, Ulrich Karlson, Maia Kivisaar, Peter C.K. Lau, Dietmar Pieper, Martin Romantchuk, Eduardo Santero, Ana Segura Carnicero, Elizabeth J. Shaw, Victoria Shingler, Stefan Trapp, Peter A. Williams for their contributions to a stimulating dialogue atmosphere throughout the duration of the Workshop.

Last but not least, we would also like to thank NATO Science Committee for selecting our meeting, NATO-ARW No. 980838, for the financial support by NATO.

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BIOREMEDIATION OF SOILS CONTAMINATED WITH AROMATIC COMPOUNDS: EFFECTS OF RHIZOSPHERE, BIOAVAILABILITY, GENE REGULATION AND STRESS ADAPTATION

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Environmental biotechnology, which was in its infancy in the early 80's, has evolved thanks to the revolution brought about by molecular biology. Multiple successes in the biological cleanup of civil and industrial wastewater and, of hydrocarbon soil pollution demonstrate the vast power of clean technologies. In addition, the buildup of information on the activities of microorganisms as catalysts in all sorts of natural, industrial and animal environments has flourished. Since environmental biotechnology has matured, it is ready to tackle bigger challenges: the scaling up of many bioremediation systems still in progress, the search for novel biocatalysts for industrial applications, the continuing effort against common human life-threatening processes such as the accumulation of hormone-mimicking substances (endocrine disrupters), the deposition of air-borne pesticides in the environment and, the degradation of recalcitrant contaminants. These endeavors will help prevent the contamination of food chains, protect human life and allow for human activity and economic development that do not compromise environmental sustainability.

Environmental biotechnology has to deal with an immense variety of compounds to be degraded, with varying characteristics of environments to be treated and, with a multitude of organisms with potential bioremediation activities. We have thus to develop a fully integrated knowledge base to tap

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the full potential of microbes as instruments for environmental management. The many concurrent developments in nanotechnologies, genomics and other approaches to complex biological systems will help us decipher and exploit the otherwise intractable *black boxes* of environmental biocatalysis. It is vital to develop conceptual, biological and computational tools for the systematic exploration of microbial diversity and for integration of disparate data. Furthermore, it is imperative to develop expert systems and models for the prediction of metabolic processes under various conditions. And most importantly, we need to integrate knowledge into a cohesive information system. Such integration will produce a true 21st century tool for both management and research purposes. Managed data become information and information fed to modeling and predictive systems becomes knowledge. This knowledge is an aid to decision-making and is critical for policy makers, scientists and industrial stakeholders. Most importantly, it allows them to work together.

The objective of this workshop was to bring together scientists from NATO and Partner countries to establish collaborative research on bioremediation. Bioremediation has become a generally accepted means of cleaning up polluted sites, particularly ones contaminated with various xenobiotic compounds. The main topic of the workshop will be bioremediation of soils contaminated with aromatic compounds (such as different herbicides, phenolic compounds, etc.). Definitely, the greatest potential for developing new bioremediation technologies, and improving existing ones, respectively, lies in increasing our knowledge of the metabolism, molecular biology and ecology of microorganisms. The development of a technology base for minimizing environmental and human health risks associated with the spread of environmental contamination by xenobiotic compounds requires networking of a critical mass of experts in the bioremediation and integration the research centers which have been omitted previously from this networking by historical/political reasons. Experts representing the major issues of environmental microbiology (microbial ecology and biodiversity, molecular microbiology and microbial physiology, biotechnology, biochemistry) were giving lectures on major facets of bioremediation.

The meeting was held on 1-3 July 2004 in Tartu, Estonia. The invited speakers included specialists (many of them are top scientists in the world) from different NATO countries (Belgium, Denmark, Germany, Italy, Spain, United Kingdom, USA, Canada), and from partner countries (Estonia, Finland, Latvia, Lithuania, Russia, Sweden, Switzerland).

The meeting was including different sessions:

1. Session 1 will cover different aspects of rhizoremediation, and bioavailability and transport of pollutants. Rhizoremediation is an attractive process since plant roots provide a large surface area for a significant population of bacteria and transport the root-colonising, remediating microorganisms to pollutants 10 to 15 m deep in the soil. Bioavailability of contaminating compounds is an important factor in determining efficiency of bioremediation.
2. Session 2 will discuss different aspects of molecular mechanisms of gene regulation in soil bacteria and genetic adaptation of microorganisms in the presence of environmental pollutants. Both specific and global regulatory mechanisms controlling aromatic catabolic pathways will be discussed.
3. Session 3 is focused on biodiversity and environmental genomics. Study of changes in microbial community composition in contaminated sites, exploration of catabolic potential of microorganisms, monitoring of gene transfer and measurement of catabolic genes expression *in situ* – all these approaches have a great outcome in assessment of pollutant discharge in natural environment.
4. Session 4 will discuss microbial stress adaptation. Natural environmental conditions always include different kinds of stress (e.g., starvation, temperature shock, changes in pH and osmolarity). Many xenobiotic compounds are also very toxic to the microorganisms. Thus, the investigation of adaptive responses of bacteria (and microbial communities) to environmental stress is necessary to get better insight in the function of microorganisms in contaminated sites.

Peter A. Williams from University of Wales gave an opening lecture titled "*Acinetobacter* sp. ADP1: a model biodegradative organism".

In the following two days, 18 internationally respected experts in their field presented summaries of their recent work. They represented the major issues of environmental microbiology: microbial ecology and biodiversity, molecular microbiology and microbial physiology, biotechnology, biochemistry. In detail, the workshop contained of 4 major sessions entitled:

Rhizosphere, Bioavailability and Transport

Molecular Biology, Gene Regulation and Genomics

Biodiversity and Environmental Genomics

Stress Adaptation

The second day of the workshop firstly dealt with several aspects of interaction of biodegrading micro-organisms with plant roots in the rhizosphere as well as transport and bioavailability of the xenobiotic substrates. This session was followed by one on the major future aspects of environmental ecology and microbiology, molecular biology, gene regulation and genomics. The third day of the meeting was dedicated to Biodiversity and Environmental Genomics. This was followed by presentations of the major aspects of Stress Adaptation.

Next to the oral presentations, the workshop contained of 33 posters that were discussed in two extra long sessions allowing especially young scientists to getting into contact with the international experts in their field of research. The programme included 18 oral presentations and a poster session.

A particular location of the workshop in the conference hall in Tartu University Library of Tartu, Estonia, was an ideal environment for an effective exchange of ideas and knowledge. This allowed an easy communication of the speakers and the participants during the workshop to establish personal contacts between people attending the conference and the formation of future collaborations and projects in the field of bioremediation. The participation of East European countries in EU proposals of the momentary running 6th Framework Programme is already strongly encouraged and will surely play a major role in future EC programmes. Therefore, contacts between already existing West European Network with East European scientists in the field of Bioremediation is of elementary interest for both sides.

The workshop demonstrated the importance and necessity of interdisciplinary research in modern environmental biotechnology. Next to the classical fields (microbiology, biochemistry, molecular biology) also other aspects as genome analysis and bioinformatics will become much more important in the near future. Especially from this point of view the meeting can be seen as a major step forward as it fostered an intensive discussion between scientists of all these fields of environmental biotechnology. The workshop helped to induce collaborations of scientists who had never been in contact before and can therefore be seen as a great success.

INFLUENCE OF THE RHIZOSPHERE ON THE BIODEGRADATION OF ORGANIC XENOBIOTICS – A CASE STUDY WITH 2,4-DICHLOROPHENOXYACETIC ACID

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Abstract: The rhizosphere is the unique environment at the plant-soil interface and a zone of intense soil-plant-microbe interaction. One recorded consequence of this complex interaction is the enhanced microbial biodegradation of organic xenobiotics. Mechanisms suggested to explain rhizosphere enhanced biodegradation range from the direct role of rhizodeposits acting as xenobiotic structural analogs to more indirect possibilities such as increased bioavailability in the rhizosphere. Our aim was to learn more about the mechanisms of enhanced biodegradation in the rhizosphere using the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) as a model xenobiotic. We report that 2,4-D mineralization is enhanced by root exudates and root debris of legume species. This rhizosphere enhancement may be due to the action of a rhizodeposit component in inducing the 2,4-D pathway. We discuss the possibility that the stimulatory component belongs to the chemical class flavonoids.

Key words: Biodegradation, 2,4-dichlorophenoxyacetic acid, rhizosphere, rhizoremediation, rhizodeposition

1. THE RHIZOSPHERE AND RHIZODEPOSITION

The existence of a distinctive microbial plant root environment, the rhizosphere, has been recognised for over a century (Hiltner, 1904). This spatially and temporally dynamic environment, when viewed in cross-section, may be categorized further to recognise sub rhizosphere zones: the

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ectorhizosphere (soil influenced by the root), endorhizosphere (intercellular space between the root tissues) and rhizoplane (root-soil interface). Influences of the plant root that define ectorhizosphere soil are numerous and include both physical (mechanical deformation of soil by the growing root; Bennie, 1991) and chemical (e.g. changed concentrations of inorganic ions and gases as a result of root activity; Bowen and Rovira, 1991; Jones *et al.*, 2004) effects. Perhaps paramount among the root influences that define the ectorhizosphere as a microbial environment is the process of rhizodeposition.

Rhizodeposition is defined as the total output of organic carbon compounds from the plant roots into the soil. Rhizodeposits may be grouped into two fractions: (i) exudates comprising active secretions (e.g. enzymes and mucilage) and compounds passively leaked from intact cells (e.g. sugars, amino and organic acids); and (ii) root debris comprising cell structural materials (e.g. lignin and cellulose and their breakdown products) and cell contents released by the decay of sloughed root cells and turnover of dead roots (Shaw and Burns, 2005). Estimates are variable and it is reported that between 2% (Jones *et al.*, 2004) and 40% (Paterson *et al.*, 1997) of carbon photosynthetically fixed is deposited in the ectorhizosphere soil as a result of exudation and root turnover. Out of the myriad of rhizodeposit compounds, most will be available (immediately or after extracellular enzyme attack) as a source of carbon and energy for microbial growth. Thus, the ectorhizosphere is characterized by elevated microbial numbers and activities in comparison to non-rhizosphere or bulk soil (Buyer *et al.*, 2002; Shaw and Burns, 2004); a phenomenon known as the 'rhizosphere effect'. Rhizodeposits not only serve as a source of carbon for microbial growth but also contain secondary metabolites that function as signalling molecules in the initiation of plant-microbial symbioses (Dakora and Phillips, 2002; Walker *et al.*, 2003; Gray and Smith, 2005) and are important in plant defence against pathogens (Singer *et al.*, 2004). In addition, plant roots exude allelopathic chemicals in an attempt to inhibit the growth of neighbouring plants (Walker *et al.*, 2003). Thus, the rhizosphere is a zone of intense rhizodeposit-mediated plant-plant and plant-microbe communication.

The quality and quantity of rhizodeposition varies between plants and, for a given species, is dependent on a host of biotic and abiotic factors (Jones *et al.*, 2004). These include plant nutritional status (Dakora and Phillips, 2002; Bertin *et al.*, 2003; Nguyen, 2003) the number and composition of microbes present (Meharg and Killham, 1995; Nguyen, 2003) and plant age (Uren, 2001; Bertin *et al.*, 2003). It has been estimated that the outer edge of the ectorhizosphere may be from 1.5 mm (Toal *et al.*, 2000) to 9 mm (Kandeler *et al.*, 2002) away from the root surface, meaning

that for densely rooted soil the ectorhizosphere of one root will overlap with that of another resulting in a rhizosphere continuum (Toal *et al.*, 2000).

2. FATE OF ORGANIC XENOBIOTICS IN RHIZOSPHERE SOIL AND MECHANISMS OF ENHANCED BIODEGRADATION

Long ago Hsu and Bartha (1979) hypothesized that the microbial biodegradation of insecticides would be enhanced in the rhizosphere in contrast to non-planted soil. They reasoned that the rate of cometabolism of diazinon and parathion would be accelerated by the presence of the plethora of potential carbon substrates released by rhizodeposition. Indeed, in experiments, they showed that the amount of insecticide carbon mineralised was more than doubled in the presence of bush bean, than in the control. Since this seminal study, numerous other studies (summarized in Shaw and Burns, 2003) have described enhanced biodegradation of organic xenobiotics in planted compared to non-planted soil. However, much of the research is solely descriptive as opposed to mechanistic; authors report the phenomenon of rhizosphere enhanced biodegradation and broadly attribute the enhancement to the elevated microbial numbers and activity without any attempt to scrutinize further the interactions between plants and microorganisms and the mechanisms which bring about increases in xenobiotic disappearance.

However, some authors (Siciliano and Germida, 1998; Shaw and Burns, 2003; Singer *et al.*, 2003) have speculated on the possible mechanisms of rhizosphere-enhanced xenobiotic biodegradation. Suggested mechanisms range from the direct role of rhizodeposits acting as xenobiotic structural analogs to more indirect possibilities such as increased bioavailability in the rhizosphere.

2.1 Rhizodeposits as xenobiotic structural analogs

It has been noted (Gilbert and Crowley, 1997; Siciliano and Germida, 1998; Dunning Hotopp and Hausinger, 2001; Shaw and Burns, 2003; Singer *et al.*, 2003) that some compounds released by rhizodeposition have structural similarity to xenobiotics or their breakdown intermediates (Figure 1). It can be predicted that such compounds will accelerate xenobiotic degradation, either by being used directly as an additional growth substrate by biodegradative microorganisms or, by acting as an inducer or

cometabolite in biodegradation. In support of the former idea that rhizodeposits act as substrates for biodegradative microorganisms, Leigh *et al.* (2002) have shown that mulberry (*Morus rubra*) root flavones can be used as sole carbon sources by the polychlorinated biphenyl degrading bacterium *Burkholderia* sp. LB400 and Rentz *et al.* (2004) demonstrated growth of *Pseudomonas putida* ATCC 17484 (a phenanthrene degrader) on *Salix*, *Thespesia* and *Cordia* spp. root exudates. Sandmann and Loos (1984) have noted that 2,4-D degrading microorganisms were selectively enriched in the pristine rhizosphere of sugar cane, an observation also supportive of the idea that rhizodeposits act as growth substrates for xenobiotic degraders.

It is well known that the expression of many xenobiotic catabolic pathways is regulated at the transcriptional level. A common theme in regulation is the involvement of an activator or repressor protein and the presence of an effector molecule, usually either the primary pathway substrate or an intermediate. For example, expression of the archetypal 2,4-D pathway (described in *Wautersia* (formerly *Ralstonia*) *eutropha* JMP134 pJP4) requires the presence of a LysR-type transcriptional activator (TfdR) and the inducing pathway intermediate, 2,4-dichloro-cis,cis-muconate (Filer and Harker, 1997). Similarly, the phenol and naphthalene catabolic operons can be activated in the presence of the regulatory proteins CatR and NahR with cis,cis-muconate and salicylic acid as the inducers, respectively. In addition to LysR-type transcriptional regulators, a diversity of other transcriptional regulatory protein families exists for mediating expression of catabolic operons (reviewed by Shingler, 2003; Tropel and van der Meer, 2004). Some transcriptional regulators of xenobiotic catabolism have been shown to display a certain degree of promiscuity, i.e. in addition to the native pathway substrate or intermediate effector molecule, they are able to interact with a (sometimes recalcitrant) structurally analogous compound to activate transcription (Shingler, 2003). For example, Cebolla *et al.*, 1997 have investigated interactions between NahR, the transcriptional regulator of the naphthalene degradative operons of catabolic plasmid NAH7, and potential aromatic effector molecules. They found NahR activated transcription from *Psal* (the promoter of the lower naphthalene pathway) not only in the presence of the native salicylate, but also in the presence of other aromatic compounds. It is possible that regulatory proteins of other catabolic pathways may also be able to accept a wider variety of chemical stuctures as effectors than currently explored. Thus, we suggest that derivatives of muconate, salicylate and possibly other compounds produced in the rhizosphere (whether by the plant directly or as a result of microbial attack on aromatic rhizodeposits) may contribute to the induction of

catabolism and explain accelerated biodegradation kinetics of some xenobiotics in the rhizosphere.

It should be mentioned that, in addition to the action of specific transcriptional regulators, the catabolic activity of a degradative bacterium is also under the control of global regulatory systems. These respond to nutritional signals and environmental stress and adjust the level of transcription to reflect the physiological condition of the cell (reviewed by Shingler, 2003). It can be assumed that the nutritional and stress conditions prevailing in the rhizosphere will be distinct from those in bulk soil. For instance, even though organic substrate supply may be more plentiful in the rhizosphere, microbial cells may be under greater toxic stress than their counterparts in the bulk soil due to the presence of allelopathic chemicals released by the plant root (Bertin *et al.*, 2003). Integration of these apparently contradictory environmental cues by global regulatory systems will undoubtedly impact the level of transcription of catabolic genes in the rhizosphere.

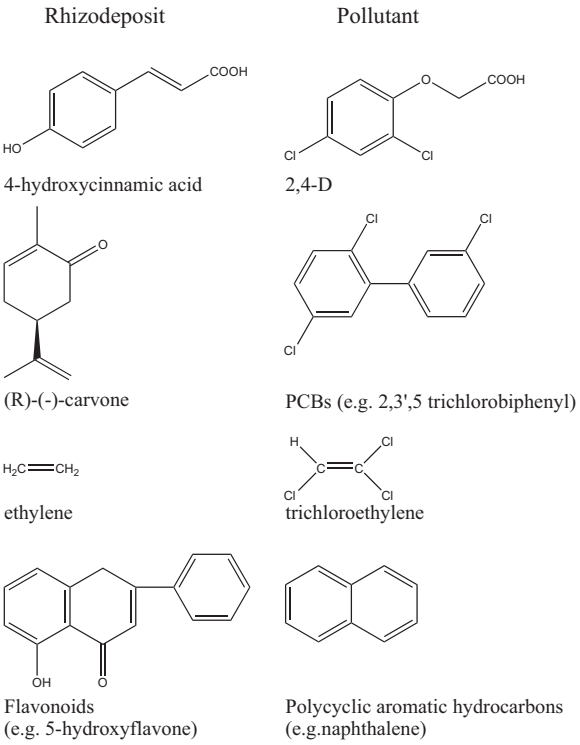

Figure 1. Structural similarity between rhizodeposit compounds and organic pollutants. Reproduced from Shaw and Burns (2003) with permission from Elsevier.

2.2 Genetic exchange

Horizontal gene transfer of xenobiotic catabolic genes *via* conjugative plasmids and other mobile genetic elements probably plays an important role in the adaptation of bacteria to degrade xenobiotics (Springael and Top, 2004). For conjugation to occur, donor and recipient cells must be in intimate contact and form a mating pair to initiate DNA transfer (Dale, 1998). However, processes that depend on cell activity and contact between cells may be restricted in the bulk soil since this environment generally has lower concentrations of available organic nutrients than does the rhizosphere and this will restrict the numbers and activity of the microorganisms and their likelihood of interaction. Indeed, microorganisms in bulk soil may exist in comparative spatial isolation, restricted in water films and sequestered in soil pores. In comparison, the probability of cell-cell contact in the rhizosphere is much greater: nutritional limitations are less as a result of rhizodeposition and, therefore, microbes are not only in higher densities but also may accumulate in biofilms on the rhizoplane (Ramey et al., 2004). For this reason, the rhizosphere has been referred to as a 'hot spot' for gene transfer (van Elsas and Bailey, 2002) and this view has been supported by experimental findings with (albeit non-catabolic) plasmids (Troxler *et al.*, 1997; Sorensen and Jensen, 1998).

The transfer of catabolic conjugative plasmids at detectable frequencies in soil (especially in the selective presence of the target pollutant) and the positive effect this has on pollutant biodegradation rates has been reported frequently (De Rore *et al.*, 1994; Top *et al.*, 1998; Dejonghe *et al.*, 2000; de Lipthay *et al.*, 2001). However, to our knowledge, no study has attempted to quantify horizontal transfer of catabolic genes in the rhizosphere in comparison to bulk soil although, significantly, de Lipthay *et al.* (2001) have reported enhanced transfer of a plasmid carrying the *tfd*A gene (encoding 2,4-D dioxygenase) in the barley 'residuesphere' (i.e. the environment associated with decaying straw).

2.3 Bioavailability

In soil, a xenobiotic may be considered to be in dynamic equilibrium between three potential phases: (i) freely dissolved in the soil solution; (ii) adsorbed to water-soluble humic material; or (iii) adsorbed to the soil organo-mineral solid colloidal phase. Of that proportion of the xenobiotic that is adsorbed, further distinction can be made between that which is surface-adsorbed and that which is sequestered (i.e. absorbed within three-dimensional structures) (Fig. 2). A bioavailable compound can be defined

simply as one which can interact with a biological system (Robinson and Lenn, 1994), but more complex definitions, which consider the configuration of adsorbed xenobiotic and the fluctuating pH of the microenvironment amongst others, have been provided (Burns and Stach, 2002). For xenobiotics that are biodegraded intracellularly, the chemical must be available for (passive or active) transport into the cell; the usual belief being that for this to occur the pollutant must be in solution (Mihelcic *et al.*, 1993). On the other hand, some suggest that it may be possible for bacteria to take up water-soluble humic material (WSHM)-associated compounds and release the xenobiotic in the cytoplasm for subsequent metabolism (Shaw *et al.*, 2000).

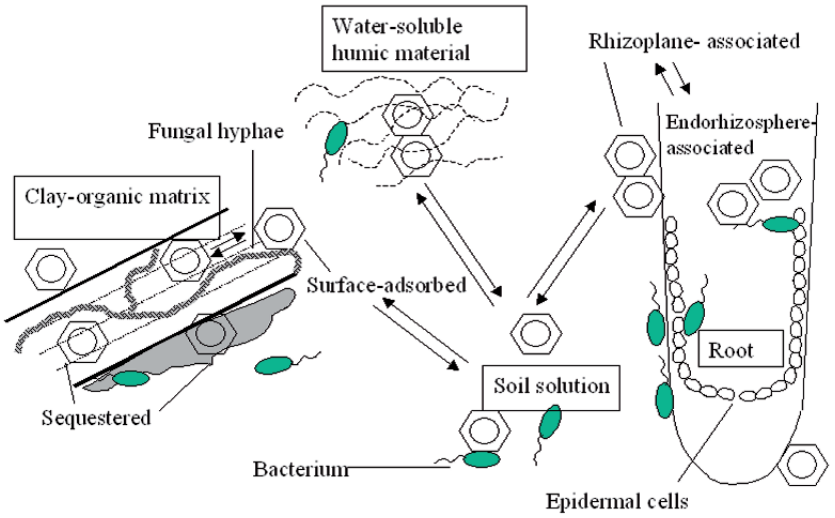

Figure 2. Xenobiotic partitioning between four phases (root, clay-organic matrix, water soluble humic material, and soil solution) in the rhizosphere. Pollutants in the root phase may be rhizoplane-associated or endorhizosphere-associated whereas pollutants in the clay-organic matrix phase may be sequestered within the three dimensional structure and its associated biofilm, be structural components of the humic molecules (i.e. bound residues) or adsorbed to the outside surface of the matrix. Reproduced from Shaw and Burns (2003) with permission from Elsevier.

In the intact rhizosphere many biological, chemical and physical factors will combine to alter the partitioning behaviour of a xenobiotic between soil, WSHM and solution phases, this, in turn, having implications for its bioavailability. To begin with, rhizosphere soil contains an additional sorptive entity when compared to non-rhizosphere soil, the root itself (Fig. 2). Organic pollutants in soil may reversibly or irreversibly adsorb to root

mucigel, cell wall constituents (such as hemicellulose and lignin) and membrane lipids; the tendency to adsorb depending on the hydrophobicity of the xenobiotic (Dietz and Schnoor, 2001).

In addition to acting directly as a sorptive surface, it is envisaged that the plant root also indirectly impacts on xenobiotic partitioning in its rhizosphere in a number of ways. For example, plant-induced pH change in the rhizosphere, which may be by as much as two pH units (Brimecombe *et al.*, 2001), will have consequences for the bioavailability of ionisable compounds whose sorption is largely dependent on the pH-dependent dissociation of the ionizable groups (Schellenberg *et al.*, 1984). Another way in which plant roots may alter the bioavailability of a xenobiotic is through the production of phytosurfactants (e.g. phosphatidylcholines), which, by lowering surface tension and forming micelles, act to increase dispersion and the apparent concentration of hydrophobic xenobiotics in the aqueous phase (Read and Gregory, 1997; Read *et al.*, 2003). Activity of mycorrhiza may also influence the bioavailability of xenobiotics through enrichment of organic matter in the rhizosphere (Joner and Leyval, 2003). Additional to the influence of the root on soil chemical properties, water potential in the rhizosphere will be strongly influenced. A significant volume of water will flow into and through the rhizosphere to the plant due to the transpiration stream. As a result, the transport of dissolved xenobiotics in the rhizosphere will be accelerated *via* convection and lead to their concentration at the root surface, thus increasing bioavailability by lessening spatial and diffusional constraints.

3. RHIZODEPOSIT-ENHANCED BIODEGRADATION OF THE HERBICIDE 2,4-D – A CASE STUDY

3.1 Evolutionary relationships between of 2,4-D degrading bacteria and relevance to the rhizosphere

One xenobiotic for which rhizosphere-enhanced biodegradation has been reported is 2,4-dichlorophenoxyacetic acid (2,4-D) (Boyle and Shann, 1995). The authors, using field-collected soil, showed that both the rate and extent of mineralization increased in the order non-planted < dicot rhizosphere < monocot rhizosphere.

2,4-D is a widely-used xenobiotic herbicide in the control of broad-leaved weeds with both intensive agriculture and small scale garden applications. Its microbial degradation is well described and bacteria

capable of using 2,4-D as a carbon and energy source have been isolated both from 2,4-D-exposed (Don and Pemberton, 1981; Ka *et al.*, 1994) and, interestingly, non-exposed (i.e.pristine) (Kamagata *et al.*, 1997; Itoh *et al.*, 2000) soils. 2,4-D-degrading isolates can be divided into three groups according to their experimentally measured growth characteristics and hypothesized evolutionary relationships (Kamagata *et al.*, 1997; Itoh *et al.*, 2004).

Group I contains copiotrophic bacteria, isolated from 2,4-D contaminated environments, and belonging to the β- and γ-*Proteobacteria*. The best-studied representative of this group, is the archetype β-*Proteobacteria, Wautersia eutropha* JMP134 (formerly *Ralstonia eutropha*) harbouring the catabolic plasmid pJP4. The initial step in 2,4-D breakdown is mediated by an α-ketoglutarate-dependent dioxygenanse, encoded by the *tfd*A gene, which cleaves the acetate side-chain to produce 2,4-dichlorophenol (Streber *et al.*, 1987; Fukumori and Hausinger, 1993a, b). The *tfd*B and *tfd*CDEF genes encode enzymes involved in the subsequent catabolism of 2,4-dichlorophenol through 3,5-dichlorocatechol and *ortho*-ring cleavage to produce TCA cycle intermediates. Other group I isolates have *tfd* genes in common, with *tfd*A genes having greater than 76% sequence similarity to the archetypal *tfd*A in *W. eutropha* JMP134 pJP4 (McGowan *et al.*, 1998).

Group II contains slow growing and oligotrophic isolates from pristine soils and belonging to the *Bradyrhizobium-Agromonas-Nitrobacter-Afipia* (BANA) cluster in the *α-Proteobacteria* (Kamagata *et al.*, 1997; Itoh *et al.*, 2000; Itoh *et al.*, 2002). Group III also contains members of the *α-Proteobacteria*, but these are fast growing, copiotrophic sphingomonads which were isolated from contaminated environments. Group II and III isolates possess *tfd*A-like genes (distinguished as *tfd*Aα), but these have only ~60% sequence identity to the *W. eutropha tfd*A (Itoh *et al.*, 2002; Itoh *et al.*, 2004). In addition to the *tfd*Aα genes, *cad*AB genes, which have a nucleotide sequence similar to that of *tft*AB encoding 2,4,5-T oxygenase, have also been identified in some of the group II and III isolates (Kitagawa *et al.*, 2002, Itoh *et al.*, 2004).

Group II degraders, because they were isolated from pristine environments, have attracted particular attention with respect to understanding the source and original function of the genes involved in 2,4-D degradation (Itoh *et al.*, 2004). It is argued that because pristine soils can mineralise 2,4-D (Fulthorpe, 1996) and that 2,4-D-degrading bacteria can be isolated from them, the genetic capacity for 2,4-D breakdown has been maintained for other reasons. Indeed, the protein encoded by the *tfd*A-like gene in the BANA cluster isolates from pristine soil has higher affinity for non-chlorinated phenoxyacids than for 2,4-D (Itoh *et al.*, 2002). Dunning

Hotopp and Hausinger (2001) have tested a range of alternative substrates for archetypal TfdA (in group I isolates) and concluded that the original substrate may have been a plant-derived compound, such as a cinnamic acid derivative (e.g. 4-hydroxycinnamic acid). Given the diversity of compounds produced in the rhizosphere, it is reasonable to hypothesize that many other natural substrates for 2,4-D degradative enzymes are found in rhizodeposits and that this may explain rhizosphere-enhanced mineralization of 2,4-D.

The aim of the experiments presented below were to: (i) confirm the rhizosphere-enhanced mineralization of 2,4-D reported by Boyle and Shann (1995) by comparing the fate of 2,4-D in non-planted soil with that collected from the rhizosphere of a dicotyledonous plant (*Trifolium pratense*) or a monocotyledon (*Lolium perenne*); (ii) test the hypothesis that rhizodeposits are acting as a growth substrate for 2,4-D degradative bacteria; and (iii) attempt to identify the stimulatory rhizodeposit components.

3.2 Mineralization of 2,4-D in pristine soil collected from the rhizosphere of *Lolium perenne* and *Trifolium pratense*

L. perenne or *T. pratense* plants were grown in boiling tubes containing moist soil. The brown forest soil used was collected from Sourhope Research Station experimental site, Cheviots, Scotland, and had not been exposed previously to 2,4-D. As controls, additional tubes were left non-planted. Four replicate tubes per plant treatment were destructively sampled 0, 25, 60 and 116 days after planting. The shoots were excised and discarded and the root chopped finely and homogenised with the soil. Sub-samples were taken to determine the 2,4-D mineralisation potential. The time at maximum rate (t_1, dependent on length of the lag phase prior to onset of exponential mineralization) and the maximum rate were estimated from the sigmoidal cumulative percent mineralization curves obtained (Fig. 3). On day 25 and 60, there was a pronounced reduction in the length of the lag phase for the *T. pratense* treatment when compared to *L. perenne* or non-planted soil. This was reflected in the parameter t_1 (Fig. 3A); e.g. t_1 = 22.0 ± 0.6 and 15.3 ± 0.7 on day 60 for non-planted and *T. pratense* planted, respectively. The rhizosphere of *T. pratense* also had a significant ($p<0.05$) effect on the maximum rate of 2,4-D mineralization on harvest days 25 and 60 (e.g. *T. pratense* = 10.0 d^{-1}, non-planted = 7.6 d^{-1} for day 25; Fig. 3B). By day 116, the *T. pratense* rhizosphere effect on both t_1 and maximum rate was no longer significant ($p>0.05$). Thus, on one level, our mineralization assays results agree with those of Boyle and Shann (1995)

who report the positive effect of the rhizosphere on 2,4-D degradation. Again, as with Boyle and Shann (1995), our rhizosphere effect was plant species specific, but conversely, our effect was more pronounced for the dicotyledon (*T. pratense*) than the monocotyledon (*L. perenne*).

3.3 Untangling the mechanisms of *Trifolium*-enhanced mineralization

Some possible mechanisms responsible for rhizosphere-accelerated xenobiotic mineralization have been discussed earlier. We decided to examine the first hypothesis (section 2.1) that rhizodeposits are xenobiotic structural analogs whose presence selectively enriches those degradative bacteria that can use the analog directly as a growth substrate. If this was the case, we would expect: (i) elevated numbers of 2,4-D degraders in the rhizosphere of pristine soil compared to non-planted pristine soil; and (ii) a rhizosphere-dependent shift in the genotypic diversity of 2,4-D degraders.

3.3.1 Are rhizodeposits a growth substrate for 2,4-D degrading microorganisms?

The most probable number of 2,4-D degraders ($MPN_{2,4\text{-}D}$) was determined for soil sub-sampled from the rhizosphere time course experiment (section 3.2). $MPN_{2,4\text{-}D}$ were low (<100 g^{-1}, and in some replicates below detectable limits) for pristine soil (Fig 4A) and not related to planting treatment ($p=0.063$) or harvest day ($p=0.33$). In contrast, soil that had been exposed to 2,4-D had significantly greater numbers of 2,4-D degraders (Fig. 4B); median values ranged from 6×10^5 g^{-1} (non-planted) to 1×10^6 g^{-1} (*T. pratense*) although plant treatment did not have a significant effect ($p=0.91$). The estimates of $\sim 1 \times 10^6$ g^{-1} in 2,4-D-exposed soil demonstrate that the $MPN_{2,4\text{-}D}$ method used was effective at detecting increases in 2,4-D degrader numbers in response to a selective pressure.

In addition to the MPN analysis, we used single strand conformational polymorphism (SSCP) analysis to examine the effect of the rhizosphere on the sequence diversity of *tfd*Aα genes. We targeted the *tfd*Aα genes (of group II and III degraders, section 3.1) as we failed to obtain amplification products with primers targeting canonical group I *tfd*A. PCR of soil extracted DNA with *tfd*Aα primers yielded faint PCR products of the expected 359 bp size from soil not previously exposed to 2,4-D and more distinct products for soil exposed to 2,4-D. Furthermore, DNA extracted from highest dilution (10^{-5} and 10^{-6}) positive $MPN_{2,4\text{-}D}$ tubes set up with 2,4-D-exposed soil (Fig. 4B) yielded amplification products in 85% of cases suggesting that the *tfd*Aα gene was numerically important. SSCP

analysis of soil PCR products followed by hierarchical cluster analysis of SSCP band patterns (Fig. 5) showed that the samples clustered according to 2,4-D exposure history, thus demonstrating the ability of the method to detect the impact of a selective pressure on the 2,4-D degrading population. However, plant treatment had no effect on the diversity of *tfd*Aα genes, either in soil not exposed to 2,4-D or in soil previously contaminated with 2,4-D.

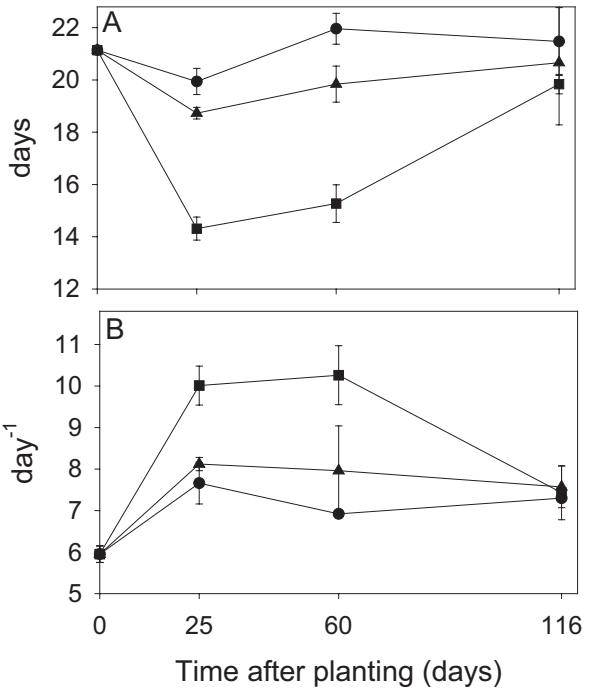

Figure 3. Effects of plant species and plant age on 2,4-D mineralization parameters in soil sampled from the rhizosphere of *L. perenne* (▲), *T. pratense* (■), or non-planted controls (●). Effects are expressed as time at inflection (A), calculated maximum rate (B). To conduct the mineralization assay, soil sub-samples (4 g) in gas-tight screw-cap vials were amended with ^{14}C-UL- 2,4-D (50 μg g^{-1} and 250 Bq g^{-1}) in a sufficient volume of sterile distilled water to bring the soil moisture content to 70 % of the maximum moisture holding capacity. A test tube containing NaOH (1 ml, 1 M) was placed onto the surface of the soil to trap the $^{14}CO_2$ released. Vials were incubated at 25 °C. On sampling days, the NaOH solution was taken for quantification of radioactivity and replaced with fresh NaOH solution. Bars represent standard error. Reproduced from Shaw and Burns (2004) with permission, American Society for Microbiology.

Both the $MPN_{2,4\text{-}D}$ and PCR-SSCP evidence suggests that 2,4-D degradative bacteria do not use rhizodeposit xenobiotic analogues directly as a growth substrate. It seems that the 2,4-D degraders, in the absence of 2,4-D, are present in soil in low numbers and are not influenced by the *T. pratense* root. However, when 2,4-D is added to soil, a sub-population of 2,4-D degraders initially present utilize 2,4-D for growth, and the composition of this sub-population (according to the diversity of *tfd*Aα of genes) is not affected by planting treatment. However, in soil previously planted with *T. pratense*, the sub-population are able to mineralize 2,4-D with a shorter lag and at a faster rate than in the non-planted and *L. perenne* treatments (Fig. 3).

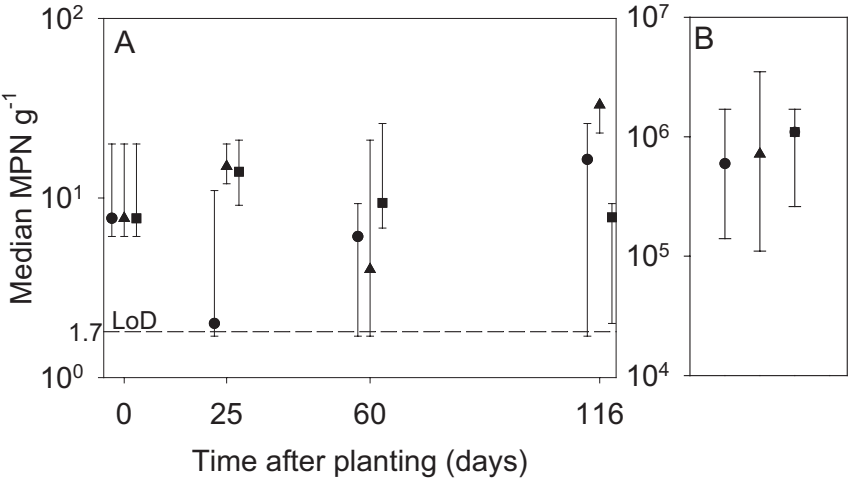

Figure 4. (A) Effects of plant species and plant age at harvest on $MPN_{2,4\text{-}D}$ in pristine soil sampled from the rhizosphere of *L. perenne* (▲), *T. pratense* (■), or non-planted controls (●). (B) $MPN_{2,4\text{-}D}$ in soil sampled from 25-day-old rhizospheres or non-planted controls and incubated in the presence of 50 μg of 2,4-D g^{-1} for 36 days. Ten-fold dilutions were inoculated to tubes (5 replicates $dilution^{-1}$) containing modified basal medium (Dorn et al., 1974) supplemented with 50 μg ml^{-1} ^{14}C-UL-2,4-D. After incubation (9 weeks, 20 °C), radioactivity remaining was quantified and tubes containing > 60% of the non-inoculated controls were scored positive. $MPN_{2,4\text{-}D}$ estimates were derived by reference to MPN tables (Alexander, 1982). Error bars represent maximum and minimum values, LoD, limit of detection. Reproduced from Shaw and Burns (2004) with permission, American Society for Microbiology.

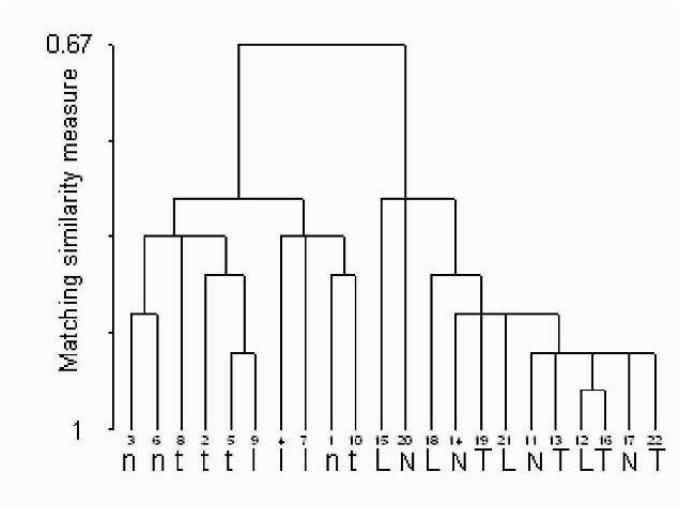

Figure 5. Hierarchical cluster analysis (using matching binary similarity distances and the single linkage method) of *tfd*Aα SSCP banding patterns. N, L and T, replicate sample lanes from non-planted, *L. perenne* planted and *T. pratense* planted soils, respectively. Lowercase letters, non-2,4-D exposed samples; capital letters, 2,4-D exposed samples. DNA extracted from soil sub-sampled from the rhizosphere time course experiment (section 3.2) was PCR amplified using primers *tfd*Aα1 (5'-CCGGCGTCGATCTGCGCAAG-3') and *tfd*Aα2 (5'-GTTGACGACGCGCGCCGACA-3') designed to amplify a 359 bp region of the α-Proteobacterial *tfd*Aα gene (Shaw and Burns, 2004). The SSCP analysis protocol was based on the method described by (Schwieger and Tebbe, 1998). Reproduced from Shaw and Burns (2004) with permission, American Society for Microbiology.

If the mineralization kinetics of 2,4-D in the harvested *T. pratense* rhizosphere cannot be explained by increased numbers of 2,4-D degraders resulting from their use of rhizodeposits as growth substrates, another explanation must be sought. Alternative mechanisms (reviewed earlier) include the rhizosphere-enhanced horizontal transfer of 2,4-D catabolic elements or the action of phytosurfactants to increase 2,4-D bioavailability. We do not think that either of these explanations are likely. First, the *tfd*Aα gene does not appear to be readily transmissible (Kamagata *et al.*, 1997; Itoh *et al.*, 2004) and second, we could not detect any bioavailability effect after comparison of 2,4-D sorptive behaviour in *Trifolium* and non-planted soil (data not shown).

One explanation that cannot be ruled out relates to the possible role of a rhizodeposit xenobiotic analog, not as a growth substrate, but as an inducing agent (discussed in section 2.1). With specific reference to 2,4-D, we know that the archetypal pathway in *W. eutropha* JMP134 (a group I degrader) is regulated by a LysR-type transcriptional regulatory protein encoded by *tfd*R (Leveau and van der Meer, 1996; Vedler *et al.*, 2000). The

inducing effector molecule of the pathway is thought to be the intermediate, 2,4-dichloromuconate (Filer and Harker, 1997; Vedler *et al.* 2000). Whether TfdR can accept chemical structures other than 2,4-dichloromuconate as effectors has not been investigated, however, (Ogawa *et al.*, 1999) have reported that the LysR-type regulator of chlorocatechol degradation in a *Ralstonia eutropha* can be activated by both chlorinated and non-chlorinated muconate. Muconates are the product of metabolism of catechols *via* the central *ortho*-cleavage pathway. Many substituted phenols and aromatic acids (produced by rhizodeposition) could be degraded through this route. Thus, it could be suggested that muconates produced in the *T. pratense* rhizosphere activate transcription of *tfd* genes through interaction with TfdR, causing the observed decrease in the lag phase and increase in mineralization rate.

The preceding argument assumes that group I degraders were responsible for the observed 2,4-D mineralization. However, PCR analysis of DNA extracted from soil and positive $MPN_{2,4\text{-}D}$ tubes using primer sets to target group I *tfd*A and group II and III *tfd*Aα suggested that organisms possessing the *tfd*Aα gene were present in the study soil. Moreover, according to PCR-SSCP, *tfd*Aα- possessing organisms responded (increased PCR product intensity, shift in diversity) when soil was amended with 2,4-D meaning that they were probably responsible for the observed 2,4-D mineralization. Because the presence of *tfd*Aα in group II and III degraders is a recent discovery (Itoh *et al.*, 2002; Itoh *et al.*, 2004), the regulation of the pathway has yet to be characterized. Therefore, until we know more, any discussion modelled on the archetypal 2,4-D pathway, in the assumption that the mechanism of transcription induction of *tfd*A and *tfd*Aα are the same, is speculative.

In addition to *tfd*Aα, a second degradative route mediated by the *cad* genes may exist in group II and III degraders (see section 3.1). In fact, Itoh *et al.* (2004) present evidence to suggest that the *cad* system may have a greater role in 2,4-D degradation in group II degraders than *tfd*Aα. Analysis of the *cad* genes in a group II degrader (*Bradyrhizobium* sp. strain HW13) suggests that *cad*R (an AraC/XylS type transcriptional regulator) is primarily responsible for the induction of *cad*A (2,4-D oxygenase large subunit) by 2,4-D and 4-chlorophenoxyacetic acid (Kitagawa *et al.*, 2002).

Despite the uncertainty regarding the mechanisms and the microorganisms responsible, we feel that the role of a rhizodeposit inducer is a likely explanation for the *Trifolium* enhanced 2,4-D mineralization. However, further experiment is required to test this hypothesis.

3.4 The identity of stimulatory rhizodeposit components

Our next research objective was to narrow down the identity of the stimulatory rhizodeposit (putative inducer) responsible for the enhanced biodegradation of 2,4-D. Specifically, our aims were to determine: (i) the inducer's distribution among rhizodeposit fractions (exudates or root debris); (ii) the effect of the presence of microorganisms on inducer production; and (iii) whether inducer production was exclusive to *Trifolium pratense*, or a property of distinct plant types (i.e. legumes or dicotyledons).

3.4.1 Location in rhizodeposit fractions

Total *Trifolium* rhizodeposits were divided in to two experimentally-defined fractions (see section 1): (i) exudates (exuded into the external milieu and probably ranging in composition from simple organic compounds to enzymes), and (ii) root debris (washed chopped roots ranging in composition from root structural components, i.e. cellulose and lignin, to exudate-like cell contents). These two fractions were added to soil. 2,4-D mineralization experiments revealed that the stimulatory rhizodeposit component was present in both fractions (data not shown).

3.4.2 Effect of microorganisms on production

The effect of the indigenous soil microflora was investigated by comparing amendment of previously non-planted soil with *Trifolium* root debris grown in non-sterile soil with that grown gnotobiotically in autoclaved soil (Fig. 6). In comparison to non-root amended soil, the expected mineralization enhancement was seen when soil was amended with *Trifolium* root debris grown in non-sterile soil. Interestingly, amendment with roots grown under gnotobiotic conditions had no significant effect on 2,4-D mineralization parameters.

3.4.3 Effect of plant species

Rhizosphere soil sampled from plant species belonging to classes monocotyledon, dicotyledon non-legume and dicotyledon legume (3 species per class) was used in 2,4-D mineralization assays to examine whether or not enhanced mineralization was particular to *Trifolium pratense*, or could be ascribed to a broader range of plant species. Mineralization parameters (t_1 and maximum rate) are shown in Fig. 7. The legumes had by far the greatest positive impact on mineralization;

maximum mineralization rates ranged from 2.9- (*Trifolium pratense*) to 4.5- (*Lotus corniculatus*) times that of the control. Nonetheless, some of the non-legume plant species tested also gave a smaller but significant ($p<0.05$) positive impact on the maximum mineralization rate and/or t_1.

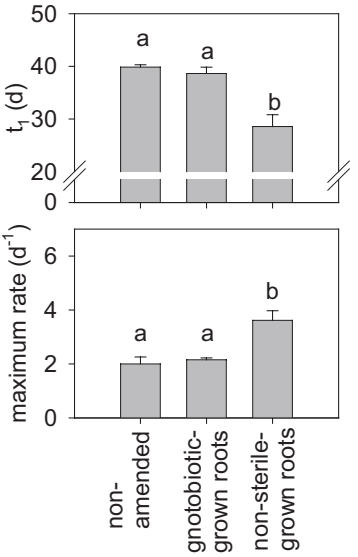

Figure 6. Effect on 2,4-D mineralization kinetics of amendment of previously non-planted soil with *Trifolium* root debris fraction prepared from plants grown under gnotobiotic conditions in autoclaved soil or in the presence of indigenous soil microorganisms in non-sterile soil. Plants were 34 days old on harvest. Mineralization assays were conducted as described in Fig. 3. Bars superscripted by the same letter are not significantly different ($p>0.05$). Values are mean ± standard error (n=3). Reproduced from Shaw and Burns (2005) with permission, Blackwell Publishing Ltd.

3.5 A role for plant secondary metabolites in rhizosphere-enhanced biodegradation of organic xenobiotics?

The plant species specificity of 2,4-D mineralization enhancement (section 3.4.3) suggests that stimulatory compounds in legume rhizodeposits are of superior quality and/or quantity than for other species. It appears also that the presence of microorganisms is required for the production of the rhizodeposit stimulant (section 3.4.2) and implies that signalling between legume and microbe is an important factor in

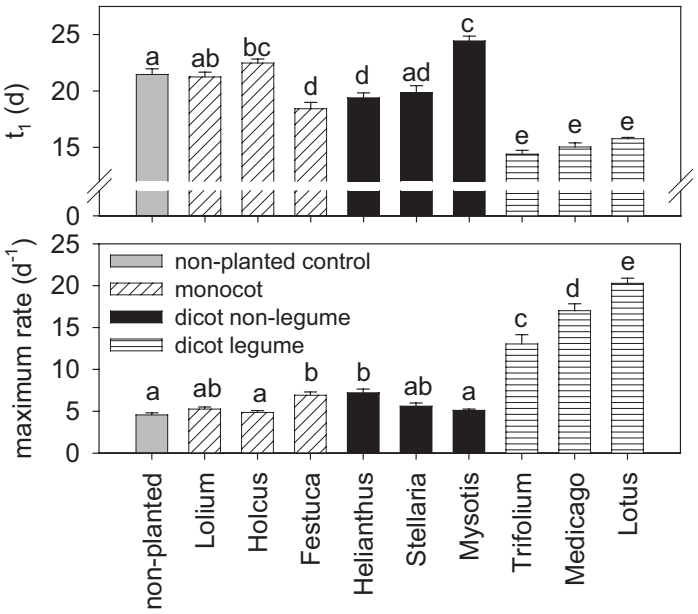

Figure 7. Effect of different plant species representative of the monocotyledons, dicotyledons (non-legume) and dicotyledons (legume) on 2,4-D mineralization parameters (t_1 and maximum rate). Replicate tubes were planted with the following numbers of seeds per tube: *Festuca arundinacea* (15); *Helianthus annuus* (3); *Holcus lanatus* (15); *Lolium perenne* (15); *Lotus corniculatus* (15); *Medicago sativa* (15); *Myosotis arvensis* (15); *Stellaria media* (15); *Trifolium pratense* (15); *Trigonella foenum-greocum* (8); *Vicia sativa* (3); *Vigna unguiculata* (3). Seeds of *Helianthus annuus* (variety 'Pacino') were obtained from WW Johnson & Son Ltd (Boston, England). All other seeds used were obtained from Herbiseed (Twyford, England). Additional replicates were left non-planted as controls but were otherwise treated identically to the planted tubes. Tubes were incubated at 20 °C in a 16 h light (4 200 lux), 8 h dark regime. Tubes were destructively sampled 33 days after sowing (except for *Helianthus annuus*, which was 18 d after sowing): shoots were excised and discarded and the root chopped finely with a sterile scalpel and homogenised with the soil. Soil-subsamples were used for mineralization assays (as described in Fig. 3). Bars superscripted by the same letter are not significantly different ($p>0.05$). Values are mean ± standard error (n=4). Reproduced from Shaw and Burns (2005) with permission, Blackwell Publishing Ltd.

rhizosphere-enhanced 2,4-D mineralization. The best-studied exchange of signals that occur between legume and microbe are those between rhizobia and their host plant during the establishment of a nitrogen fixing symbiosis (Hungria and Stacey, 1997; Broughton *et al.*, 2000; Jain and Nainawatee, 2002; Broughton *et al.*, 2003; Loh and Stacey, 2003). Signals from the host

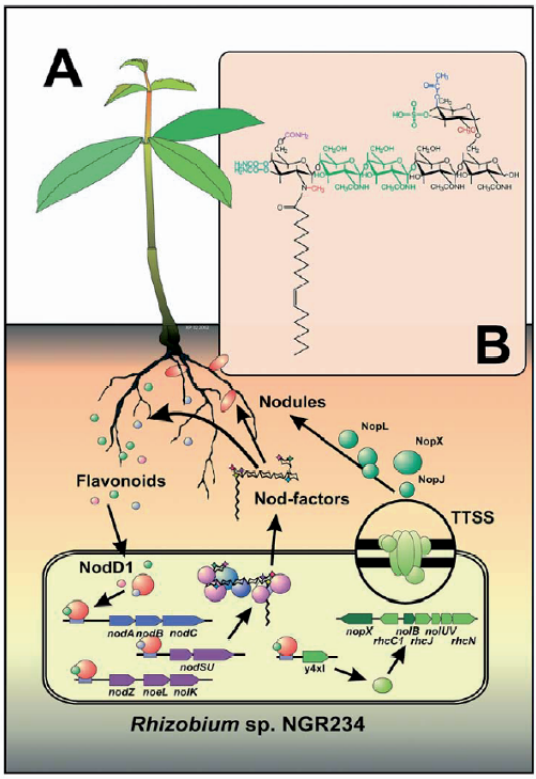

Figure 8. Signal exchange during the initiation of nodule formation in the nitrogen fixing symbiosis: *Rhizobium* sp. NGR234 as an example. (A) Flavonoids produced in the legume exudates interact with the rhizobial transcriptional regulator, NodD1, and induce the expression of the nodulation genes encoding the enzymes responsible for the synthesis of lipo-chito-oligosaccharide Nod factors (Fig. 8B). Nod-factors, in turn, trigger various plant developmental steps that culminate in the formation of a mature nodule and symbiotic N_2 fixation. In the example shown, NodD1 in conjunction with flavonoids, also controls expression of a type III secretion system. Reproduced from Broughton et al. (2003) with kind permission of Springer Science and Business Media.

plant usually belong to the chemical class flavonoids and are produced in the exudates (Fig. 8). Plant pathways resulting in increased flavonoid production are induced by microbial elicitors (Recourt *et al.*, 1991; Recourt *et al.*, 1992; Dakora and Phillips, 1996; Broughton *et al.*, 2000), consistent with our finding that the production of the stimulatory compound was dependent on the presence of microorganisms. Flavonoids are produced by the phenylpropanoid biosynthetic pathway in plants and accumulate in the vacuoles (Aoki *et al.*, 2000) for release in exudates: coherent with the detection of 2,4-D degradation stimulation by both exudates and root debris

(section 3.4.1). The release of phenolic compounds accumulated in the vacuole during root turnover and the possible role of this near constant supply of stimulatory compounds in rhizoremediation (Leigh *et al.*, 2002) has been noted. In addition, flavonoids are common to vascular plants other than legumes (Aoki *et al.*, 2000). Therefore, it is not surprising that we observed stimulatory effects with some of the non-legume species tested (section 3.4.3). Thus, perhaps the flavonoids are candidates worthy of investigation when searching for the identity of the legume-produced 2,4-D degradation stimulant.

If flavonoids are integral to legume-enhanced 2,4-D biodegradation, what would be the ecological reason? It has been suggested that during the initiation of symbiosis, such as N fixation, mutualistic microbes are likely to encounter inhibitory concentrations of flavonoids (Dakora and Phillips, 1996). In fact, the ability to combat the toxicity of flavonoids would be a likely requirement for any rhizosphere-dwelling microorganism. It has been shown that rhizobia can catabolise flavonoids producing cinnamic acid derivatives as breakdown products (Rao *et al.*, 1991; Rao and Cooper, 1994). The bacteria responsible for 2,4-D catabolism in our experiments may have been Group II degraders and closely related to the bradyrhizobia (section 3.3.1). If it this were the case, maybe the 2,4-D and flavonoid catabolic pathways in the bradyrhizobia are synergistically linked; this would explain the legume rhizodeposit enhanced 2,4-D degradation. The *Rhizobiacae* are known to be able to degrade diverse hydroxylated aromatic compounds via the protocatechuate branch of the β-ketoadipate pathway (Parke and Ornston, 1986) whereas 2,4-D degradation proceeds *via* the (convergent) catechol branch. The fact that the TfdA protein has been shown to accept substituted cinnamic acids as substrates (Dunning Hotopp and Hausinger, 2001) may be a significant finding. Research to further characterize the molecular basis of both 2,4-D and flavonoid biodegradation in model bradyrhizobial strains is required to ascertain if there is potential for convergence of, or interaction between, the two pathways.

4. SUMMARY

We report that 2,4-D mineralization is enhanced by root exudates and root debris of legume species. This rhizosphere enhancement may be due to the action of a rhizodeposit component in inducing the 2,4-D pathway. We present evidence to suggest that degraders belonging to group II and/or III (see section 3.1) are important in mediating the 2,4-D mineralization and argue that the stimulatory compound (putative inducer) belongs to the chemical class flavonoids. Further research is needed to identify positively

the interactive role of bradyrhizobia and flavonoids in the legume-enhanced mineralization of xenobiotics. Such research could have implications for understanding not only the mechanisms behind rhizosphere-enhanced xenobiotic biodegradation but also the evolution of the 2,4-D degradative pathway. The idea that plant secondary metabolites and plant-microbe interactions may be important in the evolution of new xenobiotic catabolic pathways has been discussed recently (Singer *et al.*, 2003; Singer *et al.*, 2004). Further examination of the 2,4-D pathway with this emphasis may be enlightening, and help explain why the distribution of *tfd*Aα gene homologs seems to be ubiquitous among the bradyrhizobia (Itoh *et al.*, 2004) and why phytohormone indole acetic acid biosynthestic genes have been found adjacent to 2,4-D degradative gene clusters (Poh *et al.*, 2002; Vedler *et al.*, 2004).

ACKNOWLEDGEMENTS

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REGULATION OF THE ATRAZINE DEGRADATIVE PATHWAY IN *Pseudomonas*

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Abstract: In recent times, the use of the *s*-triazine herbicide atrazine has become a major concern, due to increasing evidence of severe ecotoxicological effects. The development of strategies for bioremediation of contaminated soils and waters requires the isolation and development of strains that harbor an appropriate catabolic pathway, are competitive in the wild and display the degradative phenotype under field conditions. A limitation to the use of bioremediation for the decontamination of atrazine-polluted sites is the fact that the presence of preferential nitrogen sources, such as those used for fertilization of agricultural soils often inhibits the degradative pathway, resulting in low degradation rates. We have characterized this phenomenon in the model strain *Pseudomonas* sp. ADP. In this organism, atrazine degradation is nitrogen-repressed both in cultures and in soil. Nitrogen status is sensed from intracellular pools of metabolites. We have used this knowledge to develop a mutant unable to assimilate nitrate that displays an efficient degradative phenotype in nitrate-amended soil. The inhibitory effect of nitrogen operates at the level of gene expression. One of the targets of this regulation is the cyanuric acid degradative operon *atzDEF*, which is coordinately activated by nitrogen limitation and the presence of cyanuric acid. A complex regulatory circuit involving at least two regulators and two forms of RNA polymerase is responsible for both responses. The atrazine degradative pathway has proven an attractive model for both basic and applied studies on biodegradation.

Key words: Atrazine, Bioremediation, Nitrogen control, Gene regulation.

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1. INTRODUCTION

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is the most widely used herbicide in the world. Atrazine belongs to the *s*-triazine family and is used for broad-leaf weed control both in crop and non-crop lands. Due to its widespread use and high mobility in soils, atrazine has often been detected at concentration exceeding the legal limits in surface and ground waters (Kolpin and Kalkhoff, 1993; Kolpin *et al*., 1996; Richards and Baker, 1993; Struthers *et al*., 1998). Although generally considered as a fairly safe substance for human health, the frequent occurrence of atrazine-contaminated waters and an increasing concern about ecotoxicological properties of the herbicide (Allran *et al*., 2000; Hayes *et al*., 2003; Hayes *et al*., 2004) have prompted new research directed to the development of bioremediation strategies.

Since the mid-1990s, an increasing number of bacteria have been reported to show an atrazine biodegradative phenotype, including some that fully mineralize the herbicide (see Ralebitso *et al*., 2000, and references therein). Atrazine is most often used as a nitrogen source, since full oxidation of the carbon atoms in the *s*-triazine ring renders them unusable as an energy source. Yet, a few bacterial strains have been identified that use the isopropylamine and ethylamine side chains as a carbon source (Yanze-Kontchou and Gschwind, 1994; Strong *et al*., 2002). The best-characterized atrazine degrading strain is *Pseudomonas* sp. ADP (Mandelbaum *et al*., 1995). *Pseudomonas* sp. uses atrazine as a nitrogen source. Six hydrolytic steps are responsible for atrazine mineralization in this strain. The genes *atzA-F*, encoding the six enzymes involved in the pathway are located in the 108-Kbp conjugative plasmid pADP-1 (Martinez *et al*., 2001). Atrazine chlorohydrolase (de Souza *et al*., 1995), hydroxyatrazine ethylaminohydrolase (Boundy-Mills *et al*., 1997) and *N*-isopropylammelide isopropylaminohydrolase (Sadowsky *et al*., 1998) encoded by *atzA*, *atzB* and *atzC*, respectively, sequentially remove the substituents of the triazine ring to yield the central intermediary cyanuric acid. These genes are located at distant locations within a >40 Kbp unstable region in pADP-1. The frequent appearance of spontaneous Atr^- (non-atrazine degrading) mutants is due to loss of one or more of these genes in non-selective medium (de Souza *et al*., 1998b). The *atzA*, *atzB* and *atzC* genes have been shown to be widespread and plasmid-borne in multiple atrazine-degrading isolates from different parts of the world (de Souza *et al*., 1998a; de Souza *et al*., 1998b; Rousseaux *et al*., 1995; Topp *et al*., 2000; Sajjaphan *et al*., 2004). The *atzD*, *atzE* and *atzF* genes encode cyanuric acid amidohydrolase, biuret amidohydrolase and allophanate hydrolase activities responsible for ring cleavage and mineralization of

cyanuric acid (Martinez *et al*., 2001). These genes are clustered in a different location within pADP-1 to form the *atzDEF* operon. In addition to the genes and activities described in *Pseudomonas* sp. ADP, a number other pathways with alternative activities and intermediates have been described in other organisms (Wackett *et al*., 2002).

2. ATRAZINE DEGRADATION BY *Pseudomonas* sp. ADP IS NITROGEN REGULATED

A major issue in developing strains suitable for bioremediation is the ability of the chosen organism to display an efficient degradative phenotype under field conditions. Since most atrazine-degrading strains use it as a nitrogen source and agricultural soils are often rich in nitrogen due to routine fertilization, the effect of nitrogen amendments on atrazine degradation has been tested both in culture and in soil. Addition of inorganic and organic nitrogen has repeatedly been shown to retard atrazine degradation by indigenous populations in soils (Abdelhafid *et al*., 2000a; Abdelhafid *et al*., 2000b; Alvey and Crowley, 1995; Entry *et al*., 1993). Mandelbaum *et al*. (1993) showed that atrazine mineralization by a mixed culture was strongly inhibited by ammonium nitrate. Inhibition of atrazine degradation by added nitrogen has also been shown in *Ralstonia* sp. M91-3 (Radosevich *et al*., 1995; Gebendinger and Radosevich, 1999). However, atrazine mineralization by *Agrobacterium radiobacter* J14A was not inhibited by addition of ammonium or nitrate. Bichat *et al*. (1999) reported that degradation of atrazine by *Pseudomonas* sp. ADP was also unaffected by the presence of other nitrogen sources, albeit a close examination of their data reveals significantly reduced degradation rates in medium containing ammonium, nitrate or urea.

We have undertaken the characterization of the effect of other nitrogen sources on atrazine utilization by *Pseudomonas* sp. ADP. A simple spectrophotometric resting cell assay was developed in which cells grown in the presence of the required nitrogen sources were collected in mid-exponential phase, washed extensively and suspended in buffer. The assay was initiated by addition of atrazine, and the evolution of the atrazine concentration was monitored as the decrease of A_{225} in the supernatants (García-González *et al*., 2003). In this experimental system, cells grown on ammonium, urea, nitrate or proline as the sole nitrogen source displayed decreased degradation rates when compared to atrazine-grown cells. However, growth on cyanuric acid, biuret or serine failed to inhibit atrazine degradation. There was a clear correlation between growth rate and inhibition: nitrogen sources that support fast growth of *Pseudomonas*

sp. ADP inhibit atrazine removal, while those that limit growth of the bacterial strain have no significant effect on degradation (García-González *et al*., 2003). The sole exception to this rule was cyanuric acid. Growth rate on cyanuric acid was comparable to that on nitrate, yet fast atrazine elimination occurred in the presence of the former, but not the latter. This discrepancy may however be attributed to a specific positive effect exerted by cyanuric acid on the degradative pathway (see below). It is also worth noting that the presence of atrazine in the growth medium did not stimulate metabolization when any of the readily assimilable nitrogen sources was present. Therefore, atrazine utilization is downregulated in the presence of preferential nitrogen sources, and is not inducible by the substrate of the pathway. These results are summarized in Figure 1.

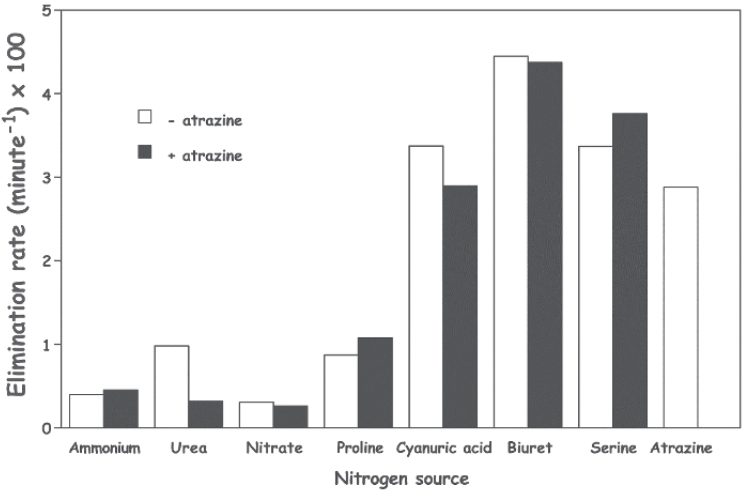

Figure 1. Effect of growth on different nitrogen sources on atrazine elimination by *Pseudomonas* sp. ADP.

The similarities between our observations and general nitrogen control (Merrick and Edwards, 1995) prompted us to test whether nitrogen availability was sensed in *Pseudomonas* sp. ADP from intracellular pools of key metabolites, as thoroughly documented in the Enterobacteria. The addition of sublethal concentrations of MSX, an inhibitor of the key ammonium assimilatory enzyme glutamine synthetase, resulted in the release of ammonium-mediated inhibition of atrazine utilization. This indicates that the presence of the preferential nitrogen source (ammonium) was not detected in the absence of efficient assimilation (García-González *et al*., 2003).

3. A READILY ISOLATED Nas$^-$ MUTANT OF *Pseudomonas* sp. ADP DEGRADES ATRAZINE EFFICIENTLY IN THE PRESENCE OF NITRATE

According to the results above, we made a simple prediction: a mutant impaired in the assimilatory pathway of an inhibitory nitrogen source should be able to degrade atrazine in the presence of that nitrogen source. We focused on nitrate, since (i) it is the major nitrogen species in fertilized agricultural soils, due to nitrification (Wild, 1998), (ii) it is clearly inhibitory in uptake assays and has been shown to inhibit atrazine degradation by soil communities (Abdelhafid *et al*., 2000a) and (iii) non-assimilating (Nas$^-$) mutants can be readily isolated due to their chlorate-resistant phenotype. A spontaneous Nas$^-$ mutant (MPO102) was isolated which grows extremely slowly on nitrate, but has no growth defect on other nitrogen sources. As expected, atrazine degradation by MPO102 is not inhibited by the presence of nitrate, suggesting that this may be a more suitable strain for atrazine bioremediation in nitrogen-fertilized soils than the wild-type (García- González *et al*., 2003).

Mineralization assays in non-sterile soil microcosms were used to prove the above hypothesis. The behavior of *Pseudomonas* sp. ADP was as predicted: the rate of atrazine mineralization in low nitrogen soil was clearly faster than in nitrate-amended soil. However, MPO102 displayed a high mineralization rate both in amended an unamended soils that was comparable to that of the wild-type in the low nitrogen soil. These results confirm that MPO102 is also a more proficient strain for bioremediation in the presence of added nitrate than the wild-type in a soil environment (García-González *et al*., 2003).

4. COMPLEX REGULATION OF THE *atzDEF* OPERON

Nitrogen control of atrazine degradation could have several possible targets. The *atzA*, *atzB* and *atzC* genes are generally regarded as constitutively expressed, based on the lack of evident regulatory regions and Northern blots of *atzA* and *atzB* (Martinez *et al*., 2001). However, *atzD*, *atzE* and *atzF* are grouped in an operon and are transcribed divergently from *atzR*, encoding a LysR-type regulator. Experiments with *atzD-lacZ* fusions showed that *atzDEF* expression is low in ammonium-grown cells, intermediate in cells grown under nitrogen limitation or in the presence of ammonium and cyanuric acid, and maximal when cyanuric acid is used as the sole nitrogen source. We concluded that the *atzDEF* operon is subjected

dual regulation involving a general physiological signal, the nitrogen status, and a specific inducer, cyanuric acid (García-González *et al*, 2004). We have characterized this phenomenon in detail by using a strain more amenable to genetic manipulation, *Pseudomonas putida* KT2442, as host for expression studies. Expression of an *atzD-lacZ* fusion in *P. putida* KT2442 faithfully reproduced the dual regulatory phenomenon observed in *Pseudomonas* sp. ADP only when the complete *atzR* gene was also provided in the construct. Basal unregulated levels were obtained when *atzR* was truncated, suggesting that AtzR is a required activator for both nitrogen- and cyanuric acid-dependent regulation. Expression of *atzR* is itself controlled from a σ^N-dependent promoter that is activated by the general nitrogen regulatory protein NtrC in response to nitrogen limitation and repressed by AtzR, and NtrC has a second, yet uncharacterized role in activation of *atzDEF* (García-González *et al*., 2004). This function of NtrC requires the presence of AtzR and operates even when AtzR is produced constitutively. Furthermore, unlike *atzR*, *atzDEF* is transcribed from a σ^{70}-type promoter, which is extremely unusual for NtrC-dependent activation. At this point we do not know the mechanism of NtrC-mediated control of *atzDEF* expression, yet it is easy to guess that it does not fit the thoroughly characterized model of activation from a distance typical of σ^N-dependent promoters (Kustu *et al*., 1989). A summary of our current regulatory model is shown in Figure 2.

The structure of the *atzR-atzDEF* intergenic region closely matches the model established for promoters controlled by LTTRs (Schell *et al*., 1993). The two divergent promoters are separated by a 31-bp spacer. A putative AtzR binding site displaying a perfect match to the LTTR binding site consensus (T-N_{11}-A within a perfect palindrome) is located at position -65 from the *atzDEF* transcriptional start. This site partially overlaps the AtzR-repressed *atzR* promoter. No evident NtrC binding sites are present at the expected locations (>100 bp from the activated promoters), which suggests that the *Pseudomonas* and enterobacterial NtrC proteins may have different DNA binding specificities. This notion is reinforced by the fact that *Escherichia coli* NtrC is unable to activate *atzR* and *atzDEF* expression (unpublished results). Studies to localize the relevant *cis*-acting sites for NtrC-mediated regulation are currently underway.

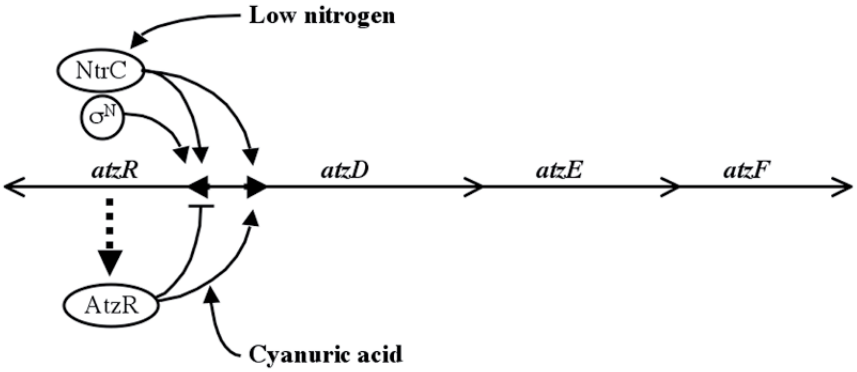

Figure 2. Working regulatory circuit for the *atzDEF* operon.

5. CONCLUSIONS AND FUTURE WORK

The atrazine degradative pathway has proven an interesting system for regulatory studies, both basic and aimed to the improvement of the catabolic capacity. Our work have shown that atrazine utilization by *Pseudomonas* sp. ADP is burdened by a regulatory trait -nitrogen regulation- that limits the use of this strain for bioremediation purposes in fertilized soils. While we characterized this regulatory phenomenon, we were able to rationally design and isolate a mutant derivative, which displays improved atrazine utilization under soil conditions in the presence of high nitrogen concentrations.

Our efforts to characterize nitrogen regulation in *Pseudomonas* sp. ADP have unveiled a peculiar regulatory mechanism that integrates nitrogen control and specific induction by cyanuric acid. As we try to put together all the pieces in the *atzDEF* jig-saw puzzle, we keep in mind that nitrogen regulation of the cyanuric mineralization operon does not suffice to account for the nitrogen regulation observed with the atrazine degradation assays. If *atzA*, *atzB* and *atzC* are indeed constitutively expressed, then conversion of atrazine to cyanuric acid should occur under all conditions, which is inconsistent with our results. We are currently exploring the possibility that a specific atrazine transport system is present and is also subjected to nitrogen control. Hopefully, new research will provide guidelines that may help improve the efficiency of this catabolic pathway under field conditions.

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THE ROLE OF PLANTS AND BACTERIA IN PHYTOREMEDIATION - KINETIC ASPECTS

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Abstract: Phytoremediation is the common name for cleaning techniques for polluted soils, sediments, and wastewaters using plants. It has been shown repeatedly that several types of pollutants, e.g., petroleum products and solvents, are degraded faster in the presence of plants. A couple of processes are known to influence the elimination of pollutants, among them transpiration of water, oxygen transport, biological stimulation in the root zone and plant uptake of chemicals. However, it is frequently unclear whether the plants directly metabolise the pollutants, or whether they only play an indirect role by supporting microbial action.

The metabolism kinetics of plant enzymes is mathematically described by the *Michaelis-Menten* kinetics. This means, that at low substrate concentrations, the degradation is first order, whereas it is linear and therefore limited at high substrate concentrations. Bacteria use the substrate for growth, and grow better at higher substrate availability. This is described by the *Monod* kinetics. Therefore, bacteria have a limited degradation capacity at low substrate concentrations. This often prohibits the biodegradation of polluted sites down to required levels. The combination of plants with bacteria might be a successful method to overcome these short-comings.

Key words: Metabolism; Michaelis-Menten; Monod; kinetics; phytoremediation

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1. INTRODUCTION

Phytoremediation is an engineering technique for remediating polluted soils, but also wastewater and sediments, by use of plants. In principle, phytoremediation is a kind of "enhanced natural attenuation", because it uses the natural clean-up mechanisms of soil, which are supported by a vegetation cover. Plants contribute to the removal of soil pollutants by a number of processes. Aside from uptake of compounds with subsequent metabolism, plants frequently participate indirectly by changing the soil conditions so that soil microorganisms can degrade pollutants (Trapp & Karlson 2001). Phytoremediation is carried out by the "team" of higher plants, bacteria and fungi, and depends on several biological, physical and chemical processes. This article discusses the kinetic aspects of the roles of bacteria and plants.

2. BASIC MATHEMATICS

2.1 Plants

2.1.1 Ecology of Plants

An estimate of the global biomass is 1841×10^9 tons worldwide (excluding bacteria), hereof are 1837×10^9 tons on continents, and 99% phytomass, 0.9% is fungi, and about 0.1 % only is animal biomass. Of the 1837×10^9 tons terrestrial biomass, more than 92% are forests, and hereof, approximately 95% is wood (all data Sitte *et al.,* 1991).

Compared to other life forms, plants have the largest genomes, with some species exceeding 10^{11} base pairs (bacteria $<10^8$) (Voet *et al.,* 1998). This corresponds with the very complex secondary metabolism of plants. More than 80 000 secondary metabolites are known today, with many more to be identified (Richter, 1998). However, only limited knowledge on the degradation pathways and rates of xenobiotics is available. There are indications that enzymes targeting endogenous plant compounds also co-metabolize xenobiotics (Messner *et al.,* 2003).

Most plants are autotrophic organisms, which means that they can form complex carbon compounds from simple inorganic precursors (carbon dioxide, water) and with sunlight as energy source. Thus, plants do not rely on organic compounds as substrate for growth. Although some pollutants, e.g., cyanide, can be used by the metabolism of plant cells (Larsen *et al.,*

2004), most xenobiotics may be expected to be either inert to the plant cell metabolism or have a negative effect.

The metabolism of xenobiotics, which may occur in plant cells, mainly targets detoxification. For the detoxification of xenobiotics, such as herbicides, cytochrome P-450 monooxygenases and glutathione-S-transferases (GST) seem to be the most important enzyme types (Pflugmacher and Schröder, 1995). P-450 enzymes catalyze phase I transformation reactions, frequently hydroxylation, but also sulfoxidation, and *N*-and *O*-dealkylation. GST are responsible for phase II conjugation reactions, which play a central role in detoxification of herbicides in plants. Unlike animals, plants cannot excrete conjugates formed via urine. Instead, phase III of plant xenobiotic metabolism involves storage and compartmentation of soluble conjugates in the vacuole and of insoluble conjugates in the cell wall (Komossa *et al.,* 1995). This may lead to so-called "bound residues".

2.1.2 Growth and Metabolism Kinetics of Plants

The growth velocity of plants depends on factors such as the availability of the resources sunlight, nutrients, and water and on environmental constraints (e.g., temperature). Growth rates of plants are between 0.2 d^{-1} (doubling time 3 days, e.g., maize under favorable conditions) and 0 (ecosystems in equilibrium conditions). A meadow in Central Europe has growth rates of about 0.035 d^{-1} (doubling time of about three weeks). However, these growth rates are related to the exponential growth phase of plants. Towards ripening, the growth of plants stops, and the plant mass may even be decreasing, due to wilting.

The growth of (annual) plants is usually described by a sigmoid curve of the general form

$$M_P = \frac{K}{1 + b \times e^{-rt}}$$

where M_P is the mass of the plant, K is the maximal plant mass, b and r are kinetic parameters and t is time.

Plants do not use xenobiotics as growth substrate, their growth velocity is in most either cases not affected or slowed down by exposure to xenobiotics. Figure 1 shows an illustrative example of plant growth - and of a reduced growth, due to toxic impact of the chemical.

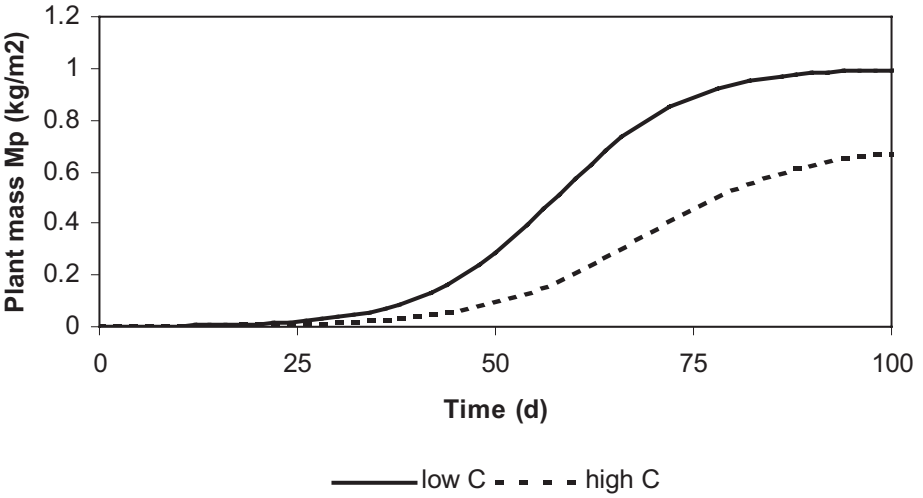


Figure 1. Plant mass vs. time at different xenobiotics concentrations in soil.

The metabolism kinetics of enzymatic reactions can be described by the *Michaelis-Menten* kinetics (Cornish-Bowden 1995):

Equation 1:

$$v = \frac{v_{\max} \times C}{K_M + C}$$

where v [mg (kg plant)$^{-1}$ d^{-1}] is the removal rate per plant mass of the substrate concentration C (mg/L), v_{max} is the maximal removal velocity and K_M (mg/L) is the half-saturation constant.

The overall removal velocity of xenobiotics by plants therefore depends on

– the mass of plant
– the velocity of uptake of the xenobiotic
– the enzymatic reaction rate

The plant mass has an upper limit, K, and the velocity of the enzymatic reaction, too (v_{max}). If follows that there will always be an upper limit for xenobiotics' degradation by plants, which is

Equation 2:

$$\frac{dm_{\max}}{dt} = -\frac{v_{\max} \times C}{K_M + C} \times K$$

where K is the maximum plant mass.

If C >> K_M, this reduces to the constant rate

$$\frac{dm_{\max}}{dt} = -v_{\max} \times K$$

2.2 Bacteria

2.2.1 Ecology of Bacteria

Most bacteria are heterotrophic organisms, that means, they need an organic substrate to feed on. This substrate can be xenobiotics, which are then used as nutrient source by degrader bacteria.

Bacteria have developed a wide range of enzymes that can chemically alter xenobiotics. Xenobiotics can hereby be used as electron acceptor, electron donator, as energy source or as precursor for other molecules (Schlegel 1993).

2.2.2 Growth and Metabolism Kinetics of Bacteria

The growth of bacteria depends on the availability of substrate. The bacterial growth or decay is described by the *Monod* kinetics plus a decay term:

Equation 3:

$$\frac{dB}{dt} = \frac{\mu_{\max} \times C \times B}{K_S + C} - k_{death} \times B$$

where B is the bacterial mass (kg), μ_{max} is the maximal growth rate of the bacteria, C is the substrate concentration (mg/L), K_S is the half-growth concentration (i.e., the concentration where the growth is half of the maximum) and k_{death} is a first order rate describing the death of bacterial cells by arbitrary events, e.g., by grazing protozoa. The growth curve for bacteria may be negative (i.e., the number of bacteria declines) when the death rate is higher than the growth rate. Because the growth depends on

the substrate concentration, but not the death, the number of degrader bacteria will increase at high substrate concentrations, but decline when the substrate is no more available. High substrate concentrations may also have a inhibition effects (Edwards 1970), which is not considered here.

During growth, the bacteria metabolize the substrate. The kinetics of the enzymatic reaction can again be described by the *Michaelis-Menten* kinetics.

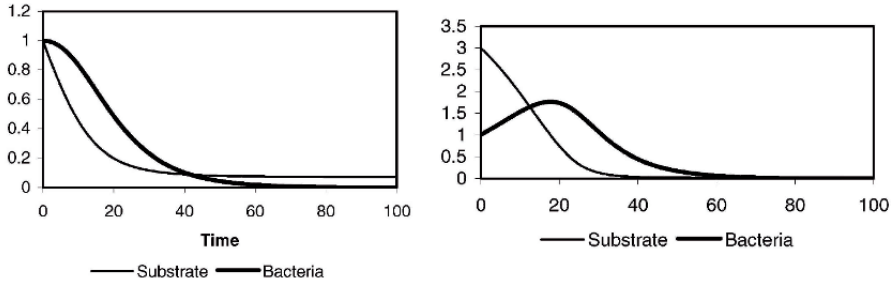
The mass balance equation for the substrate mass *m* (mg) is then

Equation 4:

$$\frac{dm}{dt} = -\frac{v_{\max} \times C}{K_M + C} \times B$$

where v_{max} has the unit mg (kg bacteria)$^{-1}$ d^{-1}. As for plants, this enzymatic reaction velocity has an upper limit. However, the loss of mass has no upper limit, because the number of bacteria increases, as long as substrate is available (and some other resources, such as nutrients etc.). Therefore, the reaction velocity of the bacterial degradation has (mathematically) no upper limit. In reality, there might be a inhibitation of the bacterial growth at higher xenobiotics' concentrations.

However, from an inspection of the bacterial growth equation it can be seen that the degradation by bacteria has a *lower* limit: If the substrate concentration is from the beginning too low to allow a growth of degrader bacteria, the number of bacteria will decline, and thus also the bacterial degrader capacity.



Figures 2 and 3. Bacterial population *B* and substrate concentration *C* at low (left) and high (right) initial *C*.

Therefore, higher xenobiotics pollution might be degraded faster and more complete, than low contamination. This is illustrated in the Figures 2 and 3, which show solutions of Equation 3 and 4 for a start concentration C = 1 mg/L and C = 3 mg/L. All other parameters remained unchanged. As can be seen, at C = 1 mg/L, the bacterial population decreases from the start. After 60 days, the population is erased, but a rest C of about 0.1 mg/L is still present. When starting at C = 3 mg/L, the bacterial population grows, and the contamination is completely degraded - then the bacteria die, too.

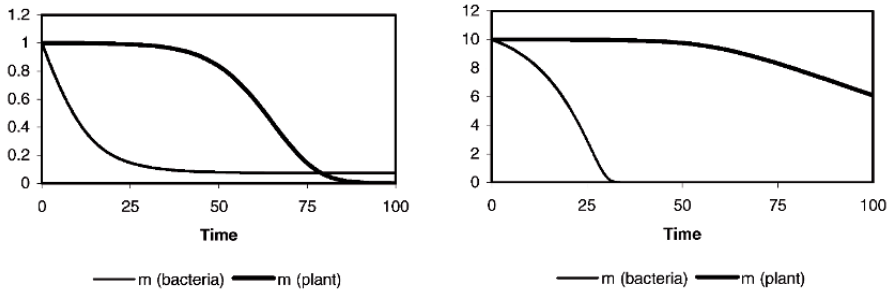
2.3 Comparison of Mass Balances

It is an interesting exercise to compare the degradation of a xenobiotic, which can be metabolized by both bacteria and plants. The next two Figures show the mass (or the concentration) remaining in soil for a low starting concentration (as before), and for a high starting concentration. The *Michaelis-Menten* parameters for plant and bacteria are the same (v_{max} 0.1 mg per kg and per d and K_M was 0.5 mg/L). The growth of plants for low C is taken from Figure 1, for bacteria from Figure 2.

For a low start concentration (C = 1 mg/L), Figure 4, bacteria initially degrade a fraction of the pollution, before the degradation stops (bacteria have starved). For bacteria, this is the same case as depicted in Figure 2. Initial degradation by plants is very low, because the plant mass is very low (see Figure 1). With time, when the plants have established most of their final size, and the absolute amount of chemical removal is peaking (after 64 days, dm/dt = -0.03 mg/d). From then on, the degradation is rapid, and after 100 days, the pollution has been degraded completely.

The picture changes completely for a higher initial concentration (C = 10 mg/L), Figure 5. The simulated bacteria can well grow at this pollution level, and soon a bacterial population is established, which degrades the pollutant completely. However, plants are less effective. Even when the vegetation cover is established, the degradation is too slow, compared to the level of pollution: the maximum degradation is dm/dt = - v_{max} x plant mass = - 0.1 mg (kg plant)$^{-1}$ d^{-1} x 1 kg plant = - 0.1 mg/d. Therefore, after 100 days, more than 50% of the pollution is still present.

If the pollution level was even higher, e.g., initial C = 100 mg/L, bacteria would need about 50 days for a complete degradation (all other parameters constant). Plants would probably die from toxic effects, but even if not, their contribution to degradation would be absolutely negligible.



Figures 4 and 5. Substrate concentration *C* at low (1 mg/L, left) and at high initial *C* (10 mg/L); degradation either by plant or by bacteria.

3. CONCLUSIONS

Even though the simulations made above were not based on real data, some conclusions can be made:

– It is not only the presence of degrader pathways in plants or bacteria, that decides about the role the organisms play in phytoremediation. Kinetic aspects need to be considered, too.

– Even if plants are able to detoxify a xenobiotic substrate, plants always have an *upper limit* for their detoxification capacity.

– Bacteria, which depend on the availability of substrate for their growth, have a *lower limit* for their degradation capacity. Below this limit, a growth on that substrate is no more possible.

– Plants are not suited to treat "hot spots" of pollution: First, because toxic effects are to be expected; second, because their metabolism is limited and slow at high pollution levels.

– Bacteria are well-suited to treat "hot spots". However, at low substrate concentrations, e.g., pesticides in the nanogram/L level in groundwater, bacteria may fail to degrade to "null"-levels (Toräng *et al.,* 2003).

– Plants might be favorable for low contamination levels (e.g., after initial clean-up of a site, as final polishing step), because their metabolic capacity does not decrease with the pollution level.

– A combination of bacteria and plants might be most useful - e.g., in form of the ENDEGRADE concept (Barac *et al.,* 2004).

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REGULARITIES IN THE OXIDIZING METABOLISM OF BACTERIA

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Abstract: It is proved that there are not any obligate aerobes. As for the aerobic bacteria, they should be identified as the anaerobes able to respire by an aerobic way. The biological redox order is shown to meet the electrochemical rules: in the first place, there are reduced these compounds the standard electrode potential of which is higher.

Key words: Aerobe bacteria, standard electrode potential, chromate reduction

A conventional subdivision of bacteria into physiological groups based on their ability to consume oxygen as a terminal acceptor of electrons is one of the most important regulations in identification of microorganisms. The aerobes consume O_2 as an oxidant. Note that some of them, to wit, the obligate (strict) aerobes, take up the only oxygen and nothing else with this end in view. At the same time, the aerobes able to an anaerobic respiration can pass on electrons on the other oxidized compounds as well, e.g. nitrogen-, sulfur-containing ones, etc. The anaerobes do not consume O_2 as a terminal acceptor of electrons.

Just due to a conviction in existence of the obligate aerobes, the aerobic bacteria (both obligate and anaerobic-respiring ones) are suggested to come into being in the course of evolution after that when photosynthesis had appeared. As for the anaerobes, they are suggested to come into being before that appearance. Taking into account these suggestions,

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microbiologists affirm that the ability to the anaerobic respiration is a secondary attribute of the aerobic bacteria[1]. According to this hypothesis, the aerobic bacteria are 'younger' than the primary (obligate) anaerobes for more than a billion years[2] .

In microbiology, it is also generally agreed a statement that use of the terminal acceptors of electrons by microorganisms comply with the following biological redox order[3] :

$$O_2 \rightarrow N^{x+} \rightarrow Me^{x+} \rightarrow S^{x+} \rightarrow CO_2.$$

Here *x* varies from 1 to 7.

This means as follows. If a population of microorganisms has an opportunity to select a terminal acceptor of electrons, it will consume oxygen in the first place, then nitrate and other nitrogen compounds, next oxides of metals and sulfur and, finally, carbonate.

In line with these concepts, the so-called demarcation limit of the aerobic bacteria into the obligate and anaerobic-respiring ones lies between oxygen and the oxidized compounds of nitrogen. Strains that do not consume nitrate as a terminal acceptor of electrons cannot take the oxidized compounds of other chemical elements (viz. those of metals, sulfur, and so on) for this purpose. The point is that the last are situated after the nitrogen compounds in such a redox order. Just these bacteria belong to the obligate aerobes. In turn, just ability of the aerobic bacteria with the oxidizing metabolism to use nitrate as an oxidant is a basis to subdivide them into the obligate and anaerobic-respiring ones.

Transfer of electrons from the oxidizing substance to the terminal acceptor comes to pass with participation of a series of carriers down to the redox-potential gradient, and it runs in the electron transport system located in the membrane of a cell. There also occurs the electrochemical transformation of energy into the internal energy of a membrane with the following synthesis of ATP[1] .

At the same time, the known electrochemical redox order (Table 1) differs of the well-established biological one. In this Table, there are shown the standard electrochemical potentials (E^0) of chemical reactions of reduction of some elements[4] which may be used by microorganisms as the terminal acceptors of electrons. The ***magnitudes*** of electrode potentials depend upon the pH of medium but the ***order*** of the reactions shown does not vary at any pH values.

Table 1. Magnitudes of the standard electrode potentials of some reactions that can be catalyzed by bacteria

No	Reaction	E^0, mV
1	$Au^{3+} + 3e^- \rightarrow Au^0$	1 498
2	$CrO_4^{2-} + 8H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O$	1 477
3	$Cr_2O_7^{2-} + 14H^+ + 6e^- \rightarrow 2Cr^{3+} + 7H_2O$	1 333
4(5)	$MnO_2 + 4H^+ + 2e^- \rightarrow Mn^{2+} + 2H_2O$	1 228
5(4)	$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$	1 228
6	$SeO_4^{2-} + 4H^+ + 2e^- \rightarrow H_2SeO_3 + H_2O$	1 150
7	$ClO^- + H_2O + 2e^- \rightarrow Cl^- + 2OH^-$	880
8	$2NO_2 + 2e^- \rightarrow 2NO_2^-$	880
9	$NO_3^- + 2H^+ + e^- \rightarrow NO_2 + H_2O$	780
10	$Fe^{3+} + e^- \rightarrow Fe^{2+}$	771
11	$TcO_4^- + 2H^+ + e^- \rightarrow TcO_3 + H_2O$	700
12	$ClO_3^- + 3H_2O + 6e^- \rightarrow Cl^- + 6OH^-$	630
13	$UO_2^{2+} + 2e^- \rightarrow UO_2$	450
14	$2NO_2^- + 4H_2O + 6e^- \rightarrow N_2 + 8OH^-$	410
15	$SO_4^{2-} + 10H^+ + 8e^- \rightarrow H_2S + 4H_2O$	311

When a few potential terminal acceptors is in the medium, in accordance with the electrochemical rules, electron bounds in the first place to that element which standard electrode reduction potential is higher. As might be seen from Table, certain reduction reactions of the oxidized metal compounds in the electrochemical redox order are located not only in front of those of the nitrogen compounds but also oxygen ones [Mn(IV), Cr(VI) etc.]. That is, the so-called obligate aerobes when respiring should likely involve these substances as terminal acceptors of electrons because E^0 of these reactions equal to that of O_2 reduction or surpasses it. The last reaction comes to pass in the microbial cell when oxygen makes use of as a terminal acceptor of electrons[5] :

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O.$$

Consequently, when the biological redox order corresponds to chemical one, the bacteria pertaining to the obligate aerobes are able to respire without O_2, i.e. in an anaerobic way. On an example of manganese dioxide E^0 reduction of which is like that of oxygen and is equal to 1228 mV (see Table, reactions 4 and 5), we have showed that the collection strains of the 'obligate aerobes' without any adaptation to MnO_2 consume Mn(IV) when respiring as a terminal acceptor of electrons. Thus, the term 'obligate (strict) aerobes' is actually groundless in view of the fact that these bacteria are able to respire in the absence of oxygen, i.e. in an anaerobic way

passing electrons on Mn(IV)[6,9]. The strains non-adapted to MnO_2 perform in fact reduction of four-valence manganese without any lag-phase. In other words, this process is not a specific one, and it comes likely to pass by virtue the same terminal cytochromoxidaze which passes electrons on O_2 in the aerobic process.

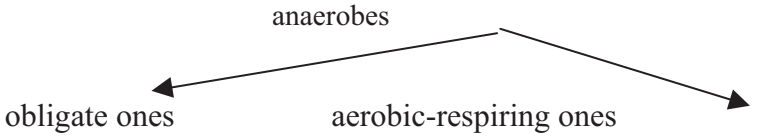
The studies described in Refs.[7,8,9] have proved that the non-adapted collection trains of bacteria that are identified as the obligate aerobes reduce the Cr(VI) of both dichromate and chromate ions in the reactions E^0 of which are higher than that of oxygen reduction, and their values are equal to 1 333 and 1 477 mV, respectively. The standard electrode potentials of these reactions are beyond the limits of the thermodynamic stability of water. It is therefore not surprising that the collection strains of bacteria reduce the six-valence chromium very slow. This appears is one of the reasons causing a low Cr(VI)-reducing ability of the microorganisms being adapted to the six-valence chromium. Alternatively, a high Cr(VI)-reducing activity of bacteria would result in decomposition of water in a cell: $2H_2O \rightarrow O_2 + 4H^+$ (the upper limit of the thermodynamic stability of water is 1.23 V)[10].

We have studied a sequence in consumption by bacteria of the compounds with variable oxidation extent as the terminal acceptors of electrons at their joint presence in the cultural liquid. The results of studies we conducted have exhibited that, in the simultaneous presence in a medium of two oxidants (nitrate and six-valence chromium), the denitrifying *Pseudomonas* bacteria bring about in the first instance reactions with the higher standard electrode potential, i.e. they reduce Cr(VI). In cases when these oxidants are in the different media, nitrate for these bacteria is the same ease of access terminal acceptor of electrons as oxygen, whereas those bacteria implement the chromate-reduction of a culture extremely slow[11]. The *Acinetobacter calcoaceticus Ac-1* collection strain, 'strict aerobe' non-adapted to Cr(VI) and Mn(IV), reduces also slow the six-valence chromium but does that extremely effectively with the four-valence manganese. At the same time, the reduction complies with the electrochemical rules when these compounds are together in the cultivation medium: the dichromate is reduced first, and then only the four-valence manganese (MnO_2) undergoes the same[9].

An analogous microbial redox order is characteristic for reactions with other elements with variable oxidation degree. So, the reduction of Fe(III) by the *Alteromonas putrifaciens MR*-1 culture begins in a medium after a complete reduction of the Mn(IV) (see Table, reaction 10)[12]. At the same time, Mn(IV)- and Fe(III)-reducing bacteria are competitive with the sulfate-reducing ones (see Table, reaction 15)[13]. In the presence of two terminal acceptors of electrons in the U(VI)- and SO_4^{2-}-containing medium, the *Desulfovibrio vulgaris* and *Clostridium sp*. sulfate-reducing bacteria

reduce sulfate in the pure and binary cultures after the complete reduction of U(VI) only (see Table, reaction 13)[14]. To summarize in short, the result we reached and the data described in the scientific literature provide us a mean to state that the biological redox order complies well with the electrochemical rules[15].

All the information stated above gives us a right to suggest that there are not any obligate aerobic bacteria in the modern meaning. In the course of the evolution process, "the aerobic bacteria" came likely into being as the fermenting and anaerobic-respiring ones together with the obligate anaerobes before the photosynthesis had appeared. When the free oxygen had appeared in the Biosphere, the anaerobic Mn(IV)-reducing bacteria were able potentially to consume O_2 as a terminal acceptor of electrons because $E°$ of the oxygen and four-valence manganese (MnO_2) reduction reactions are the same and equal to 1 228 mV. That is, the anaerobiosys of the aerobes is to all appearance a primary property with respect to aerobiosis. This means that bacteria should be identified as follows:



As for the biological redox order, it obeys the electrochemical rules in the simultaneous presence of a few oxidants in a medium, which are capable to serve as the terminal acceptors of electrons to bacteria at their respiration. In the first place, the microorganisms consume these elements with the varied oxidation degree E^0 of the reduction reaction of which is higher

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FORMATION OF MICROBIAL COMMUNITIES IN OIL SHALE CHEMICAL INDUSTRY SOLID WASTES DURING PHYTOREMEDIATION AND BIOAUGMENTATION

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Abstract: Oil shale thermal processing has resulted in solid waste dump sites containing up to 100 million tons of solid waste. The processed oil shale contains complex mixture of organic and inorganic compounds and is highly toxic. Laboratory and field experiments were carried out in order to test the effect of phytoremediation and bioaugmentation for remediation of pollutants in semi-coke. Microbial community of aged (ca 10 ten years) semi-coke is characterized by few dominant populations and possesses low diversity. Changes in microbial community structure and activity occurred in semi-coke as a result of phytoremediation and bioaugmentation. The phytoremediation increased the number of bacteria and diversity of microbial community in semi-coke as well as microbial biomass. The general trend was the increase of proportion of biodegradable bacterial numbers within microbial community due to the treatment. Highest values for all measured microbiological parameters were found in rhizosphere samples. Within a two and half year period starting from establishment of test plots, the concentration of phenolic compounds decreased up to 100% and oil products more than three times at plots with vegetation compared to control. Bacterial biomass consisting of three bacterial strains was applied to three experimental plots. These three bacterial strains *Pseudomonas mendocina* PC1, *P. fluorescens* PC24 and *P. fluorescens* PC18 degrade phenols via catechol meta, catechol or protocatechuate ortho or via the combination of catechol meta and protocatechuate ortho pathways, respectively. Bioaugmentation increased biodegradation intensity of oil products up to 50% compared to untreated planted controls and enhanced plant growth, but the effect of bioaugmentation on microbial community parameters was short-term. Our results indicate that increased biodegradation activity was due to proliferation of specific microbial groups, changes in taxonomic diversity of bacterial community and catabolic genes.

H.J. Heipieper (ed.), Bioremediation of Soils Contaminated with Aromatic Compounds, 57–66.

1. INTRODUCTION

More than 70 years of oil shale thermal processing has resulted in huge dump sites of the coke ash (semi-coke) from semi-coking of oil shale in the areas surrounding oil shale chemical industry plants in northeastern part of Estonia. The semi-coke mounds cover an area about 200 ha and contain up to 100 million tons of solid waste. Currently, about 600 000 tons of processed semi-coke is disposed annually. Semi-coke solid wastes contain several organic and inorganic compounds (oil products, asphaltenes, phenols, PAHs, sulfuric compounds), while liquid wastes (leachate) from depository area are characterized by high concentration of oil products, phenol, cresols, dimethylphenols and resorcinols. The liquid pollution from semi-coke dump area deteriorates surface water as well as the underlying aquifers (Truu *et al.,* 2002).

Phytoremediation is a new emerging environmental technology for removal of contaminants from the environment. This approach is based on the combined action of plants and their associated microbial communities to degrade, remove, inactivate or immobilize toxic compounds in soil. Plants may act directly accumulating (phytoaccumulation) and in some cases metabolising (phytodegradation) pollutants (White, 2001), but one way to achieve truly *in situ* bioremediation is to perform rhizosphere bioremediation by utilising plants (rhizodegradation or phytoremedition *explanta*) (Alkorta and Garbisu, 2001). Sometimes rhizodegradation defined as degradation of contaminants in root zone due to both microbial activity and roots (Trapp and Karlson, 2001) Vegetation facilitates bioremediation of contaminated soils due to increased microbial activity, population density and diversity on the surface and in the vicinity of root (Romantschuk *et al.,* 2000). During rhizoremediation, exudates derived from the plant roots stimulate activity of bacteria in soil resulting in a more efficient degradation of pollutants. The degradation of pollutants during phytoremediation can be enhanced by adding to the soil specific microorganisms, able to degrade the certain pollutants (Kuiper *et al.,* 2004). Another approach where bacteria are added to the environment is the use of plant growth-promoting bacteria that facilitate the proliferation of plants under stressful conditions (Glick, 2003). Phytoremediation has been proposed as an efficient, low-cost remediation technique to restore areas contaminated with chlorinated solvents, BTEX compounds, phenols and PAHs (Sarand *et al.,* 1999, Harvey *et al.,* 2002, Banks *et al.,* 2003).

The aim of current study was to assess the response of soil microbial community to the phytoremediation in combination with bioaugmentation.

2. MATERIAL AND METHODS

2.1 Phytoremediation experiment

Four test plots (each 50 m^2) were established at semi-coke depository in July 2001. Plant treatment was based on a grass mixture of four species (*Lolim perenne* - perennial ryegrass), *Poa pratensis* - Kentucky bluegrass, *Festuca rubra* - red fescue, and *Festuca ovina* - blue fescue). In addition to plants, four different treatments were utilized. The following treatments were applied: plot 1 – no treatment (grass seeds in semi-coke), plot 2 – seeds in semi-coke were covered by sand layer (1-2 cm), plot 3 - seeds in semi-coke were covered by peat layer (1-2 cm), plot 4– semi-coke was covered with the layer of sod (pre-grown lawn). In October 2001, 2002 and 2003 soil sampling was performed on treatment plots and control area. We analyzed semi-coke samples collected from the test plots at the depository area for chemical and microbiological parameters.

2.2 Bioaugmentation experiment

For the bioaugmentation experiment the set of bacteria consisting of three strains isolated from nearby polluted area was selected. These three bacterial strains *Pseudomonas mendocina* PC1, *P. fluorescens* PC24 and *P. fluorescens* PC18 degrade phenols via catechol *meta*, catechol or protocatechuate *ortho* or via the combination of catechol *meta* and protocatechuate *ortho* pathways, respectively (Heinaru *et al.,* 2000). In bioaugmentation experiments the biomass of these bacteria was supplied to the part experimental plots (each 10 m^2) in July 2002. Each treatment received 20 L of bacterial suspension with concentration 10^8 CFU ml^{-1}. The ratio of bacterial strains PC1, PC18 and PC24 was in suspension 3:1:1.

2.3 Microbiological methods

The microbial communities were removed from semi-coke and plant root surface by vortexing in sterile tap water. Heterotrophic plate count was enumerated by the spread plate method in triplicate on R2A agar (Difco). The number of phenol-degrading bacteria was determined in triplicate sets

on M9-salts agar plates supplemented with trace elements and phenol (2,5mM). The most probable number (MPN) technique was used to count alkane degrading bacteria in Bushnell-Haas medium on microtiter plates with n-hexadecane according to Wrenn and Venosa (1996). The heterotrophic activity and diversity of microbial community was measured using Biolog EcoPlates (Biolog, Inc.). Results of Biolog profiles are presented as total activity (summed well color development of all 31 wells) and by Shannon diversity index. Color development data of Biolog EcoPlates was also subject to kinetic data analysis according to method of Lindstrom *et al.,* 1998.

$$\sum OD_{590} = \frac{K}{(1 + e^{-r(t-s)})}$$

The method provides two kinetic parameters K and r that are invariant with respect to inoculum density, and reflect the composition of cultivable microbial communities. In order to estimate statistical significance, an ANOVA on the model parameters K and r from each treatment regression was performed.

Microbial biomass carbon was measured by fumigation-extraction technique (Schinner *et al.,* 1996).

Microbial DNA was extracted from soil samples with UltraClean Mega Soil DNA kit (Mo Bio Laboratories, Inc.). Bacterial community structure was assessed with primer pair 318f-GC/535r (Schäfer and Muyzer, 2001). A denaturating gradient gel electrophoresis (DGGE) system DCode (Bio Rad, Inc.) was used to separate the amplified gene fragments. DGGE gels were digitized and banding pattern analysed using Principal Coordinate Aanalysis (PCoA).

3. RESULTS

Chemical properties of the semi-coke from experimental area are shown in the *Table 1*. After retorting at 500 ºC, processed oil shale is highly saline, alkaline, biologically sterile, nutrient deficient material with no structure. Semi-coke is characterized by high organic carbon content, nearly half of which consists of asphaltens. The semi-coke contains n-alkanes and PAHs, but the concentrations of these compounds is relatively low (maximum individual alkane concentrations ca. 8 μg g^{-1} and maximum individual PAH concentrations ca. 20 μg g^{-1}; (Kronholm *et al.,* 2004)).

Table 1. Chemical properties of semi-coke from control plot.

Variable	Measured value
pH	8.0-11.0
Total nitrogen (%)	0.08
P-PO_4^{3-} (mg kg^{-1})	12.3
K^+ (mg kg^{-1})	799
Ca^+ (mg kg^{-1})	18673
Mg^+ (mg kg^{-1})	826
Total organic carbon (%)	15.0-18.0
Oil products (mg kg^{-1})	340
Volatile phenols (mg kg^{-1})	0.30-0.34

The chemical analysis of soil samples showed impact of the plant treatment on degradation rate of pollutants. Within a two and half year period starting from the establishment of test plots in July 2001, the concentration of volatile phenols was reduced up to 100%, the concentration of oil products more than three times (from 340 mg kg^{-1} to 100 mg kg^{-1}), and the total content of organic carbon decreased by 10 to 30 g per kg (from 15% to 12%). The degradation rate was highest on the plots with peat amendment and sod with the highest root density in semi-coke. In upper layer samples (0-10 cm) the reduction of oil products and phenols was even bigger being in the range from 83% to 98%.

Bacterial biomass consisting of three bacterial strains was applied to three experimental plots in June 2002. Within a three months period the concentration of residual oil shale oil in semi-coke decreased by 13.6% to

Table 2. Microbiological properties of soil from different treatment plots on the third year of the experiment.

Treatment	Microbial biomass (μg g^{-1}dw)	Aerobic heterotrophic bacteria (CFU g^{-1}dw)	Phenol degrading bacteria (CFU g^{-1}dw)	Alkane degrading bacteria (CFU g^{-1}dw)
Control	1070	$3.7*10^6$	$4.9*10^5$	$2.2*10^3$
Control with biomass	1380	$8.4*10^6$	$7.2*10^5$	$2.2*10^3$
Grass	2600	$1.8*10^7$	$1.8*10^6$	$7.0*10^3$
Grass and sand layer	1510	$7.4*10^6$	$2.1*10^5$	$1.9*10^3$
Grass and peat layer	1670	$9.4*10^6$	$5.6*10^4$	$3.1*10^3$
Grass and peat layer with biomass	1360	$6.0*10^6$	$5.5*10^5$	$4.6*10^3$
Sod	1720	$5.5*10^6$	$1.8*10^6$	$6.3*10^3$
Sod with biomass	1550	$1.8*10^7$	$1.6*10^6$	$1.2*10^4$

53.6% on plots treated with bacterial biomass compared to untreated parts of experimental plots. Bioaugmentation increased the root biomass and length.

The plant treatment increased the number of phenol-degrading bacteria by order of magnitude, while the number of heterotrophic aerobic bacteria remained on the same level compared to the untreated plot. Samples from the second year (2002) showed lower values of aerobic heterotrophic bacteria than in year 2001, which could be due to extremely dry vegetation period. During the three year monitoring period the general trend was the increase of proportion of biodegradable bacterial numbers within microbial community due to the plant treatment as well as increase in microbial biomass (Table 2). Highest values for all measured microbiological parameters were found in rhizosphere samples. While bacterial total numbers increased by order of magnitude compared to control, the number of phenol-degrading bacteria was more than 100 times higher in the rhizospheric soil. The highest values for microbial activity and diversity measured with Biolog EcoPlates were recorded in rhizosphere samples. Addition of bacterial biomass to semi-coke resulted in rise of both absolute number (up to 7.8×10^6 CFU g^{-1}) and relative abundance (up to 30 %) of phenol-degrading bacteria in the studied samples in year of application, while these values drop down to level of untreated plots next year.

We compared microbial communities from different treatments using kinetic model based on summed well color development of Biolog EcoPlates (Table 3). Model parameters estimated from these fitted curves were statistically different (ANOVA) both in 2002 and 2003 ($P<0.001$), indicating changes in microbial community structure due to plant treatment

Table 3. Comparison of kinetic model parameters for summed well color development of Biolog EcoPlates for years 2002 and 2003.

Sample	Year	K	r	s (h)	R^2 (%)
Control	2002	8.8±2.7	0.045±0.007	117.8±13.5	99.8
	2003	4.5±0.2	0.031±0.002	110.4±3.2	99.5
Control with biomass	2002	5.5±0.2	0.061±0.005	90.6±2.1	99.8
	2003	11.6±0.6	0.032±0.003	104.6±4.9	99.8
Grass and peat layer	2002	29.1±1.0	0.060±0.004	75.3±1.8	99.8
	2003	22.2±0.7	0.060±0.006	66.0±2.1	99.0
Grass and peat layer with biomass	2002	40.8±1.0	0.070±0.006	56.8±1.5	99.7
	2003	28.7±0.7	0.034±0.004	83.9±3.4	98.0

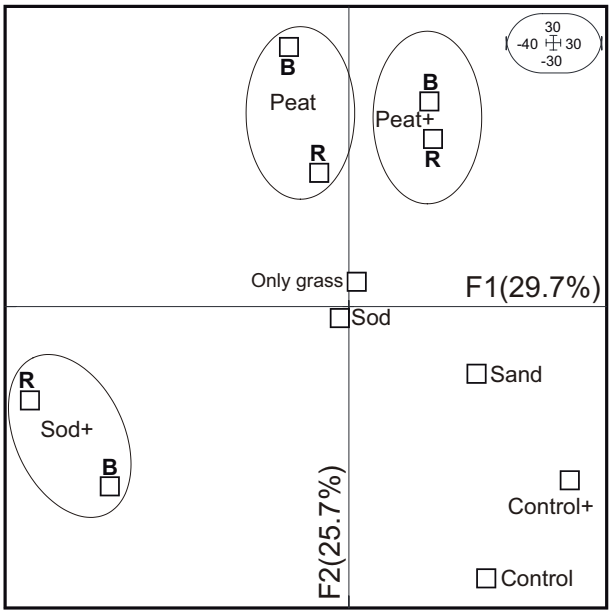

Figure 1. Ordination of bacterial communities based on Principal Coordinate Analysis of DGGE fingerprints. Plus at the end of treatment type indicates bioaugmentation.

and bioaugmentation. Like in case of microbial numbers the differences between bioaugmented and non-treated vegetated plot microbial community kinetic parameters K and r were bigger in the year of biomass application compared to values obtained year later.

A comparison of 16S rRNA gene-based DGGE fingerprints of soil samples using multivariate analysis showed variation between the bacterial community profiles from different treatments. According to PCoA plot (Fig. 1) the bacterial communities from bioaugmented plot with sod and peat cover are most distinct from the control plot. Application of sand layer with vegetation to semi-coke gave the rise to less altered bacterial community profile compared to untreated plot. The PCoA plot also indicates that shifts in bacterial community structure due to bioaugmentation are opposite in case of sod and peat amendment.

4. DISCUSSION

Biodegradation processes of organic pollutants in semi-coke are more difficult to achieve than those in natural soils. High initial pH value, elevated concentration of Ca and Mg as well as sulfuric compounds limit

microbial activity in semi-coke. Another group of factors affecting the biodegradation activity is susceptibility of organic pollutants to microbial attack. Among organic compounds found in semi-coke, asphaltenes are the most resistant fraction for bioremediation (Mana Capelli *et al.,* 2001; Peressuttia *et al.,* 2003). Asphaltenes are also known for the inhibition of some physiological groups of microorganisms in soil like actinomycetes as well as microbes participating in the processes of nitrogen and phosphorous transformations (Muratova *et al.,* 2003).

Degradation rates of pollutants did not differ significantly between plots with vegetation except for sod, showing negligible effect of soil amendment type during study period. This suggests that establishment of vegetation on semi-coke was a key factor for acceleration of degradation of pollutants. Phytoremediation had a substantial impact on bacterial numbers, activity, biomass and diversity in semi-coke. Our previous studies have shown that introduction of plants caused changes in the structure of catabolic genes. In the plots with plants dominated two different multicomponent phenol hydroxylases (LmPH) belonging to low- and moderate K_s kinetics groups, indicating more efficient degradation of aromatics at these plots (Truu *et al.,* 2003). There was only one dominant LmPH at untreated plot, belonging to high-K_s group. Analysis of 16S rDNA DGGE fingerprints shows that the effect of phytoremediation is not associated only with rhizosphere soil, but plant treatment affected the microbial community structure in bulk soil.

Enhancement of pollutant degradation rate using bioaugmentation has been successfully applied in several cases with non-vegetated soil (Madsen and Kristensen, 1997, Ruberto *et al.,* 2003;) and planted soil (Siciliano and Germida, 1998; Singer *et al.,* 2003). Typically during the time course of bioaugmentation process the improvement of the bioremedation activity and increase of microbial counts is temporary and return to initial values is observed (Margesin and Shinner, 1997). The same kind of time dynamics of measured microbial community parameters was observable in our experiments. According to Dejonghe and coworkers (2001) bioaugmentation should aim at the rearrangement of the group of organisms dominantly involved in the overall energy flux, so that specific catabolic traits necessary for the clean up of pollutants are part of that active group. The rhizosphere is considered as a habitat that allows higher frequency of catabolic gene transfer as well as higher metabolic activity compared to bulk soils, both of which are necessary for a successful plasmid-mediated bioaugmentation approach (Top *et al.,* 2002). Our data do not give evidence what was the mode of action of bioaugmentation in case of semi-coke – transfer of catabolic genes (plasmid-mediated bioagmentation) or survival of introduced strains. Two out of three bacterial strains used in our

bioaugmentation experiment contain plasmids, which carry catabolic genes. Among these genes are catechol 2,3-dioxygenase (C23O) gene, in strain PC18 and the *p*-cresol methylhydroxylase gene, in strains PC18 and PC24 (Heinaru *et al.,* 2001). Further research is needed to identify the mechanism how inoculation enhances degradation of pollutants in semi-coke.

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STUDIES ON BIODEGRADATION OF AROMATIC POLLUTANTS BY *TRICHOSRORON CUTANEUM* YEAST STRAIN

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Abstract: Yeast strain *Trihosporon cutaneum* R57 was studied for its abilities to grow and utilize some organic compounds (phenol and phenol derivatives, acetophenone, acetone, α-methylstyrene, benzoic acid, dimethyl phenyl carbinol, methanole and izopropylbenzene) as a sole carbon and energy source. The degradation and development abilities of *T. cutaneum* R57 applied to some of enumerated aromatic compounds are presented in this work. It was established that the strain could degrade and assimilate completely up to $1gl^{-1}$ phenol for a period of 18 h, 0.4 gl^{-1} α-methylstyrene, acetophenone and p-cresol were degraded for 24-27h.

The intracellular specific activities of phenol hydroxylase [EC 1.14.13.7] and catechol 1,2-dioxygenase [EC 1.13.11.1] in *Trichosporon cutaneum* R57 strain and its mutants are measured. The comparison of data for phenol hydroxylase and catechol 1,2-dioxygenase activities in cell free extracts obtained by ultrasonication and in permeabilized cells showed that the method of cell permeabilization was more favorable for enzyme analyses. The obtained specific activity of phenol hydroxylase in *Trichosporon cutaneum* R57 was 0.8 Umg^{-1} protein while those in 2R and 4R mutant strains were 1.47 Umg^{-1} protein and 1.28 Umg^{-1} protein respectively. The value of catechol 1,2-dioxygenase activity showed quite less variability and was kept about 0.2 Umg^{-1} protein in all investigated strains. The results received in these experiments demonstrated that the increased rate of the initial phenol degradation reaction enhanced phenol utilization by cells and *vice versa.* We present some preliminary data for enzyme activities in the process of degradation of hydroxyl phenol derivatives.

H.J. Heipieper (ed.), Bioremediation of Soils Contaminated with Aromatic Compounds, 67–74.

1. INTRODUCTION

There is a great interest in the microbial degradation of toxic or inhibitory carbon sources in wastewater treatment facilities [1]. Phenol and its various derivatives, as well as many other aromatic compounds, are known as hazardous pollutants.

Various phenol-degrading microorganisms have been extensively studied for the purpose to innovate and improve the technological processes of biodegradation. A number of studies with prokaryotic microorganisms have been carried out [2,3,4,5]. Only some members of yeast genera *Candida, Rodotorula* and *Trichosporon* that can metabolize phenolic compounds as a sole carbon and energy source are described in the literature [6,7,8].

The strains used for decontamination of waste waters from phenol production should not only be highly active to one of the contaminants but they should also be resistant enough to the reminder. Their resistance can be ensured by the degradation activity of the strains used towards most of the waste products present in the waste water [9,10]. Few publications devoted to the degradation of toxic compounds in waste waters from industrial phenol production were found in the available scientific literature. Strains of the genera *Nocardia* and *Arthrobacter* which use acetophenone as the sole source of carbon and energy were described [11]. Microorganisms degrading α-methylstyrene [9,12,13] have been the subject of investigations and *Pseudomonas* sp. selected which utilized dimethyl phenyl carbinol [9,10,14].

The genus *Trihosporon* represents a taxon comprising microorganisms with a unique set of enzyme capabilities for aerobic biodegradation of diverse organic compounds including unsubstituted phenol [15]. The first step in aerobic metabolism is phenol hydroxylation to catechol by phenol hydroxylase [16]. This enzyme was described as a NADPH-dependant flavoprotein in *Trihosporon cutaneum* [16,17]. Exclusively NADPH-dependant phenol hydroxylase have been reported for *Bacillus stearothermophilus* [18] and *Pseudomonas sp. CF600* [19]. Catechol, the product of the reaction catalyzed by phenol hydroxylase, is a central intermediate in the degradation pathways of various aromatic compounds. It is metabolized by different strains via either the *ortho*- or the *meta*-fission pathway. Previously, we have isolated and described a strain of *Trihosporon cutaneum* which could grow aerobically and assimilate $1gl^{-1}$ phenol for a period of 18 - 20h [20]. This is the shortest period of time reported for phenol biodegradation by non-induced cells. The investigated strain has been immobilized on polyamide carriers and has shown good capacity for long-term degradation of high phenol concentration [21,22].

2. MATERIALS AND METHODS

2.1 Microorganisms and growth conditions

The yeast *Trichosporon cutaneum* R57 (N2414/1994 NBMICC) and its mutants were used in all experiments. The cultures were grown in Erlenmeyer flasks (100 ml culture volume) on a New Brunswick rotary shaker at 28-30^0 C and 200 rev.min^{-1}. The cultivation was carried out on the medium for yeast containing 6.7 gl^{-1} Yeast nitrogen base without amino acids (YNB w/o AA, Difco) supplemented with each one of the investigated compounds as single carbon source. Cell density was monitored spectrophotometrically by measuring the optical density at λ = 610 nm.

2.2 Enzyme assay

Enzyme activities were determined in cell-free extracts and in permeabilized cells. Cells were harvested in logarithmic phase, washed twice in 50 mM Tris-SO_4 buffer, pH=7.6 and broken by sonication (6 cycles of 30 s interspaced with 1 min periods of cooling of ice) and then cells debris were removed by centrifugation at 5000g for 30 min. The cleared supernatant solution was used both for enzyme and total protein assays.

Permeabilization procedures were essentially similar to those described for *Yarrowia lipolytica* [23]. In our experiments, maximal permeabilization of the cells was achieved with 0.1% of the non-ionic surfactant Triton X-100.

Phenol hydroxylase [EC 1.14.13.7] activity was assayed spectrophotometrically (LKB UV-Vis Ultraspec 1000), following NADPH absorbance at 340 nm [24]. The activity of catechol 1,2-dioxygenase [EC 1.13.11.1] was determined by measuring the rate of *cis*,*cis*-muconic acid accumulation at 260 nm [25]. One unit of activity is defined as the amount of enzyme transforming 1 μmol of substrate in 1 min under the assay conditions. Specific activities were expressed as units (U) per mg total cell protein. Protein content of the permeabilized cells was determined as described by Herbert [26].

2.3 Analytical methods

The cell free supernatants were analyzed by HPLC performed on a reversed phase C18 column (Lichrosorb RP18, Perkin Elmer) with methanol-water (50:50) liquid phase by using UV detector at 220 nm. The phenol concentration was also determined by a residual calorimetric method, described by Lurie with basic reagent 3,4-dimethyl amino antipyrine [27].

3. RESULTS AND DISCUSSION

The present study demonstrates the growth abilities of *Trichosporon cutaneum R57* and its properties of an efficient microorganism for degradation of toxic organic compounds. The results confirming the growth and degrading characteristic of investigated strain are shown on Fig. 1 and Fig. 2.

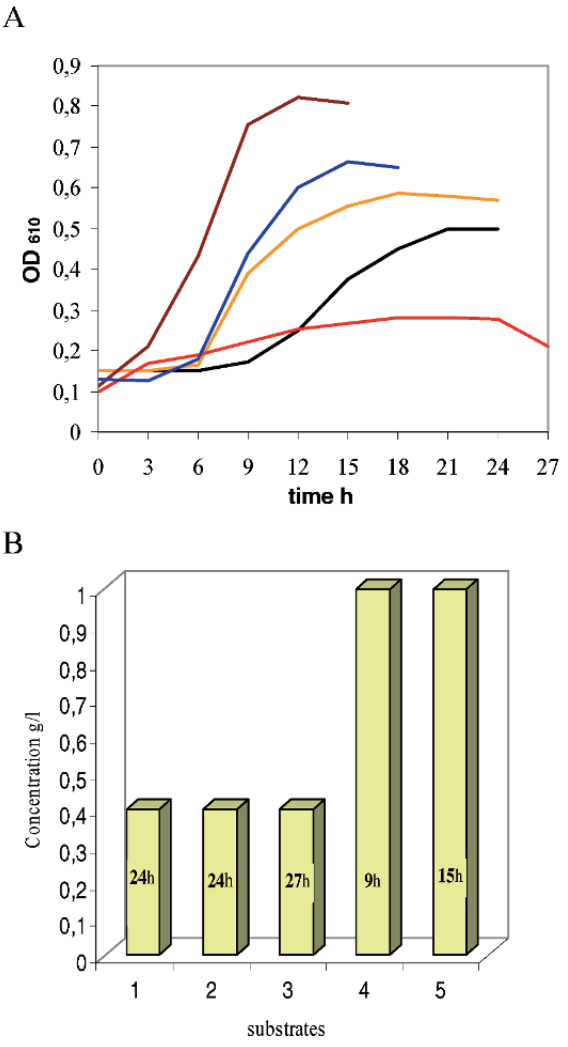

Figure. 1. Growth curves (A) and degradation abilities (B) of ***Trichosporon cutaneum R57*** in Yeast Nitrogen Base medium supplemented with each one of the investigated compounds as single carbon source: 1 - acetophenone; 2 - α- methylstyrene; 3 - p-cresol; 4 - resorcinol; 5 - phenol. The cell free supernatants were analyzed by HPLC performed on a reversed phase C18 column (Lichrosorb RP18, Perkin Elmer).

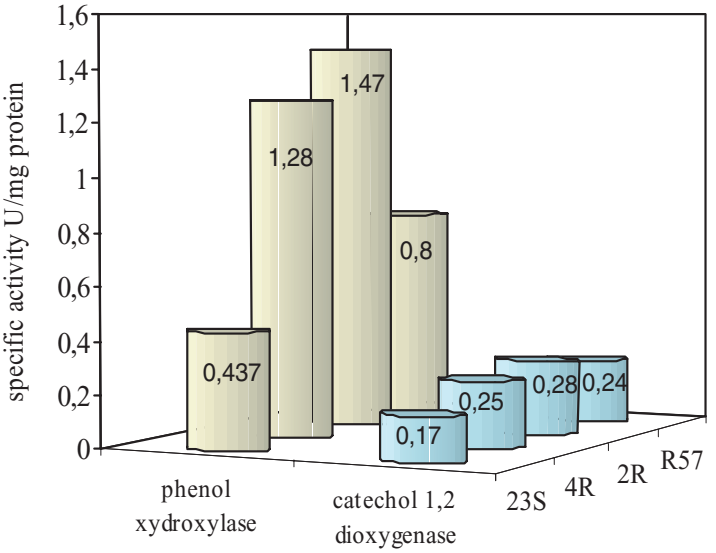

Figure 2. Enzyme activities determined in permeabilized cells of ***Trichosporon cutaneum*** **R57** and isolated mutants – 2R, 4R, 23S. Phenol concentration is 0.5.

The efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme(s). The specific activities of the first two enzymes degrading phenol were investigated. As it has been reported earlier, both enzymes showed higher activities in cells cultivated on complete medium (YEPD) supplemented with phenol than these grown on synthetic minimal medium [28]. In the present paper, the results from experiments carried out on a medium without other carbon compounds included (YNB w/o AA) are discussed.

Despite of the most favorable conditions applied for the cell disruption by ultrasonication, there is some risk of protein destruction and consequent damage of the investigated enzymes [29]. The method of permeabilization of cells gives an opportunity to avoid the mentioned above influence on the measured enzyme activities. (Fig. 2). The obtained data showed higher activities of phenol hydroxylase in the mutants 2R and 4R which were able to grow in the presence of 1.2 gl^{-1} phenol. For the 23S mutant, which is less resistant to phenol (0.6 gl^{-1}) than the parent strain (1.0 gl^{-1}), the same enzyme showed the lowest activity. The differences observed for catechol 1,2- dioxygenase activity in the investigated strains are not so significant. The results received in these experiments demonstrated that the increased rate of the initial phenol degradation reaction enhanced phenol utilization

by cells and lead to increased (2R and 4R), respectively decreased (23S) resistance of mutants to phenol.

We have measured the activities of the first two enzymes in the cells grown with hydroxyl phenol derivatives. The data obtained from experiments with resorcinol are presented on Fig. 3.

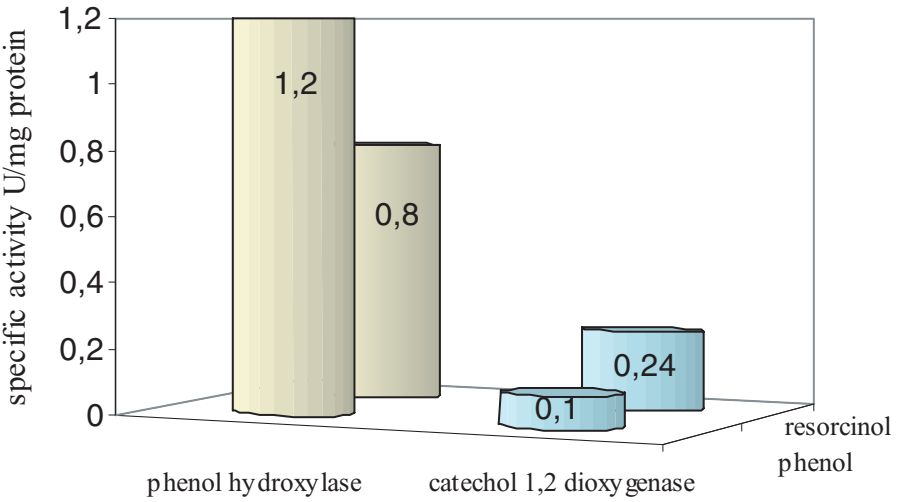

Figure 3. Comparison of enzyme activities determined in permeabilized cells of *Trichosporon cutaneum* R57 grown with resorcinol (1g l-1) and phenol (0.5 g l-1)

4. CONCLUSIONS

The enzymes phenol hydroxylase and catechol 1,2-dioxygenase of *Trichosporon cutaneum* studied exhibited certain similarities and differences compared to similar enzymes isolated from other microorganisms. It should be pointed out, that the levels of phenol hydroxylase activities established in the present research are higher than the results cited in the literature [29,30,31]. On the basis of our previous and recent analyses, the investigated strain and some of its mutants were considered to have a good potential for application in remediation of phenol contaminated environment and improvement of phenol removing treatment of industrial wastewaters.

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THE POTENTIAL OF KERATINOLYTIC AND KERATINOPHILIC FUNGI FOR DEGRADATION OF PETROLEUM HYDROCARBONS IN SOIL

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Abstract: The goal of work was to determine potential of keratinolytic fungi for degradation of crude oil. Two strains belonging to species *Chrysosporium keratinophilum* and *Trichophyton ajelloi* were isolated form sewage sludge and oil waste-contaminated soil from a refinery. Preliminary experiment was performed in liquid media with increasing concentration of peptone with and without presence of hair(source of keratin). The mean value of hydrocarbons loss was 39,8% during degradation of peptone and 49,2% during degradation of peptone and hair. The ability for removal of hydrocarbons was characteristic for fungal strains and probably associated with their high degree of adaptation for living in habitats heavily contaminated with petroleum hydrocarbons. It was observed that petroleum hydrocarbon removal rates depended on fungal proteolytic activity, biomass production, and easily degradable protein content in the medium.

The main goal of work was determination of effect of fungi inoculum on the petroleum hydrocarbon removal rate in soil covered and not covered with hair. The hair was the major nutrient for keratinolytic fungi and other soil microorganisms. The fungal inoculum accelerated the petroleum hydrocarbon biodegradation process during the first month of the experiment. The highest petroleum hydrocarbon removal rate was observed in soil inoculated with native fungal strains. The lowest removal rate was observed in soil not inoculated with fungi (60%). The hair applied as additional nitrogen, sulfur and carbon source did not impact, or slightly inhibited, the petroleum hydrocarbon biodegradation process. The fungal inoculum caused dramatic changes in soil fungal qualitative composition.

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Key words: biodegradation; keratinolytic fungi; keratinophilic fungi; petroleum hydrocarbons;

1. INTRODUCTION

One of the serious soil pollution problems is connected with the distribution of petroleum hydrocarbons. The hydrocarbons are released to the environment by purposeful introducing of petroleum wastewater or by accidents during production, transport and use of chemicals.

Among the soil bioremediation methods, bioaugmentation is one solution. Indigenous or extraneous bacteria are often added to soil as inoculum. In the last years, also fungal inoculation is more and more frequently applied (Lestan *et al.,* 1997). The potential of fungi for biodegradation of TNT and other explosives was an aim of the investigation by Johnston *et al.,* (1994). Subsequently, Takada *et al.,* (1994) studied the degradation of polychlorinated dibenzofurans and dioxins by fungi. The potential of different fungi for degradation of hydrocarbons was also the subject of studies by Davies and Westlake (1979), Watkinson and Morgan (1990), McGillivray and Sharis (1993), MacGallivray and Skiaris (1993), Field (1994,1995), McGughan (1995), Ravelet *et al.,* (2000), Giraud *et al.,* (2001), and Chavez-Gomez *et al.,* (2003).

However, little is known on the potential of keratinolytic and keratinophilic fungi for degradation of petroleum hydrocarbons (Ulfig *et al.,* 2000). Keratinolytic fungi are specialized in keratin decomposition, while keratinophilic fungi accompany keratinolytic fungi and utilize non-keratin components of keratinous substrata or the products of keratin decomposition. Soil is the main environment of fungal occurrence and activity. The abovementioned fungi occur not only in natural environments but also in polluted man-made sites. They are especially abundant in organic waste-contaminated areas rich in keratin substrata of human and animal origin. Such areas are often contaminated with different resistant compounds, e.g., aliphatic hydrocarbons, BTEXs and PAHs (Ulfig, 2000).

The study was to determine the ability of keratinolytic and keratinophilic fungi for removal of petroleum hydrocarbons from liquid medium and soil.

2. MATERIALS AND METHODS

2.1 Liquid medium experiment

Two strains, i.e., *Chrysosporium keratinophilum* PS14 and *Trichophyton ajelloi* PS16, were isolated, with the hair baiting method (Vanbreuseghem, 1952; Prochacki, 1975), from sewage sludge and oil

waste-contaminated refinery soil, respectively. The strains were tested for removal of petroleum hydrocarbons from liquid medium. The test was performed in 300-ml Erlenmeyer flasks. Liquid medium with different peptone (Bacteriological peptone, Difco) concentrations, supplemented and non-supplemented with hair, was used. The composition of the medium was as follows: $MgSO_4*7\ H_2O$ (0.25 g/L), $CaCl_2*6\ H_2O$ (0.025 g/L), $FeSO_4*7\ H_2O$ (0.015 g/L), $ZnSO_4*7\ H_2O$ (0.005 g/L), KH_2PO_4 (1,5 g/L), dextrose (1 g/L), microelement solution (1 ml), and chloramphenicol (0.1 g/L). The microelement solution included: $ZnCl_2$ – 0.07 g/L, $Na_2SeO_3*5H_2O$ – 0.026 g/L, $MnCl_2*4H_2O$ – 0.1 g/L, $NaVO_3*H_2O$ – 0.01 g/L, $CoCl_2*6H_2O$ – 0.2 g/L, $NaWO_4*2H_2O$ – 0.03 g/L, $NiCl_2*6H_2O$ – 0.1 g/L, HCl 25% - 1 ml/L, $CuCl_2*2H_2O$ – 0.02 g/L, and $NaMoO_4*5H_2O$ – 0.05 g/L. The final pH of the medium was 6.8. 50 ml of the liquid medium was added to each Erlenmeyer flask. The modifications of the experiment were as follows: P0 (medium without peptone), P0H (medium without peptone but with hair), P2 (medium with 2 g/L of peptone), P2H (2 g/L of peptone and hair), P4 (4 g/L of peptone), P4H (4 g/L of peptone and hair), P8 (8 g/L of peptone), P8H (8 g/L of peptone and hair). Each modification of the experiment was performed in six repetitions. At the end of the experiment, three repetitions were taken for extraction of hydrocarbons and the remaining three for other analyses. 0.1-g portions of defatted (in chloroform: methanol 2:1 mixture), cut into 0.5-cm pieces and autoclaved (20 min. at 121° C and 1.2 atm.) children's hair were added to H flasks.

The inoculum was prepared from 14-day old fungal cultures on MEA supplemented with chloramphenicol 0.1g/L to avoid bacterial contamination. The solid medium was cut into 0.5 x 0.5-cm blocks and 6-8 blocks were put into 10 ml of sterile physiological saline. Tubes with blocks suspended in physiological saline were intensively shaken for 1 minute. The fungal propagule concentration of the inoculum was 10^6 per ml. Except for control flasks, 200 µl of the inoculum was added to each flask. Samples were incubated 43 days in the dark on a rotary shaker (100-rpm) at 22°C.

At the end of the experiment, TPH (Total Petroleum Hydrocarbons included non-polar petroleum hydrocarbons) and TPOC (Total Petroleum Organic Carbon included non-polar petroleum hydrocarbons + their polar derivatives) were measured by the IR method. The extraction of samples in flasks was performed with carbon tetrachloride. The extract was quantitatively measured after calibration with five standards. The standard substance was a mixture of 37.5% (v/v) n-hexadecane, 37.5% (v/v) isooctane and 25% (v/v) benzene, and the spectrum was recorded between the 3000-2800 cm^{-1} range. The absorbency value was measured at 2926 cm^{-1} with an IR spectrophotometer Unicam SP1000 (UNICAM, UK). The TPH content was related to the CH_2 group number. The dry mass of samples was measured with a gravimetric method. Reaction (pH) was measured with an

electrometric method. Protein and thiol concentrations were determined with the methods by Lowry (Harlow and Lane, 1988) and Saville (1958), respectively. Sulfate concentrations were measured with ion chromatography. The Kjeldahl method was used for ammonium concentration determination (PN-75/A-04018).

3.2 Soil experiment

The experiment was performed with soil from a cattle farm. The soil was classified as clay (BN-78/9180-11). After sampling, the soil was cleaned from stones and plant remnants, thoroughly crumbled, mixed, dried on open air and sieved through a 2-mm sieve mesh. In the soil, the composition of keratinolytic and keratinophilic fungi was preliminarily determined with the hair baiting method (Vanbreuseghem, 1952; Prochacki, 1975). Soil samples (144 dishes) supplemented and non-supplemented with actidione (500 mg/L) were surveyed for fungi. Then, the soil was supplemented with sterile crude oil (autoclaved two times for 20 minutes at 121^{o} C and 1.2 atm. at a 24-hour interval). The oil concentration was 15 g per kg of soil dry weight. The experiment was performed in 10-cm glass Petri dishes. In the dishes, 30-g portions of oil-supplemented soil were placed. In half of the dishes set up, soil was covered with 0.3 g of hair in each dish. The hair was prepared in the manner described in the liquid medium experiment. Two inocula were prepared. The first inoculum (S1) consisted of extraneous keratinolytic strains *Chrysosporium keratinophilum* PS14 and *Trichophyton ajelloi* PS16, which were used in the liquid medium experiment. A mixture of indigenous *Chrysosporium keratinophilum* and *Trichophyton ajelloi* strains (earlier isolated from the examined soil and purified) was the second inoculum (S2). The dishes were inoculated with the fungal inocula. Each modification of the experiment was performed in 18 repetitions. The dishes were incubated for two months at 26^{o} C in the dark. The soil moisture was maintained at ca. 30% during the experiment.

Growth of keratinolytic and keratinophilic fungi on hair was observed every week. The scale of the fungal growth on hair was as follows: 0 – no growth; 1 – fungi covered ca. 25%; 2 – fungi covered ca. 50%; 3 – fungi covered ca. 75%; and 4 – fungi covered ca. 100% of a Petri dish. Samples for other measurements were taken at the beginning, after one and two months of the experiment (at its end). TPH and TPOC concentrations were measured by the IR method. Total, ammonium, nitrate and nitrite nitrogen was measured with methods recommended by Hermanowicz (1976). Sulfates and pH (in H_2O and 1M KCl) were measured every month with methods recommended by Nowosielski (1974). Statistical analysis of the data was performed using the Statistica 5.1 program.

3. RESULTS

3.2 Liquid medium experiment

During the liquid medium experiment, significant influence of peptone concentration on the petroleum hydrocarbon removal rate was observed for both fungal inocula (Table 1). The increase of peptone concentration 0-4 g/L resulted in the TPH removal increase from ≈11 to ≈70% and from ≈2 to 76% for *Chrysosporium keratinophilum* and *Trichophyton ajelloi* inocula, respectively. The same influences were observed for TPOC removals. Further increase of peptone concentration up to 8 g/L did not influence TPH and TPOC removal rates. The mean removal rates were 70 and ≈54% for *Chrysosporium keratinophilum* and *Trichophyton ajelloi* inocula, respectively.

Table 1. Removal of petroleum hydrocarbons from liquid medium.

Modification of the experiment	*Chrysosporium keratinophilum* PS14				*Trichophyton ajelloi* PS16			
	TPH		TPOC		TPH		TPOC	
	%	St. Dev.	%	St. Dev.	%	St. Dev.	%	St. Dev.
P0	0	1.5	0	0.2	5.7	15.0	6.5	13.1
P0H	26.4	16.5	26.0	16.1	0	1.8	0	2.5
P2	30.8	14.0	27.1	14.4	42.4	10.9	46.5	10.8
P2H	31.9	10.5	36.4	15.9	42.0	11.1	44.7	14.3
P4	59.4	7.8	59.2	1.2	66.9	3.4	65.4	1.8
P4H	80.4	1.5	79.1	0.9	85.1	3.5	81.9	6.5
P8	73.6	5.8	71.1	7.8	43.6	0.8	42.4	4.6
P8H	66.5	10.1	63.5	12.6	63.8	9.7	63.6	8.6

St. Dev. – Standard Deviation

The medium supplementation with hair generally increased hydrocarbon removal rates but the rates depended on the specified inoculum and peptone concentration. The mean TPH removals from samples non-supplemented and supplemented with hair was 39.8 and 49.2%, respectively. The highest hydrocarbon removal was observed in samples inoculated with *Trichophyton ajelloi* PS16 and in the medium supplemented with 4 g/L of peptone and hair (85.1 and 81.9% of TPH and TPOC removal, respectively). Under the same conditions, the TPH and TPOC removals from samples inoculated with *Chrysosporium keratinophilum* PS14 were 80.4 and 79.1%, respectively.

Table 2. Physico-chemical properties of media at the end of experiment with liquid medium.

Modification of the experiment		pH		N-NH_4 [mg/sample]		Proteins [mg/sample]		Thiols [μg/sample]		S-SO_4 [mg/sample]		Dry mass [mg/sample]	
		AV	S.D.	AV	S.D.	AV	S.D.	AV	S.D.	AV	S.D.	AV	S.D.
Control	P0	5.0	0.5	0.2	0.1	0.9	0.1	0.0	0.0	3.8	0.6	6.0	2.0
	P0H	5.7	0.3	0.1	0.1	1.5	0.3	0.0	0.0	3.7	0.6	75.7	8.0
	P2	6.5	0.1	0.9	0.0	13.2	0.6	0.0	0.0	4.1	0.4	6.0	2.0
	P2H	6.5	0.0	0.7	0.1	13.9	0.4	0.0	0.0	4.2	0.0	80.0	4.4
	P4	6.2	0.2	1.2	0.1	17.8	0.6	14.3	1.5	2.0	0.9	14.3	1.3
	P4H	6.1	0.0	1.2	0.2	18.7	1.2	15.7	1.3	4.9	0.5	99.8	8.4
	R8	7.1	0.0	2.6	0.1	33.4	0.8	0.0	0.0	2.6	0.1	10.0	2.0
	P8H	7.0	0.0	2.5	0.1	35.0	1.6	0.0	0.0	2.6	0.3	84.7	14.3
Inoculum PS14	P0	5.1	0.0	0.3	0.0	0.7	0.3	0.0	0.0	3.9	0.3	3.7	0.6
	P0H	8.5	0.1	4.4	0.3	5.2	0.8	0.1	0.0	7.7	0.5	48.3	4.7
	P2	8.8	0.1	3.6	0.2	4.5	0.4	0.1	0.0	4.7	0.5	43.5	21.9
	P2H	9.0	0.0	5.1	0.0	6.6	0.7	0.2	0.0	5.5	0.5	89.7	4.6
	P4	7.5	1.4	10.1	1.7	14.6	5.7	7.4	0.7	4.6	1.0	36.8	24.2
	P4H	8.1	1.3	9.7	0.7	11.6	3.2	4.7	0.8	3.4	0.1	159.3	11.4
	R8	9.3	0.1	9.1	1.7	16.8	1.4	0.1	0.0	6.2	0.3	78.3	7.6
	P8H	9.4	0.1	9.9	0.8	19.1	2.6	0.3	0.0	6.9	1.2	131.0	7.2
Inoculum PS16	P0	4.6	0.1	0.2	0.0	1.4	0.4	0.0	0.0	3.5	0.5	3.7	2.1
	P0H	8.0	0.4	3.1	0.8	5.6	0.7	0.1	0.0	4.8	0.3	67.7	2.5
	P2	8.9	0.0	3.9	0.2	5.4	0.2	0.0	0.0	4.1	0.4	38.0	8.7
	P2H	8.9	0.1	7.5	0.4	6.7	0.4	0.1	0.0	4.4	0.2	88.0	3.6
	P4	8.6	0.1	7.9	0.7	9.5	0.8	3.2	1.3	4.4	1.0	80.8	14.5
	P4H	8.7	0.1	8.5	1.3	10.2	0.7	3.1	1.4	4.2	1.3	159.3	13.4
	R8	9.3	0.1	10.2	0.8	15.2	0.4	0.1	0.1	5.0	0.5	87.7	2.9
	P8H	9.2	0.1	8.7	0.5	16.3	1.5	0.1	0.0	5.7	0.2	142.7	10.5

AV – average, S.D. – standard deviation

Petroleum hydrocarbon removal values correlated with physico-chemical changes in the liquid medium (Table 2). In samples with high hydrocarbon removal rates, high dry mass was determined. The highest dry mass was found in the medium supplemented with hair. The protein concentration positively correlated with pH and ammonium nitrogen content. The medium supplementation with hair increased pH. Generally, pH

increased with increasing peptone concentration in the medium. Thiols occurred in the medium at very low concentrations. The medium supplementation with hair significantly increased the sulfate concentration only in some samples, e.g., control/P4/P4H and PS14/P0/P0H.

3.2 Fungal composition in soil

The qualitative and quantitative composition of keratinolytic and keratinophilic fungi in the soil examined is presented in Table 3. Altogether, 48 fungal appearances belonging to 11 species were observed. Six species

Table. 3. The occurrence of keratinolytic and keratinophilic fungi in soil.

Species of fungi	Number of appearances		Sum of appearances
	With actidione	Without actidione	
Chrysosporium keratinophilum D.Frey ex Carmichael*	7	4	11
Fusarium solani (Mart.) Saccardo	2	7	9
Trichophyton ajelloi (Vanbreuseghem) Ajello*	2	7	9
Paecilomyces lilacinus (Thom) Samson	2	3	5
Pseudallescheria boydii (Shear) McGinnis *et al.,*	0	5	5
Gliocladium roseum Bain.	2	2	4
Plectosphaerella cucumerina (Lindf.) W. Gams	1	0	1
Chrysosporium pannicola (Corda) van Oorschot et Stalpers*	1	0	1
Chrysosporium anamorph of *Aphanoascus clathratus* Cano et Guarro*	1	0	1
Chrysosporium an. *Aphanoascus reticulisporus* (Routien) Hubálek*	1	0	1
Chrysosporium indicum (Randhawa et Sandhu) Garg*	1	0	1

* - keratinolytic species

were keratinolytic. *Chrysosporium keratinophilum* (22.9%), *Fusarium solani* (18.8%), *Trichophyton ajelloi* (18.8%), *Paecilomyces lilacinus* (10.4%), and *Pseudallescheria boydii* (10.4%) were the prevailing species. The other species occurred with frequencies <10%.

The numbers of fungal appearances in soil samples supplemented and non-supplemented with actidione were 20 and 28, respectively. The soil supplementation with actidione increased the number of *Chrysosporium keratinophilum* appearances and the number of keratinolytic species but decreased the number of keratinophilic fungi appearances.

3.3 Soil experiment

Strains *Chrysosporium keratinophilum* and *Trichophyton ajelloi* added to the soil examined as extraneous (S1) and indigenous (S2) inocula, increased the hydrocarbon removal rates (Table 4; figures 1 and 2). This effect was observed after the first month of the experiment, while after the second month the differences in hydrocarbon removal rates equalized. After the first month of incubation, the hydrocarbon removal rate in the inoculated soil was almost two times higher than in the non-inoculated soil. The inoculum S1 enhanced the TPH removal rate from 39 to 62.5%. The inoculum S2 enhanced the TPH removal rate even to 70.7%. The hair addition to the soil did not have significant impact on hydrocarbon removal. TPH changes were similar to TPOC changes in the soil during the experiment.

Table 4. TPH and TPOC removal rates in the soil experiment.

Modification of the experiment	TPH		TPOC	
	% removal		% removal	
	After 1 month	After 2 months	After 1 month	After 2 months
S	39.0	60.1	43.6	49.3
SH	29.2	60.1	26.6	38.6
SS1	62.5	59.1	45.2	47.0
SHS1	61.9	59.8	58.3	50.5
SS2	70.6	76.6	58.2	72.0
SHS2	64.8	63.7	52.6	59.3

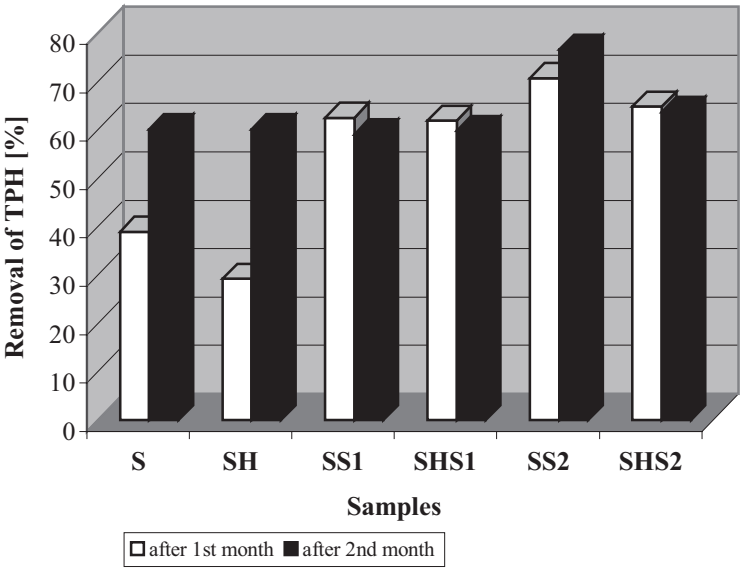

Figure 1. Removal of TPH from soil during experiment.

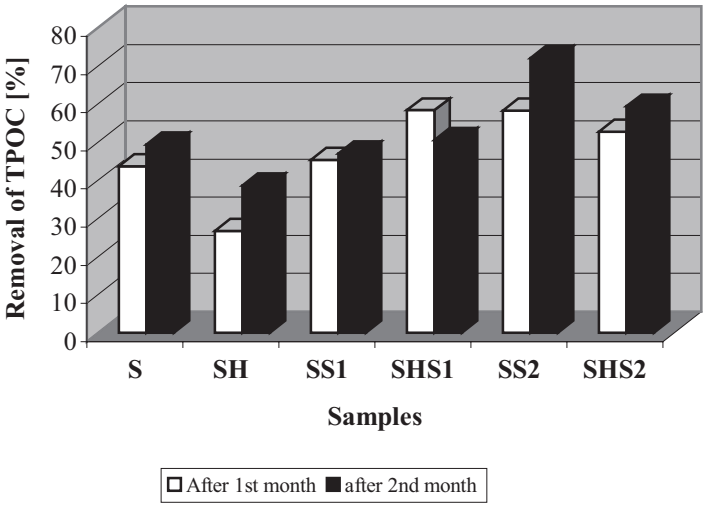

Figure 2. Removal of TPOC from soil during experiment.

The best growth of keratinolytic and keratinophilic fungi was observed in soil samples covered by hair and supplemented with inocula. After 3 weeks, almost 100-% cover by mycelium and spores was noted in samples SHS1 and SHS2 (figure 3). Mycelium and spores covered the non-inoculated soil SH only in 50% after 6 weeks of incubation. At the 9th week of the experiment, the biomass atrophy was observed. The intensity of fungal growth did not correlate with TPH and TPOC removal rates. During the experiment, *Trichophyton ajelloi* and *Chrysosporium keratinophilum* prevailed in the non-inoculated soil. *Trichophyton ajelloi* also prevailed in the S2-inoculated soil, whereas *Microsporum gypseum* was the predominating species in the S1-inoculated soil.

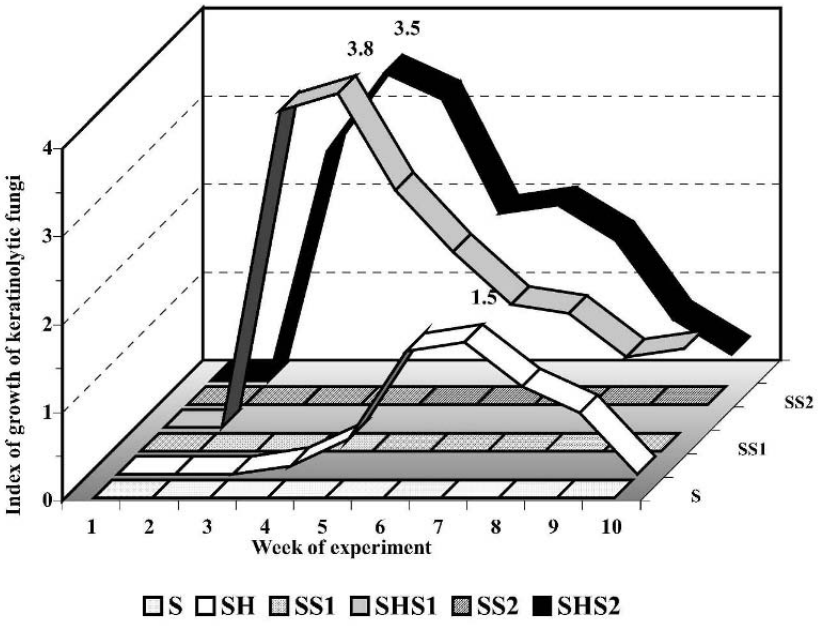

Figure 3. The fungal growth index changes on petroleum-contaminated soil and on hair laid on petroleum-contaminated soil.

Changes in soil physico-chemical parameters are presented in Table 5. Reaction (pH in H_2O) ranged between 8.9-9.6 at the beginning of the experiment. After the first month of the experiment, pH decreased and ranged between 7.0–7.9. After the second month, further pH increase was observed and the range was 6.5-7.7. The lowest pH values were observed in samples SHS1 and SHS2, while the highest was in sample SS2. Similar changes were found for pH in 1M KCl.

Table 5. Soil physico-chemical characteristics.

Modification	measure-	Day of experiment	pH		$S\text{-}SO_4$	Nitrogen			
			H_2O	KCl		N total	NH_4^+	NO_2^-	NO_3^-
					% (d.m.)	g/kg d.m.	mg/kg d.m.		
S	0	1	9.6	8.9	0.018	7.0	19.9	0.0	70.5
	1	31	7.8	7.4	0.026	5.6	47.4	0.0	21.8
	2	63	7.2	7.3	0.024	6.4	38.4	0.9	15.7
SH	0	1	9.1	8.4	0.013	7.8	38.1	0.0	30.0
	1	31	7.8	7.5	0.053	8.2	51.4	0.0	24.6
	2	63	6.8	6.8	0.096	7.3	38.5	0.8	0.0
SS1	0	1	9.2	8.4	0.020	8.1	25.8	0.0	0.0
	1	31	7.8	7.7	0.022	7.9	49.3	0.0	6.8
	2	63	7.5	7.4	0.029	7.5	39.6	2.5	63.4
SHS1	0	1	8.9	8.5	0.016	8.6	25.6	0.0	4.1
	1	31	7.0	7.0	0.065	7.8	48.1	0.0	13.5
	2	63	6.5	6.5	0.033	6.9	29.1	1.6	24.2
SS2	0	1	9.1	8.5	0.022	6.5	25.6	0.0	9.9
	1	31	7.9	7.6	0.019	6.6	43.9	0.0	22.3
	2	63	7.7	7.4	0.028	8.3	45.1	1.1	21.8
SHS2	0	1	9.0	8.3	0.019	6.4	20.1	0.0	25.5
	1	31	7.2	7.1	0.041	5.7	48.4	0.0	25.3
	2	63	6.6	6.6	0.050	5.9	42.3	1.3	65.3

d.m. – dry mass

At the beginning of the experiment, sulfate concentrations varied between 0,013-0,022% d.m. After the first month of incubation, the sulfate concentration was found to be much higher, especially in the hair-covered soil (0,041-0,065% d.m.). After the second month of the experiment, further sulfate increase was observed in samples SH (0,096% d.m.) and SHS2 (0,050% d.m.). In sample SHS1, a sulfate concentration decrease was observed. A sulfate concentration increase was also observed in the soil uncovered by hair. However, this increase was not so significant compared to the increase in the soil covered by hair.

During the experiment, the total nitrogen content ranged between 5.6-8.6-gN/kg d.m. At the beginning of the experiment, the ammonium concentration ranged between 19.9-38.1 mg $N\text{-}NH_4$ /kg d.m. and after the first month of the experiment increased to 43.9 and 51.4 mg $N\text{-}NH_4$ /kg d.m. in samples SS2 and SH, respectively. After the second month of incubation, ammonium concentrations decrease to 29.1 and 45.1 mg $N\text{-}NH_4$/kg d.m. was noticed in samples SHS1 and SS2, respectively. During the first month of the experiment, nitrite nitrogen was not observed in soil. After the second month of the experiment, the nitrite concentrations were 0.8 and 2.5 mg $N\text{-}NO_2$/kg d.m. in samples SH and SS1, respectively. During the first month of the experiment, the nitrate nitrogen concentration decreased from 70.5 to 15.7 mg $N\text{-}NO_3$/kg d.m. in sample S. The same tendency was observed in sample SH. In inoculated soil, a nitrate nitrogen concentration increase was evident.

4. DISCUSSION

Davies and Westlake (1979), and Ulfig *et al.,* (2000) observed the removal of petroleum hydrocarbons by keratinolytic and keratinophilic fungi for *Microsporum* sp., *Trichophyton* sp. and *Chrysosporium* sp. All our experiments were performed to confirm this phenomenon and show that the above-mentioned fungi could play a role in the degradation of petroleum hydrocarbons in the environment.

The liquid medium experiment focused on the potential of two keratinolytic strains for removal of hydrocarbons from the liquid medium during degradation of different protein substrates. On the one hand, the hydrocarbon removal process was studied and, on the other hand, fungal biodegradation of keratin and peptone as co-substrates for removal of hydrocarbons was evaluated. Generally, the hydrocarbon removal rate increased with increasing peptone concentration in the liquid medium. The lowest hydrocarbon removal, or no removal, was observed in the medium non-supplemented with peptone. The peptone in the range of 2-4 g/L promoted the hydrocarbon removal. Further peptone concentration increase (up to 8 g/L) did not promote the process. The phenomenon could result from the substrate inhibition process (Stryer, 1995) or from leaving of less accessible substrate, i.e., hydrocarbons, for easy accessible sources of carbon, nitrogen and energy. At the end of the experiment, the atrophy of fungal biomass was observed in the medium supplemented with 8 g/L of peptone. This could also suggest a deficit of oxygen or accumulation of harmful-to-fungi metabolic products in the medium. The increase of dry mass associated mostly with biomass was observed in all samples supplemented with peptone in the range of 2-8 g/L. Differences between *Trichophyton ajelloi* and

Chrysosporium keratinophilum were observed. In the case of the first strain, the medium supplementation with hair was associated with no hydrocarbon removal and the growth of the fungus was weak. In the case of *Chrysosporium keratinophilum,* the medium supplementation with hair promoted hydrocarbon removal. The highest dry mass values were found in samples supplemented with hair. It was associated with the presence of non-degraded hair in the samples. Keratin is a difficult substrate for all microorganisms and only keratinolytic microorganisms, chiefly fungi can destroy the substrate (Kączkowski, 1974; Deshmukh and Agrawal, 1981; Safranek and Goss, 1982; Wolski, 1985; Marshal *et al.,* 1991; Onifade *et al.,* 1998). Unfortunately, till now the proper method for separation of biomass from hair has not been found (Chesters and Mathison, 1963).

The peptone and hair degradation by keratinolytic fungal strains increased pH and sulfate concentrations but decreased protein concentrations in the medium. The medium alkalization process conducted by *Microsporum* sp. and *Trichophyton* sp. was also observed by Paveia *et al.,* (1975). In samples supplemented with hair, pH and sulfate concentrations were higher than in samples supplemented only with peptone. In contrast, protein concentrations were higher in peptone-supplemented samples. Paveia *et al.,* (1975) observed that the addition of hair to the medium slightly increased pH. The same trends were observed for *Trichophyton* sp. and *Chrysosporium* sp. by Deshmukh and Agrawal (1985), for *Microsporum* sp. and *Trichophyton* sp. by Safranek and Goos (1982) and Deshmukh and Agrawal (1998), and for *Chrysosporium* sp. and *Microsporum* sp. by Nigam and Kushwaha (1992), and for *Trichophyton ajelloi* by Ulfig *et al.,* (2000). In our experiment, the covering effect of proteolysis and keratinolysis could be observed in samples supplemented with peptone and hair. Which of the processes was more important, we could only try to explain based on observations of samples. Only when the deficit of peptone was observed in the medium, the fungi started to utilize hair as a substrate. This can be an explanation of insignificant differences in thiol concentrations between samples supplemented and non-supplemented with hair. The hair degradation starts from the disruption of keratin fibers by mycelium and the disruption of disulfide bonds of cysteine (Kunert, 1992, 2000; Onifade, 1998; Ulfig *et al.,* 2000). Such denatured keratin is more susceptible for proteolysis that results in deamination and alkalization of the medium (Deshmukh and Agrawal, 1981, 1985; Safranek and Goos, 1982; Ulfig *et al.,* 2000). The final products of the keratin sulfur compound decomposition are sulfites, sulfates and sulfur (Kunert, 1992, 2000; Ulfig *et al.,* 2000).

The soil inoculation with extraneous and indigenous keratinolytic fungi was performed to enhance the petroleum hydrocarbon removal. It is known that crude oil pollution has a considerable influence on the homeostasis of the environment. One of the most important effects is connected with

changes in C:N ratio. This ratio is higher in polluted soil and connected with the deficit of nitrogen that can slow down the bioremediation process (Malina and Szczepański, 1994; Kańska *et al.,* 1997). Addition of hair to soil should decrease this deficit and be a source of nitrogen, sulfur and carbon to keratinolytic fungi (Wolski, 1985; Marshall *et al.,* 1991; Kunert, 2000).

The growth of keratinolytic fungi was more abundant in inoculated soils. This was certainly associated with higher initial concentrations of these fungi and more beneficial environmental conditions for fungal growth. Such a beneficial influence of soil inoculation was also observed by Łebkowska (1996) and Kańska *et al.,* (1997). However, significant changes in the composition of fungal species were observed in inoculated soils. In the soil inoculated with indigenous strains (SHS2), only one species from the inoculum, i.e., *Trichophyton ajelloi*, prevailed. Subsequently, a "new" species, i.e., *Microsporum gypseum*, prevailed in the soil inoculated with extraneous strains. Such effects could result from changes in soil physico-chemical properties and quantitative and qualitative composition of microorganisms caused by inoculation (Kańska *et al.,* 1997). One of the most important factors influencing the activity and composition of microorganisms in soil is pH. Geophilic dermatophytes and other keratinolytic fungi occur in the environment within a wide pH range. However, *Trichophyton ajelloi* is acidophilic and *Chrysosporium keratinophilum* prefers alkalophilic environments (Garg *et al.,* 1985). The explanation of the *Chrysosporium keratinophilum* atrophy could be the soil acidification. *Microsporum gypseum* occurs in a wide pH range but prefers soils with high organic matter content. Extraneous strains added to the soil probably died and became a substrate for other microorganisms, including the fungus.

The hydrocarbon removal from the soil was the highest during first month of the experiment. The phenomenon was also observed by Maliszewska-Kordybach (1993) in a bioremediation experiment. After the first phase, the hydrocarbon degradation process slows down, what is chiefly associated with accumulation of toxic metabolites, soil acidification, sorption of pollutants and the deficit of easy accessible carbon and energy sources. The promotion of petroleum hydrocarbon removal during the first month of the biodegradation process was also observed by Bieszkiewicz *et al.,* (1999) in a bioaugmentation experiment. Additionally, during our experiment the hair addition to the soil did not have a positive effect on hydrocarbon removal rates. In some samples, the hair addition even slowed down TPH/TPOC removal rates. This is in agreement with results by Maliszewska-Kordybach (1993). The researcher observed that an additional source of carbon not always had a positive effect on hydrocarbon degradation processes. Additional carbon sources can promote sorption of pollutants and concentrate metabolic activity of microorganisms on easier

accessible sources of carbon end energy. The influence of nitrogen sources on hydrocarbon degradation processes also is not clear (Maliszewska-Kordybach, 1993). Another explanation of our observations could be a competition between indigenous and extraneous microorganisms while degrading hydrocarbons. The competition between microorganisms might have eliminated some microbial groups, including keratinolytic fungi from the inocula. However, the other microorganisms were not eliminated from the soil and could significantly influence the bioremediation process. Thus, the hydrocarbon removal rate is a result of the total microbial activity in the soil (Davies and Westlake, 1979; Cerniglia, 1984; MacGillivray and Skiaris, 1993; Łebkowska, 1996; Heusemann, 1997; Kańska *et al.,* 1997; Nowak and Hawrot, 1999). The keratinolytic fungi on hair could only weakly influence the hydrocarbon removal from the soil.

Significant changes in soil physico-chemical parameters were observed in all samples during the experiment. During the first month of the experiment, the increase of ammonium nitrogen was evident. This testifies that the degradation of organic matter and ammonification proceeded in the soil. However, the hair addition to the soil did not significantly influence nitrogen transformations. During the second month of the experiment, the concentration of ammonium nitrogen decreased, what was probably connected with incorporation of ammonium to the biomass, and with nitrification (Kunicki-Goldfinger, 1994; Schlegel, 2000). During the experiment, the increase of sulfates was observed especially in the soil covered by hair. The concentration of sulfur in hair (concentrated mostly in amino acids such as cysteine, cystine and methionine) used in the experiment was 3.6%. The end product of the organic sulfur oxidation by keratinolytic fungi is sulfate (Kunert, 2000). The oxidation of hair sulfur by keratinolytic fungi could only explain the increase of sulfate concentration in the soil. Nitrates and sulfates contributed to the acidification of the soil examined.

5. CONCLUSIONS

The ability for removal of petroleum hydrocarbons depended on the specified fungal strain and was probably associated with high degree of adaptation for living in habitats heavily contaminated with the pollutants. It was observed that petroleum hydrocarbon removal rates depended on fungal proteolytic activity, biomass production, and easily degradable protein content in the medium. The mean value of hydrocarbon removal was 39.8% during degradation of peptone and 49.2% during degradation of peptone and hair. The highest hydrocarbon removal from the medium was observed when the concentration of peptone was 4 g/L.

The fungal inocula significantly increased the petroleum hydrocarbon removal process during the first month of the experiment. During the second month, TPH removal rates were found to be similar for soil inoculated and not inoculated with fungi. The highest petroleum hydrocarbon removal rate was observed in the soil inoculated with native fungal strains. The TPH removal rates were about 64 and 77% in soils covered and uncovered with hair, respectively. The lowest removal rate was observed in soil non-inoculated with fungi (60%). The hair applied as additional nitrogen, sulfur and carbon source did not impact, or slightly inhibited, the petroleum hydrocarbon removal process. The fungal inoculum caused dramatic changes in soil fungal qualitative composition.

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STUDY OF CONTAMINATION AND MIGRATION POLYCHLORINATED BIPHENYLS IN THE ENVIRONMENT. BIOREMEDIATION OF CONTAMINATED SOILS AND ASSESSMENT OF THEIR IMPACT ON THE SERPUKHOV POPULATION HEALTH

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1. INTRODUCTION

According to the WHO data polychlorinated biphenyls (PCBs) are among twelve most dangerous and hardly decomposed environmental pollutants. Until recently they had been broadly used for the electro–technical and chemical industries because of extreme persistence in the environment. They cannot be decomposed photolytically, chemically, and biologically. However, polychlorbiphenyls are highly toxic, are accumulated in human organism and have carcinogenic effect.

Processes of soil self–cleanup are slow, due to high PCB concentrations and their high persistence in the environment, and they need active human interference. In our opinion, biotechnological methods have a number of advantages in comparison with physical–and–chemical ones: ecological safety, harmlessness of final degradation products for the environment, high

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adaptability and specificity towards different pollutants, acceptable labor expenditures and work cost, keeping of natural state and fertility of soils under bioremediation.

Serpukhov is a typical industrial town of the Central part of Russia with population over 150,000 people. For many years PCB contamination of topsoil of built–up zone in "Zaborie" region was # 1 problem for Serpukhov. Specially conducted studies have identified the contamination source – Scientific–and–Production Association "Condensator", at which starting from early 60–s 14 commercial PCB preparations including Arochlor 1242 and 1254 (USA), Kanchlor (Japan), Pyralen and Fenochlor (France), Klofen (Germany), Trichlordiphenyl, Sovol, and Sovtol (Russia) have been used in capacitor production technology. Average monthly PCB consumption made up 1.4 tons. As a result of the technology imperfection, great amounts of PCB have been discharged into the environment over the 20–year period.

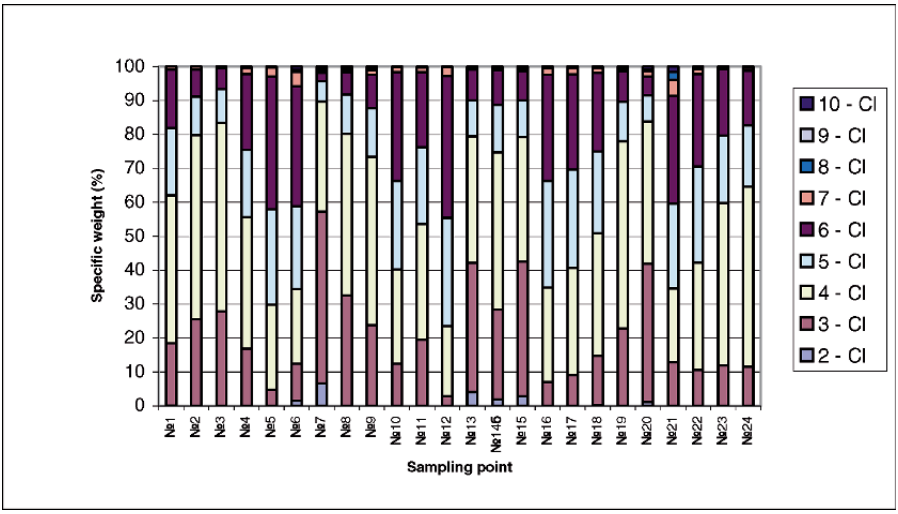

Figure 1. Specific weight of biphenyls by a number of Cl in soils of Serpukhov (starting samples).

State Sanitary and Epidemiological Surveillance carried out the first special studies for determination of the contaminated soils of Serpukhov in 1987–1988. The considerable contamination of upper soil layer, which was tens and hundreds mg of PCB/kg under approximately permissible APA amount – 0.06 mg/kg, was found. On about 30 % of the territory, soil contamination exceeded APA up to 10 times. About 30 % of city soils contain 10-50 APA of PCB. 5 % of soil is contaminated by biphenyls in

concentrations divisible by 50-100 APA. About 14 % of city territory is contaminated by more than 100 APA of PCB, and about 3 % - by more than 1,000 APA. The most PCB-contaminated area around the plant is 20 hectares.

PCBs migration in the environment. Work on ecologo-geochemical assessment that was conducted on the territory of Serpukhov during several years made it possible to draw a map of PCBs content in soil. Based on relief knowledge the conclusion was made on horizontal distribution of PCBs in soil. Migration down soil profile was observed at a depth of 10-20 cm with 6.5-9 times higher content. This kind of picture does not have a single-meaning explanation since vertical PCBs distribution in soil depends on a number of factors.

Low chlorinated PCBs prevail either in volatile fraction of outside air or in the atmosphere with the content of 84%-97%. At that more than 50% of PCBs low chlorinated portions fell on TCD. Total PCBs flow from atmosphere for any type of subjacent surface is a sum of four flows. Those are flows of wet sedimentation of gas and aerosol phases and gas phase exchange flow. The last one can be directed down to characterize sedimentation or up to characterize remission. The main contribution is made by dry sedimentation of gas and aerosol phases. Portion of gas phase wet sedimentation is very slow in comparison with other sedimentation processes as a result of poor dissolubility of gas phase of all kinds of PCBs in rain sediments.

Since main portion of PCBs is accumulated in soil it is essential to study PCBs migration in soil phase as well as to study in-depth analysis of soil contamination by polychlorinated biphenyls: heterogeneity of contamination, distribution of PCBs in soil, sorption properties and bioavailability of PCBs for microorganisms and plants, migration with subsoil waters, change in PCBs concentration during several seasons under the influence of climatic factors, saprophytic soil microflora and physical factors (aeration, ploughing, moistening).

PCBs phytotoxicity and bioavailability, sorption properties of different types of soil, coefficients of accumulation into different agricultural plants – all these data are of great practical and theoretical interest.

Study of the PCB impact on the population health. The larger part of the contaminated zone is occupied by private houses with farmlands, where crop production, traditionally used as food by population, is actively grown. Resident population is daily using the PCB–contaminated food, what results in direct PCB impact on human organisms

To determine relative risk and population attributive risk to health of preschool-aged children, living in the zone of extreme chronic PCB effect, copying out data from individual health cards, Russian forms Ф26

(«Individual card of a child going to child preschool institution (CPI) and school»), and Ф112 («Children health card») of children on sickness rate for the period from 1996 to 1999 was done. Children sickness rate was registered by Russian form МКБ-Х.

Extracts of health condition of the population, living on contaminated territory, were made from personal medical records.

On the basis of collected medical information computer database was developed on the population health condition, on the basis of which the risk of sickness rate was evaluated.

Calculation of carcinogenic risk resulted from using of PCB–contaminated vegetable production was done in accordance with the existing techniques for quantitative evaluation of risk resulted from using of contaminated food, EPA "RISK ASSESSMENT" [*PCBs: Cancer Dose–Response Assessment and Application to Environmental Mixtures// EPA/600/P–96/001F. – National Center for Environmental Assessment. Office of Research and Development. USEPA, Washington D.C., September 1996*]. Affecting PCB doses were calculated on the basis of comparative analysis of data on contamination of soil and data on PCB monitoring in foodstuff in "Zaborie" microdistrict (where “Condensator” plant is situated and where most contaminated soils were revealed). As it was revealed during analysis, using corresponding coefficient of PCB migration fron soil into agricultural products allow to obtain amounts of biphenyl content in agricultural products (vegetables, potatoes, and greens), comparable to real data of monitoring of production quality.

Analysis of health status of the population inhabiting the PCB–contaminated territory showed evident abnormalities expressed in elevated morbidity of some organs and system. Typical for this population is increased mortality rate resulted from blood circulation system diseases, malignant growths, and respiratory organ diseases. Noted is elevated epidemiological attributive risk of sickness with blood circulation system, respiratory organs, skin diseases, digestion organs, endocrine system, musculoskeletal system, malignant growths, women morbidity and total morbidity.

Extremely hazardous ecological situation in Serpukhov provoked the urgency to conduct the work for remediation of territories contaminated by PCBs.

The biotechnology for bioremediation of soils contaminated by PCB was developed at the RCT&HRB within the ISTC Project # 228 in 1996–1999. As a result of investigations at RCT&HRB, 2 natural strains of microorganisms–PCB degraders – *Hansenulla californica* strain *AT and Alcaligenes latus* strain *ТХД–13*– were obtained from soils contaminated by polychlorbiphenyls. Isolated microorganisms are aerobes and can be

applied directly in upper soil layers (*in situ*). According to the results of laboratory and field trials on PCB degradation in soil, as well as toxicological trails, the given microorganisms strains can be used for bioremediation.

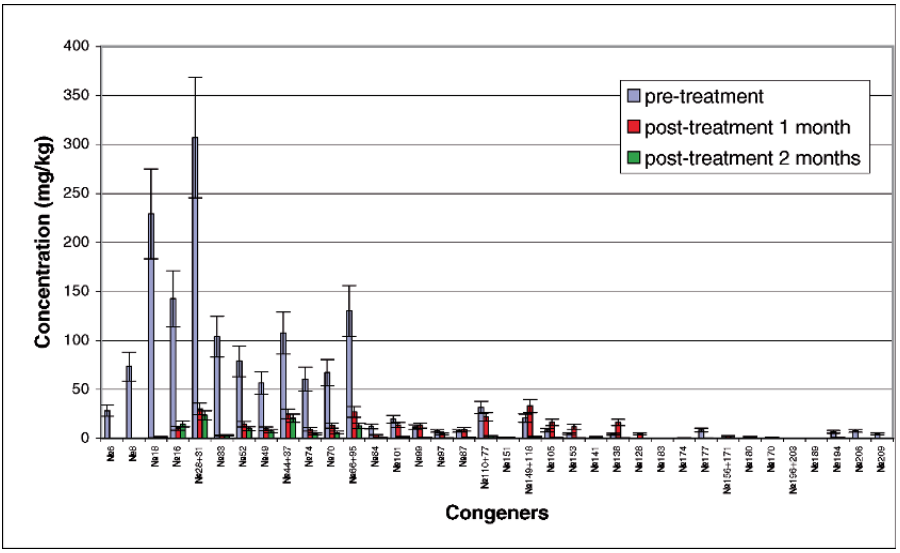

Figure 2. PCB congener's profiles in soil on the territory of sewer outlet of "Condensator" plant during bioremediation.

The microorganisms were deposited to All–Russia Collection of industrial depositing. 2 international patent applications PCT – RU/98/00036, and PCT – RU/98/00037, as well as Russian (# 2155803 and 2155804 from 13.02.98) and American patents (# 6.284.521 and 6.287.842 from 20.09.2000) have been drawn up on them.

Results of laboratory investigations of microbiological polychlorbiphenyls destruction showed that the optimal factors for deliberate degradation speed are high humidity (60–80%) and temperature (20–30°C) of soil. Development of microorganisms–PCB degraders of aboriginal soil microflora is not suppressed.

Technology of biotesting of soil on integral toxicity was suggested for the express–control of bioremediation process. Daphnia for which LD_{50} is 2,8 mg PCB/kg of soil showed high sensitivity for soil contaminated by PCB. Biotesting allows assessing integral toxicity before and after bioremediation.

Technology for bioremediation of *«in situ»* soils, contaminated by PCB, was worked out on the basis of conducted laboratory and field investigations.

1.1 Demonstration testing of technology for bioremediation of soils contaminated by PCB

Trails of developed technology for bioremediation of soils, contaminated by PCB were conducted on the territory of Serpukhov within the framework of ISTC Project # 2093 during summer of 2001. Serpukhov administration rendered active assistance in the work conducting.

8 most contaminated Serpukhov sites with total area – 2 hectares were selected for trials of the bioremediation technology. 4 of them were on the territory of SPA «Condensator», and the other 4 were on the adjoining living territory

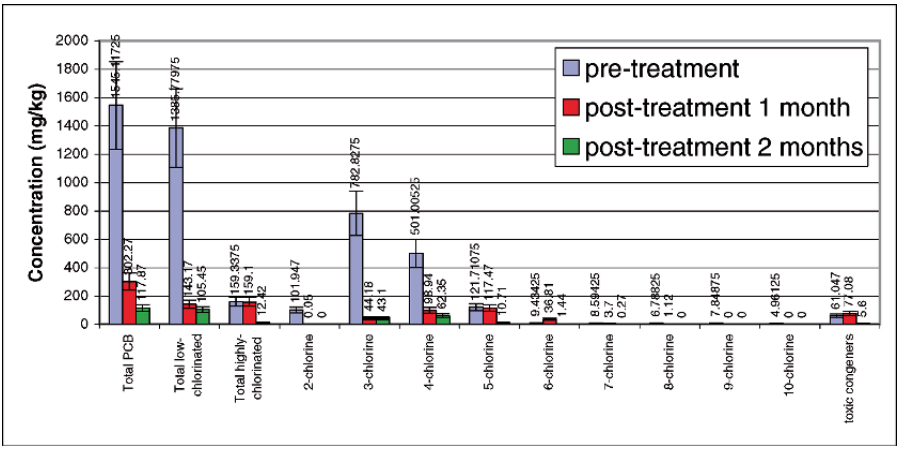

Figure 3. Change of PCB congeneres content by chlorine atoms number in soil on the territory "Condensator" plant during bioremediation.

In June 2001 sites intended for bioremediation were prepared (scavenging, ploughing and harrowing). On the base of RCT&HRB accumulated was microbial biomass (100 litres per each strain) of two strains of microorganisms-PCB degraders (strain AT and strain TCD–13 with concentrations 10^7 and 10^9 respectively). From July 2, 2001, degrader microbial biomass was introduced in contaminated soil on the sites with the help of farming machinery. Microbial suspension was 100–fold diluted by trap water, and introduced in soil on the basis of 1 liter per $1m^2$.

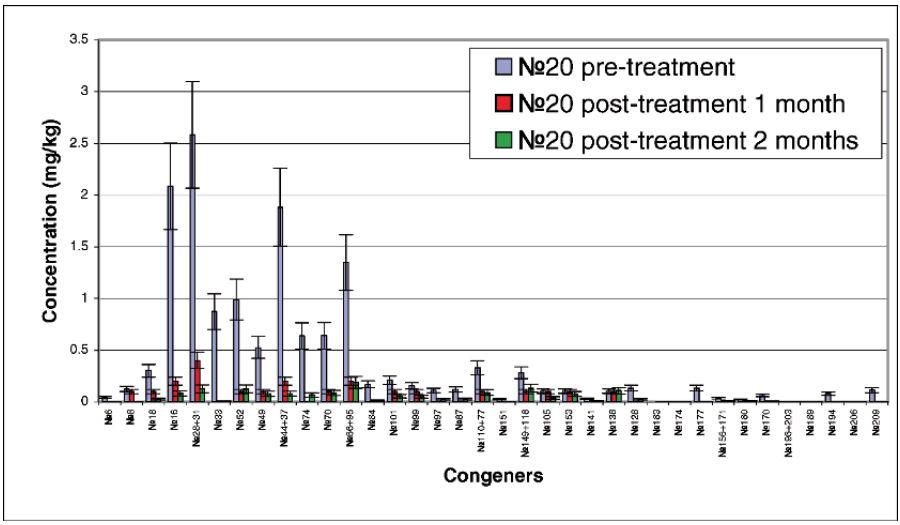

Figure 4. PCB congener's profiles in soil on the territory of the "Besymyanny" lane during bioremediation.

During July and August, due to extremely high temperature for this region and rain absence, soil was moistened with the help of washing machinery. To study soil contamination by polychlorinated biphenyls and assessment of soil integral toxicity, averaged soil samples were taken monthly from the upper most contaminated layer (0–20 cm).

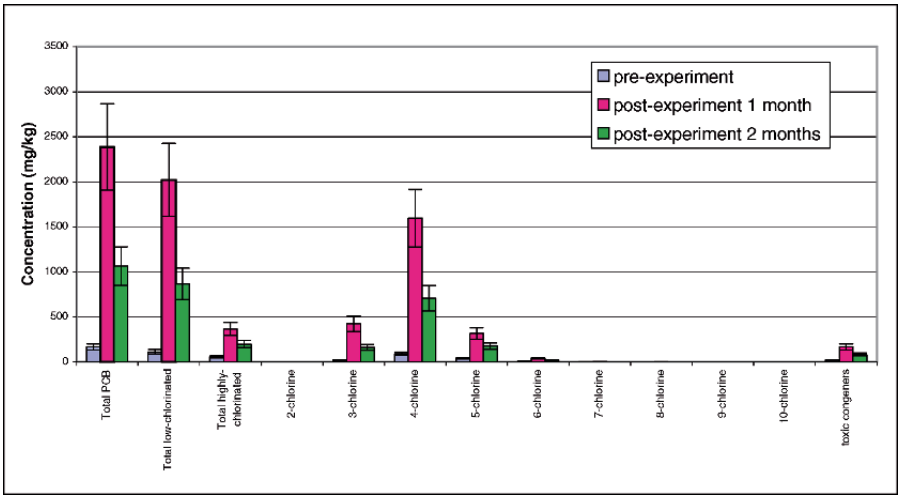

Figure 5. Change of PCB congeners content by chlorine atoms number in soil on the territory of garden cooperative society "Yurievka" during bioremediation.

Soil samples chemical analysis was conducted at chemical-and-analytical laboratory SPA "Taifun" (Obninsk), certified by the International Standard MAPEP-98-W6 and Russian Standard ROSS RU.0001.512774. Detection of PCBs in test samples was performed on gas chromatograph Hewlett Packard 5790 A with electronically capturing detector.

Analysis of contamination by PCB was conducted by:

- Total pollutant concentration,
- Congener profiles (38 congeners),
- Amount of low- and highly chlorinated biphenyls,
- Content of toxic isomers (7 congeners).

The chosen sites were remarkable for peculiar contamination by polychlorinated biphenyls. PCB contamination varied from 10 mg/kg up to 1600 mg PCB/kg. Maximal xenobiotic content was registered in soil on the territory of the plant in the area of waste disposal plants, minimal contamination was registered at the top of the site in Bezyimyanni lane.

Congener analysis of soil contamination by PCB showed that in connection with great diversity of those commercial preparations, as well as with different physical-and-chemical properties of soil, depth of ground water flowing and site profiles, contamination picture was quite diverse. Soil contamination by PCB was of "mosaic" character. It correlates well with literature data on congener composition of PCB commercial mixtures.

The sites for cleanup also had qualitative contamination composition. Speaking about specific position of low- and highly chlorinated biphenyls, there were sites where low-chlorinated biphenyls dominated, and there were also sites where highly chlorinated ones prevailed. Mostly, low-chlorinated biphenyls dominated in soil 60-83 %.

Heterogeneity of PCB contamination on the bioremediation sites appeared on level of isomers with the same amount of chlorine atoms as well. Isomers with 4 chlorine atoms dominated (39-55% of total PCB amount). Trichlorinated and pentachlorinated biphenyls occupied the second and the third place in pollutant composition.

It was determined that the sites differ from each other by content of toxic congeners. Toxic congeners came to 4-37 % in total PCB content, and 39-48 % in composition of highly chlorinated biphenyls. Using correlation analysis, reverse non-lineal strong connection between content of low-chlorinated biphenyls and high toxic congeners was determined. The more part of highly chlorinated biphenyls in pollutant composition is, the more specific weight of toxic congeners is in it. No doubt, PCB mixture toxicity is determined by many parameters including complex impact of all congeners independently of their initial toxicity; however, such approach is

a sort of reference mark when determining toxicity of soil contamination by chemical analysis data.

One should remember that polychlorinated biphenyls are compounds easily migrating with ground waters and reacting with organic soil components, followed by formation of differently-stable complexes (easily-decomposing – sorbed at the expense of hydrogen bonds on the surface of soil particles; mid-decomposing – forming complexes with soil humic substances; and hardly-decomposing – forming chemical compounds with soil substances). Besides, different PCB congeners (due to their structure and chemical properties) generate different by stability compounds with soil. That is why it is hard to assess real integral toxicity of soil, contaminated by PCB on the basis of only chemical analysis data. PCB may be strongly bounded with soil, and high toxicity will be impossible to reveal or vice verse.

1.2 Biotesting of soil on integral toxicity

At the same time with the chemical analysis on soil toxicity before, during, and after bioremediation, relative (indirect) method for assessment of integral toxicity by biotesting in daphnia was used. This technique allowed to determine total toxicity of all substances in soil, including toxicity of initial PCB and products of their microbial decomposition. The method allowed to show that no highly-toxic products of PCB decomposition form during bioremediation.

Investigations on assessment of soil integral toxicity showed that soil on the territory under bioremediation was highly toxic for biotests. Based on the general (integral) toxicity test, dependence between percent of died daphnia and PCB concentration in soil was noted practically for all sites. This dependence was of direct non-lineal character (average correlation coefficient was 0.62).

1.3 Bioremediation results

In two months soil samples were taken to register bioremediation results. The results of chemical analysis of soil samples, showed that during summer microorganisms actively decomposed PCB being in soil. Level of polychlorbiphenyl decomposition varied in dependence on PCB initial concentration in soil, as well as on soil humidity during bioremediation. At that, not only polychlorbiphenyls in soil solution, but also those sorbed on soil particles were subjected to decomposition. Thus, according to the chemical analysis data, on some sites in one bioremediation month PCB concentration considerably increased at the expense of released by

microorganisms PCB part bounded in helate complexes with humic acids of soil particles. That correlates well with literature data on bioremediation.

On the territory of "Condensator" plant, where most contaminated soils were revealed under regular watering (site near sewer outlet), PCB concentration at constant moistening lowered from 1,600 down to 160 mg/kg, and decomposition degree amounted to 90 %. According to the congener analysis data, mainly 2-, 3-, 4-, and 5-chlorinated biphenyls were subjected to degradation.

Where soil was not watered, only partial PCB decomposition was observed, and PCB concentration lowered by 20-30 %.

On the territory of the Besymyanny lane (slightly contaminated site), which was watered constantly, PCB concentration lowered from 12-14 mg/kg of soil down to practically maximum permissible values (to 0.1 mg/kg).

On the territory of garden cooperative society "Yurievka", where soil contained highly-chlorinated biphenyls, microorganisms decomposed not only 2-, 3-, and 4-, but also even 5- and (inconsiderably) 6-chlorinated biphenyls. Also, as on the plant territory, release of PCB from soil colloids by microorganisms-degraders followed by partial pollutant decomposition was revealed.

Investigations on assessment of integral toxicity of soil showed that after bioremediation soil integral toxicity on all sites lowered considerably and reached permissible level.

Results of chemical analysis and biotesting in daphnia showed the formation of little toxic compounds during the process of microbial PCB degradation.

Microbiological investigations of soil samples revealed that during bioremediation there is practically no change in concentration of aboriginal soil microflora. On the contrary, after introduction in soil, microorganism-degrader concentration, as PCB decompose, lowers considerably and reaches ecologically sound level.

The mathematical calculations showed 30-fold reduction of individual carcinogenic risk for population living in bioremediation zone. Under all ambiguous evaluations of detoxication processes revealed was lowering of PCB concentration in soil, and, consequently, reduction of toxicant getting in the growing agriculture production, what is certainly favorable for health of population in "Zaborie" region.

2. CONCLUSIONS

The results of the demonstrated tests in Serpukhov show that the biotechnology, we have developed, as well as microorganisms-destructors are very promising in respect to their practical use for bioremediation of soils "*in situ*", contaminated with polychloride biphenuls. It should be noted that bioremediation *"in situ"* has always been a quite difficult technological method, as it is hard to create equal conditions for technological process due to differences in place relief, temperature-and-humidity differences, soil nature, etc. on vast territories. Nevertheless, we managed to get satisfactory results even under such conditions.

Biotechnology has high effectiveness (PCB decomposition degree amounted to 90 %), low laboriousness (not require barking of soil, contaminated by PCB with the following camping, the processes go *"in situ"* directly) and ecological safety (during PCB degradation process destruction toxic products are not formed, soil structure and its biological properties are not changed).

Biotechnology can be applied for sanitations of contaminated soils at factories of electrical industry, for bioremediation of contaminated territories.

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SHEDDING LIGHT ON THE BIOAVAILABILITY OF ORGANIC POLLUTANTS

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Abstract: A new solvent-responsive gene locus, designated *sepABC* and a divergently transcribed *sepR*, (*sep* for solvent efflux pump), was found downstream of the two-component *todST* signal transduction phosphorelay system that regulates toluene degradation (the *tod* pathway) in *Pseudomonas putida* F1 (*Pp*F1). We have made use of this new property, a non-catabolic promoter, in combination with a *luxCDABE* gene cassette to create a second generation whole-cell bioluminescent biosensor, of name *Pp*F1G4. The response of this new biosensor to a wide range of aromatic hydrocarbons as well as a number of ubiquitous multicomponent non-aqueous phase liquids (NAPLs), including gasoline, JP-4 jet fuel, diesel, coal tar creosote and three varieties of crude oil was demonstrated. *Pp*F1G4 was also assessed as a new environmental tool for a direct measurement of the bioavailability of hydrophobic organic compounds (HOCs) partitioned into surfactant micelles. In general, the results of this study demonstrated the utility of a novel bioreporter system capable of direct measurement of bioavailability of HOCs by the judicious choice of non-ionic surfactants.

Key words: Bioavailability, bioremediation, bioreporter, chemluminescence, transcriptional regulator

1 *PSEUDOMONAS PUTIDA* F1 CONTINUES TO AMAZE

Bioavailability of organic pollutants refers to the ability of microorganisms to reach and desorb pollutant molecules from the soil

H.J. Heipieper (ed.), Bioremediation of Soils Contaminated with Aromatic Compounds, 105–124.

matrix in a bioremediation setting where biodegradative microorganisms such as those represented by numerous *Pseudomonas* spp. are used to detoxify or attenuate the culprit pollutants (Alexander, 1994). Approximately 20-30% of the bacteria listed in the Biodegradative Strain Database of the Michigan State University (http://bsd.cme.msu.edu) and the Biocatalysis/Biodegradation Database of the University of Minnesota (http://www.umbbd.ahc.umn.edu) are of the *Pseudomonas* genus that presently consists of at least 85 validated species (Palleroni and Moore, 2004).

P. putida F1 (PpF1), originally known as *P. putida* biotype B and initially isolated on ethylbenzene as the sole source of carbon and energy for growth (for a historical perspective: Gibson *et al*. 1990), is one of the best studied degrader of aromatic hydrocarbons for a number of attributes at both the molecular and biochemical level. This fluorescent soil microorganism has provided the first example of: (i) a toluene degradation (*tod*) pathway in which the multicomponent toluene dioxygenase (TodC1C2BA) enzyme system exemplifies one of the five aerobic modes of degrading toluene via a dioxygenation step to form *cis*-toluene dihydrodiol (Zylstra and Gibson, 1989); (ii) an unusual hybrid histidine kinase (TodS) as part of a two-component signal transduction system (TodST) that regulates the *tod* pathway (Lau *et al*., 1997); (iii) a membrane protein, of name TodX, that may be involved in the delivery of exogenous toluene inside the PpF1 cells, if not partaking as a membrane component of the TodST signal transduction process (Wang *et al*., 1995); and (iv), a *p*-cymene and *p*-cumate pathway that is adjacent to *tod* but independently controlled by a repressor CymR, whose specificity does not include toluene (Eaton, 1996, 1997; Patel, A. *et al*., 1997).

As in several pollutant-degrading microorganisms, PpF1 is chemotactic to toluene as well as to benzene, ethylbenzene, trichloroethylene (TCE, a non-growth substrate) and other aromatic hydrocarbons in an inducible manner (Parales *et al.,* 2000). The response is coordinately regulated with the *tod* genes although the specific chemoreceptor has not been identified. Shitashiro *et al*., (2005) has brought forward the possibility that methyl-accepting chemotaxis proteins, e.g., PctA may be responsible for the chemoattractant phenomenon.

By virtue of the catabolic pathways for toluene and *p*-cymene/*p*-cumate in PpF1, in each case, solvent- or pollutant-responsive promoter sequences have been identified (Lau *et al*., 1997, Eaton, 1996, 1997; Patel *et al*., 1997). One of the earliest use of the catabolic promoter elements that control the corresponding degradation pathways is the construction of whole-cell biosensors for detecting the presence of environmental pollutants such as aromatic solvents or hydrocarbons (King *et al*., 1990).

The *nahG-lux* gene fusion in *P. fluorescens* HK44 designed for naphthalene sensing is a prototype of such constructs which generally consist of two obligatory elements: a regulator region that would interact and thereby sense the presence of a culprit pollutant; a reporter gene, e.g., *lux* encoding luciferase that imparts a measurable response, in this case, light production. The many ways of constructing biosensors and their use in environmental applications have been the subject of several reviews (Heitzer *et al*., 1998; Daubert *et al*., 2000; Keane *et al*., 2002; van der Meer *et al*., 2004).

2.2 A new bioreporter and its versatility

A new generation of a whole-cell biosensor was exemplified by *P. putida* strain F1G4, a derivative of PpF1, that contained a chromosomally integrated *sep-lux* transcriptional fusion capable of sensing a broad spectrum of aromatic compounds (Phoenix *et al*., 2003).

Table 1. Strains TVA8, F1G4 and UT93 are chromosomal integration of the *luxCDABE* cassette.All other strains shown contain the full *lux* cassette.

Inducer(s)	Strain	Gene Fusion	Ref.
Naphthalene, salicylate	*P. fluorescens* HK44	*nahG-lux*	King et al. (1990)
Toluene, xylene	*P. putida* RB1401	*xylR-lux*	Burlage et al (1994)
Alkylbenzenes, alkanes, Chlorinated solvents, Naphthalene	*E. coli* HMS174 (pOS25)	*ibp-lux*	Selifonova and Eaton (1996)
BTEX	*P. putida* B2	*tod-lux*	Applegate et al. (1997)
BTEX	*P. putida* TVA8	*tod-lux*	Applegate et al. (1998)
Polychlorinated biphenyls	*R. eutropha* ENV307 (pUTK60)	*bph-lux*	Layton et al. (1998)
2,4-D, 2,4-dichlorophenol	*R. eutropha* JMP143-32	*tfd-lux*	Hay et al. (2000)
Aromatic compounds	*P. putida* F1G4	*sepR-lux*	Phoenix et al. (2003)
p-Cymene	*P. putida* UT93	*cymB-lux*	Ripp et al. (2003)

The novelty in this design resides in the use of a non-catabolic promoter, in lieu of the commonly used catabolic promoter sequences fused to the *lux* gene cassette (Table 1). The non-catabolic promoter, inducible by solvents, is derived from the *sepR-sepABC* gene locus that encodes a tripartite solvent-efflux pump system and a repressor (SepR) of the isocitrate lyase (IclR) regulator family that controls it (Phoenix *et al*., 2003).

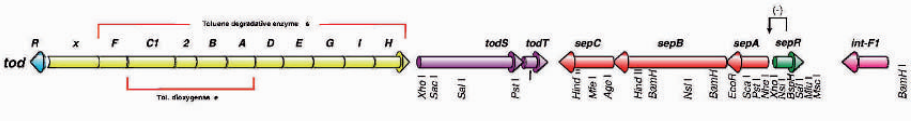


Figure 1. The gene context of the solvent efflux pump system (*sepCBA-sepR*) in *P. putida* F1 downstream of the toluene degradation (*tod*) pathway genes (*todFC1C2BADEGIH*) and its regulatory two-component *todST* system. (-) indicates the site of action of repressor SepR. Some restrictions sites are shown. Int-F1 is a putative integrase; todX, a possible facilitator of toluene transport; and todR, a non-functional (truncated) regulator of the LysR-type (Wang *et al.*, 1995).

The SepABC proteins are members of the RND-MFP-OMF (RND = resistance-nodulation-division); MFP = major facilitator protein; OMF = outer-membrane factor) family of efflux pump systems that include the first to be reported proton-dependent solvent efflux system, SrpABC, of *P. putida* S12 (Kieboom *et al.*, 1998), the three Ttg (toluene-tolerant gene) systems (TtgABC, TtgDEF and TtgGHI) of *P. putida* DOT-T1E (Rojas *et al.*, 2001), and the many clinically relevant multidrug efflux systems (e.g., MexAB-OprM) involved in antimicrobial resistance (for a review: Poole, 2001). For simplicity, we refer the SepABC proteins to as the periplasmic, inner membrane and outer membrane efflux proteins, respectively, adopting the nomenclature of Johnson and Church (1999). Modeled after the MexAB-OprM efflux systems and the AcrAB-TolC of *E. coli*, of which there are much structural information, the solvent efflux is believed to occur via: first, accumulation of the solvent molecules by SepB, the largest protein component (1046 amino acids) in the cytoplasmic inner membrane, and subsequent extrusion or transport of the solvent through SepC (480 amino acids) in the outer membrane, possibly in participation with a porin. The periplasmic SepA (382 amino acid) is expected to associate with both SepB and SepC as a "membrane bridge" protein in analogy with the MexA protein; besides, SepA may also function in the opening of the SepC "channel" as a result of its conformation flexibility, if eventually found to behave similarly as AcrA (Mikolosko *et al.*, 2006).

Figure 2 shows the unusual property of SepR as a chemical-effector responsive molecule in PpF1G4 in its capacity to respond to a wide range of compounds – from simple aromatic hydrocarbon, e.g, benzene to biphenyl, and polycyclic aromatic hydrocarbons (PAH), e.g, naphthalene

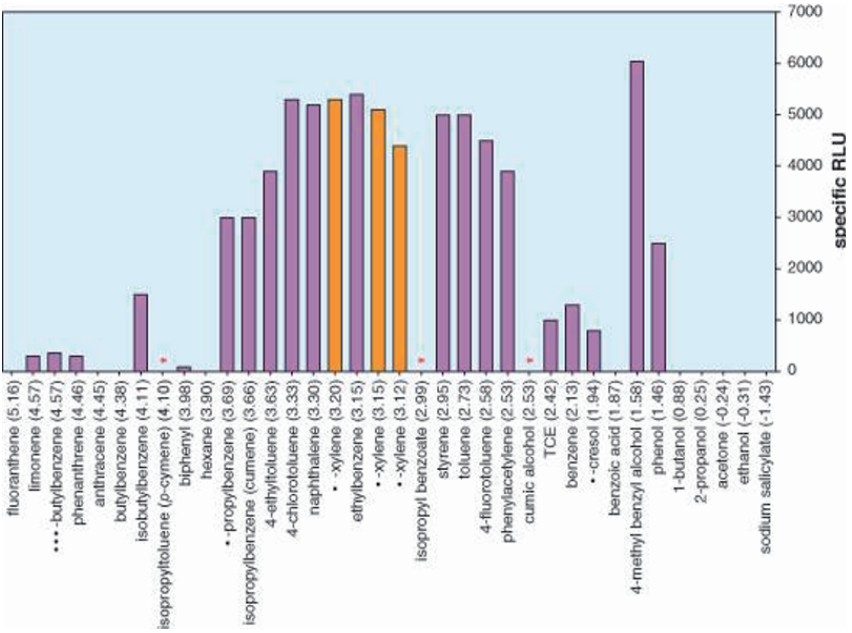


Figure 2. Response of PpF1G4 whole-cell biosensor to a variety of compounds with their log*P* values (octanol-water partition coefficients) provided in parentheses. Adopted from Phoenix *et al*., (2003). RLU is relative light unit measured in a Dynex MLX Microtiter Plate Luminometer as described in Phoenix *et al*., (2003). The specificity of PpF1G4 response includes all three isomers of xylene, and excludes *p*-cymene and its derivatives, isopropyl benzoate and cumic alcohol, shown by asterisks. TCE is trichloroethylene.

and phenanthrene. The chemical effectors also include TCE, styrene, phenol (and several other aromatic alcohols), and limonene (a monocyclic monoterpene), although at a low level. Unlike currently available biosensors based on *lux* fusion (Table 1), PpF1G4 is unique in being able to respond to all three isomers of xylene, notably *o*-xylene. The *tod-lux* fusion in *P. putida* TVA8, based on the *tod* "catabolic" promoter, was unable to respond to *o*-xylene, but otherwise *m*-xylene, *p*-xylene, benzene, ethyl among other aromatic hydrocarbons are good effector molecules (Applegate *et al*., 1998). Recently, Kim *et al*., (2005) reported *o*-xylene sensing by a XylR regulatory protein of the *P. putida* strain KCTC1643, a degrader of toluene, *m*-toluate, *p*-toluate, *m*-xylene and *p*-xylene. This appears to be exceptional, since the classical XylR system of *P. putida* mt-2 strain is not able to do so (Tizzard *et al*., 2006).

We are particularly interested in knowing whether PpF1G4 could respond to a number of ubiquitous multicomponent non-aqueous phase liquids (NAPLs) like gasoline, JP-4 jet fuel, diesel, coal tar creosote and

crude oil. As a result, all were found to be good effectors with the following preference: creosote > gasoline > jet fuel > diesel > Brent Blend > Isthmus Maya > Menemota Venezuela. The latter three are varieties of crude oil. However, there appears to have no correlation with the actual BTEX content, since on a % weight basis gasoline and diesel are estimated to be 19.3 and 0.72, respectively; and for the three crude oil varieties, the % weight ranges from 0.6 to 2.5. Suffice to say that NAPLs are complex mixtures, hence the light response may have been enhanced or suppressed by the presence of unidentified components in the mixtures. On the other hand, in response to individual components of BTEX, a dose-dependent curve was observed for the various solvents at concentration of at least up to 3 mM until toxicity sets in.

2. ADDRESSING BIOAVAILABILITY ISSUES

Needless to say, in a successful bioremediation setting, it is quinnessential to be available, not only the appropriate electron acceptors or donors, but the presence of requisite biodegradative microorganisms with the appropriate catabolic pathways and enzymes to carry out the various hydrolytic, reductive or oxidative reactions. However, it is possible that the pollutants may simply not be bioavailable to the microorganisms due to such hindering factors like: low aqueous solubility of hydrophobic organic compounds (HOCs); their entrapment in micropores; sequestration or strong binding to solid organic matter as a function of time, effect of ageing, etc.(Alexander, 2000; Bosma *et al*., 1997; Semple et al., 2003, 2004) (Fig. 3).

Hence, bioavailability as a rate-controlling factor is an important consideration. Thus far, there is limited understanding of this phenomenon. Conventional wisdom is that microbial uptake of sparingly soluble organic compounds occurs only in the aqueous phase, and that compounds such as NAPLs, including those in sorbed states, are assimilated only after they have been partitioned to the aqueous phase after physical dissolution or desorption. The majority of studies aimed at assessing bioavailability and modes of bacterial uptake have relied upon quantification of microbial degradation rates in comparison to rates of equilibrium partitioning and dissolution or desorption in corresponding abiotic systems. A direct uptake mechanism is said to be operational when the rate at which microorganisms acquiring organic compounds exceeds the mass transfer rate in the absence of bacteria, i.e, abiotic state. However, mass transfer processes in abiotic

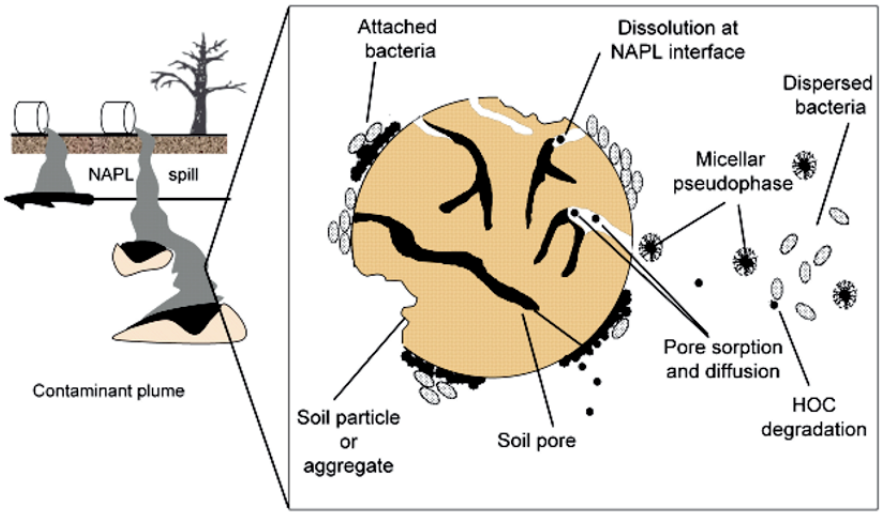


Figure 3. Many facets of a soil particle or aggregate in a contaminant plume illustrating the potential bioavailability of HOCs. HOC is hydrophobic organic compound. Adopted from Ramaswami and Luthy (1997).

systems may be different from those in biotic systems. Conventional analytical techniques to assess bioavailability is inadequate. Hence, a direct measurement technique for assessing microbial bioavailability is needed. The following will show that a whole-cell bioluminescent biosensor, PpF1G4, is a promising tool for this purpose, because the light signal emitted by the microorganism in response to target compounds partitioned into surfactant micelles is easily detected in a manner that is non-destructive to cell physiology, allowing for continuous monitoring in real-time. PpF1G4 has the added advantage that the range of HOCs that can be tested is not limited to its growth substrates since the biosensor response is based on the activation of solvent efflux pump. Besides, glucose can be used as a growth substrate, preventing the biodegradation of test compounds, e.g. toluene, during bioluminescence assays. There is no need to analyze interphase phase mass transfer or biodegradation kinetics since the aqueous concentration is constant.

2.1 Bioavailability in surfactant solution: single-solute systems

Surfactants are amphipathic molecules that form aggregates known as micelles at concentrations above a threshold, referred to as the critical micelle concentration (CMC). In aqueous solutions, surfactant micelles solubilize HOCs and increase the apparent solubility of these sparingly soluble compounds. The potential of surfactant in enhancing bioremediation is not well understood. Some studies reported inhibition of HOC biodegradation in the presence of surfactants, whereas others observed enhancement of biodegradation. We wished to evaluate whether micellar-phase compounds are bioavailable to PpF1G4 using three non-ionic surfactants, Triton X100, Brij 30 and Brij 35 (CMC values of 43, 10.6 and 39.6 mg/L, respectively), and test compounds: toluene, naphthalene and phenanthrene. Non-ionic surfactants were selected because they are generally less toxic to bacteria (Volkering *et al*., 1998).

A priori, it was essential to determine the micelle-water equilibrium partition coefficients (K_{mc}) for Triton X100, Brij 30 and Brij 35 and each of the three test compounds in order to calculate the mass of target compound to be added to each reactor in the bioluminescence assays (Fig . 4) so that the true aqueous concentration would be the same in each reactor, regardless of the concentration of surfactant. Following the methods of Guha and Jaffe (1996ab), the respective coefficients (in L/mg) were calculated for naphthalene and phenanthrene in the presence of the respective test detergents, and these values were found to be very close to those previously reported by Guha and Jaffe, 1996ab, 1998). For the experiments, 3.3 mg/L and 1.2 mg/L of naphthalene and phenanthrene, respectively, representing the true aqueous concentrations of the two substrates, were used in each reactor (Keane, 2003). In the case of toluene, as it is a liquid vs crystals of the two PAHs, a reliable value for its K_{mc} could not be obtained. As a result, the same amount of toluene was added to each reactor. The bulk concentration of toluene, at 20 mg/L, was kept constant in all reactors, noting that this is well below its aqueous solubility limit at 526 mg/L, i.e., no toluene would present as NAPL in any of the reactors.

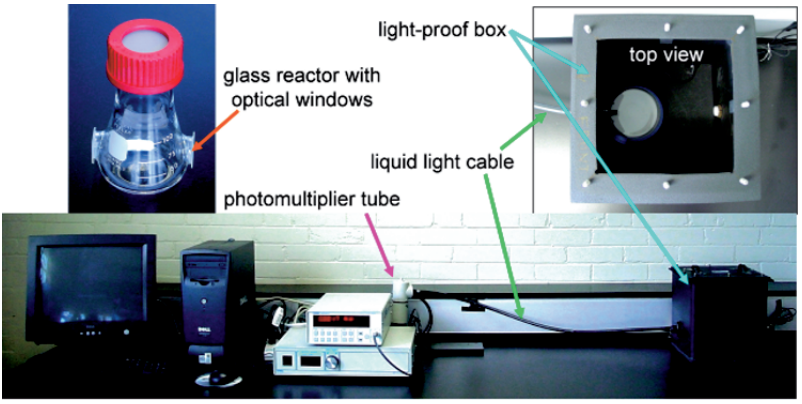
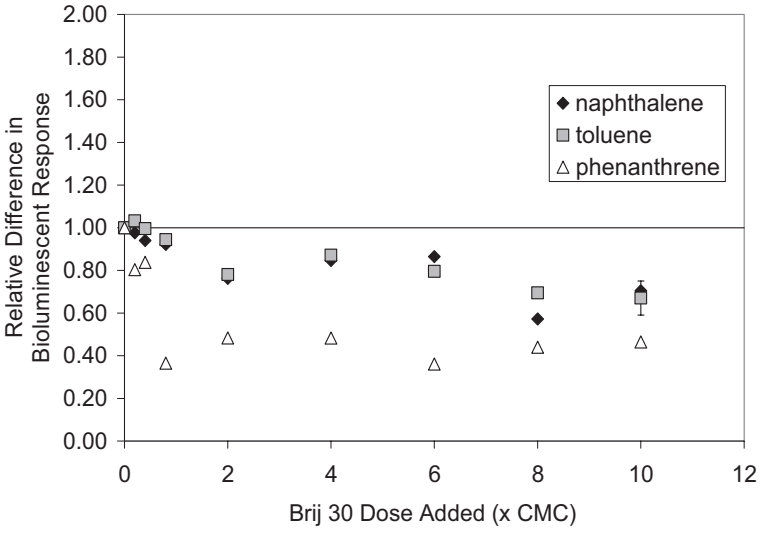


Figure 4. Components for measuring bioluminescence. Adoted from keane (2003). The set up includes an Optical Power Meter (OPM, Oriel Instruments, Stratford, CT), that consists of a photomultiplier tube, power supply, and readout. To measure the intensity of the light emitted by the biosensor cells, the reactors were placed inside a light-proof box with one of the optical windows flush up against the tip of a liquid light cable that is connected to the OPM. A data acquisition program was used to collect 20 readings at 5-second intervals for each sample, and the mean bioluminescence was calculated from these values. In order to supply adequate oxygen to the cells to ensure a steady bioluminescent output, the reactors were continuously stirred with a magnetic stir bar when placed in the light-proof box, which was equipped with a magnetic stir plate. Stirring of the cell suspensions provided sufficient aeration to avoid decay of the light signal during the measurement process. After the bioluminescence readings were taken, the A_{600} of the cell suspensions were measured to verify that the cell numbers in each reactor were comparable. The concentrations of the test compounds were measured after the bioluminescence readings to ensure that no losses had occurred. In all these experiments, an overnight culture from a frozen glycerol stock of *Pp*F1G4 was used to prepare 350 mL of subculture, which was grown to an A_{600} of 0.3 (~ 5 x 10^9 cfu/mL). The cells were then harvested and resuspended in 2.5 mL minimal M9 medium.

Figure 5A shows that Triton X-100 significantly enhanced the bioluminescent response of *Pp*F1G4 to naphthalene and phenanthrene. The horizontal line in the figure represents the bioluminescent response to the test compounds in the absence of surfactants and to the true aqueous phase concentration of 3.3 mg/L for naphthalene and 1.2 mg/L for phenanthrene. Data points above this line indicate a response greater than for the true aqueous phase concentration of the two PAHs. The increasing bioluminescence with surfactant dosage suggests that the PAHs partitioned into the micellar phase were available to the *Pp*F1G4 cells and were



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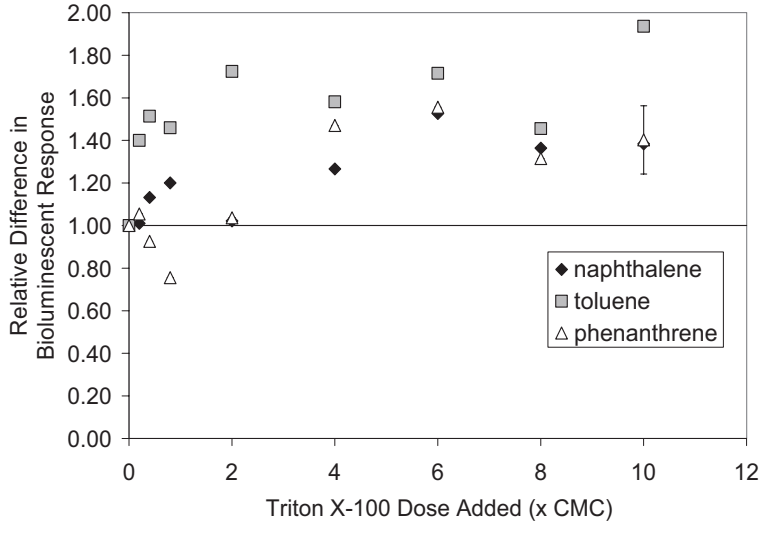

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Figure 5. Change in bioluminescent response of *Pp*F1G4 to target compounds in single-solute systems as a function of surfactant concentration. The horizontal line indicates the response of *Pp*F1G4 to the target compounds when no surfactants are present. The error bars represent the average standard error for that data series.

inducing the *sep* genes. The bioluminescence response would have not changed with surfactant dose, if micellar phase PAHs were not bioavailable since the pure aqueous phase PAH concentration was identical in all surfactant systems and in the surfactant-free systems. The bioluminescent response to toluene was somewhat different in that it increased rapidly to the maximum levels at sub-CMC surfactant doses. This is expected because the bulk phase toluene concentration of 20 mg/L was unchanged in all systems. Again, if micellar phase toluene was not bioavailable, the bioluminescent response would have decreased with increasing surfactant concentrations. The plateau in the bioluminescent response to toluene and to the two PAHs that occurred at doses above the CMC could be caused by a limitation in the mass transfer of oxygen required for the bioluminescence reaction, or because the bioluminescence genes were maximally expressed under those conditions.

The pattern of response on the effect of Brij 35 on PpF1G4 bioluminescence is virtually the same as Triton X100 (not shown). On the other hand, Brij 30 actually inhibited the bioluminescence response, as shown by the data points that lie below the horizontal line (Fig. 5B).

The basis for this inhibition is not known since in a biodegradation test when toluene is used as a substrate, the same inhibition was observed at all concentrations of Brij 30, compared to when no surfactant was used (Fig 6A). On the other hand, in the case of Triton X100 (Fig. 6B) and Brij 35 (not shown) the biodegradation rate of toluene by PpF1G4 was enhanced by all concentrations (greater than the CMC) of the added surfactant.

However, biodegradation of toluene was slightly inhibited at sub-CMC concentrations of both these surfactants, suggesting that the enhancement in bioavailability is associated with the presence of micellar-phase toluene. It should be noted that PpF1G4 strain did not produce a bioluminescent reponse when exposed to any of the surfactants. Besides, none of the surfactants at the doses used inhibited cell growth, and PpF1G4 is unable to use any of the surfactants as carbon source.

The above toluene biodegradation experiments were carried out as follows: Overnight cultures from a frozen glycerol stock of *Pp*F1G4 were grown with 100 μL toluene as the sole source of carbon in 300 mL of minimal M9. After 16 hours growth, the cells were harvested and resuspended in 1.25 mL of M9. A series of 50-mL culture tubes was filled with 30 mL of solution, consisting of M9 and the appropriate volume of stock surfactant solution (100 CMC) to attain various surfactant concentrations (0, 0.2, 0.8, 4, or 8 CMC). Each tube was inoculated with

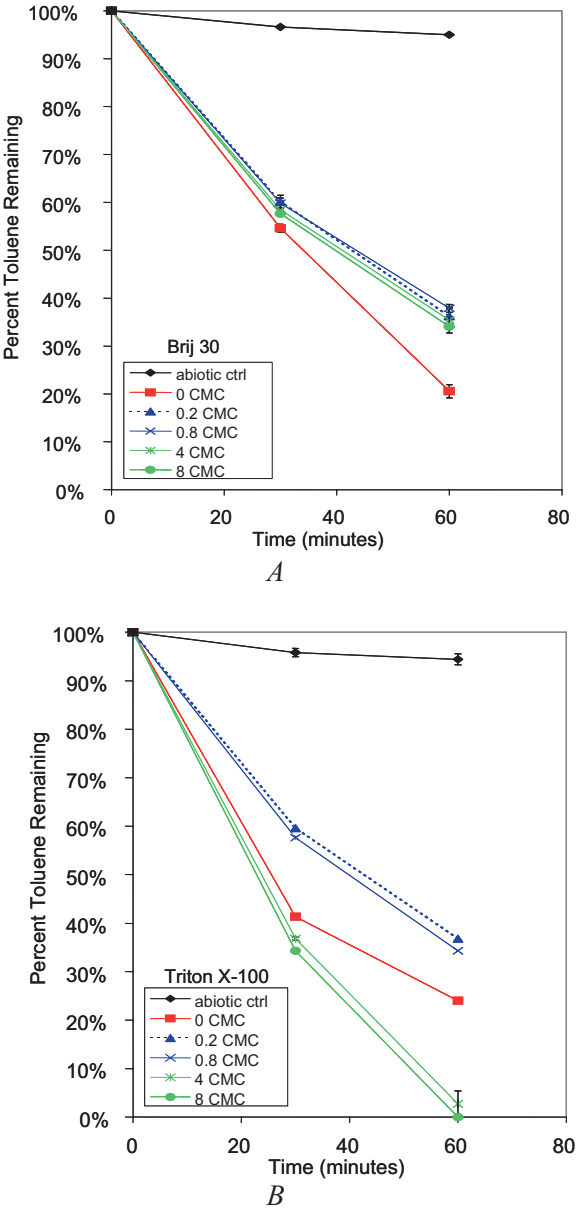


Figure 6: Effect of surfactant concentration on the biodegradation of toluene by *Pp*F1G4. The error bars represent the standard error for replicate experiments.

100 μL of cell suspension (except for the abiotic controls), resulting in a cell density with an A_{600} of 0.3, and spiked with toluene, yielding an initial aqueous concentration of 45 mg/L (approximately double the concentration of toluene used for the bioluminescence assays). The tubes were tightly sealed with open-top caps and teflon-lined septa, and then well-mixed on a vortex. Each septum was punctured with a syringe in order to extract an aliquot to determine the exact initial concentration of toluene. The holes were immediately sealed with a drop of fast-drying glue (Seal-All, Eclectic Products, Inc., Pineville, LA), to prevent losses due to volatilization. The tubes were incubated at 30^0C and 250 rpm, and samples were extracted with a syringe at 30 minutes and 60 minutes. After each sampling event, the same precautions were taken to prevent losses.

The concentrations of the test compounds in aqueous or micellar solutions were quantified with an Agilent 1100 series high-pressure liquid chromatograph (HPLC), fitted with a Vydac 201 TP52, 5 μm, 250 x 2.1 mm specialty reverse-phase column. Toluene was detected with a diode array UV detector at 262 nm for concentrations above 50 mg/L, and at 203 nm for concentrations below 50 mg/L. Naphthalene was detected with the diode array UV detector at 220 nm, and phenanthrene was detected using the fluorescence detector with excitation at 280 nm and emission at 389 nm. The mobile phase consisted of acetonitrile and water. When surfactants were present, the run time for each sample was extended to 33 minutes from 10 minutes. All aqueous samples were diluted 3:1 in HPLC grade methanol and centrifuged at 4000 rpm for 10 minutes prior to analysis. The dilution in methanol served several purposes: to precipitate the salts in the Min M9 media, to release HOCs from surfactant micelles, and to lyse any suspended bacterial cells.

Absorbance measurements to estimate cell numbers were performed on a Pharmacia Biotech Ultrospec 2000 UV/Visible spectrophotometer at 600 nm. If necessary, cell suspension samples were diluted with M9 media prior to analysis to obtain absorbance measurements in the linear range between 0.1 – 0.5.

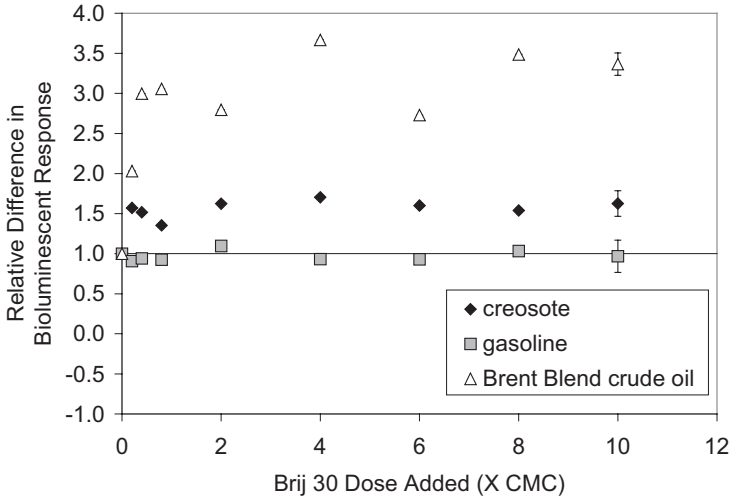
2.2 Bioavailability in surfactant solutions: complex mixtures of HOCs

In contrast to the previously described bioluminescence assays involving only a single test compound, we also evaluated the response of PpF1G4 to surfactant solutions equilibrated with complex multicomponent NAPLs. The experimental design consisted of first equilibrating the surfactants solutions with the multicomponent NAPLs (gasoline, creosote, or Brent Blend crude oil) in order to allow for partitioning of the HOCs

from the NAPL phase to the aqueous phase.The equilibrated aqueous solution containing multiple dissolved HOCs was then removed and then introduced into the reactors where the cells were added and the bioluminescence assays were carried out. In this way, the biosensor cells never came into contact with a NAPL phase in order to avoid possible toxic effect due to a direct exposure of the biosensor cells to a NAPL phase; also, this is to avoid dispersed NAPLs from causing possible light quenching effects that could interfere the bioluminescence measurements; moreover, the presence of a possible light non-aqueous phase liquid layer at the air-water interface could impede the mass transfer of oxygen from the headspace to the cells in the aqueous phase. Figure 7 shows the bioluminescent response of PpF1G4 to surfactant solutions that were pre-equilibrated with either creosote, gasoline, or Brent Blend crude oil. In contrast to the single-solute systems described previously all three surfactants, Triton X-100, Brij 30, and Brij 35 (not shown), produced very similar results in enhancing the bioavailability of HOCs partitioned from creosote and crude oil. However, this is not the case with gasoline. It is noteworthy that the bioluminescent response to the controls (growth medium pre-equilibrated with gasoline, no surfactants present) was very high. Hence, the surfactants did not produce any significant further enhancement. Moreover, the relatively soluble BTEX content in gasoline readily partitions into the aqueous phase, yielding concentrations high enough for the biosensor to detect even without the presence of surfactants.

3. DISCUSSION

The bioluminescent response of PpF1G4 to the individual test compounds toluene, naphthalene, and phenanthrene (single-solute systems) as well as complex mixtures of HOCs represented by creosote, gasoline and Brent Blend crude oil, in surfactant solutions provides a positive indication that whole-cell biosensor is a valuable tool to assess bioavailability. It is noteworthy that the response of PpF1G4 to phenanthrene in the absence of surfactant, is relatively low compared to that of the more soluble toluene and naphthalene (Fig. 4). As surfactants are known to enhance solubility of PAHs, (Tiehm, 1994; Mulder *et al*., 1998) it may not come to a surprise that they can enhance the bioavailability of single solutes as well as HOCs partitioned from multicomponent NAPLs, one mechanism being the enhancement of the mass flux of compounds from micellar solutions to the cell through membrane permeabilization. It is known that nonionic surfactants bind to cell membranes and that this interaction alters membrane



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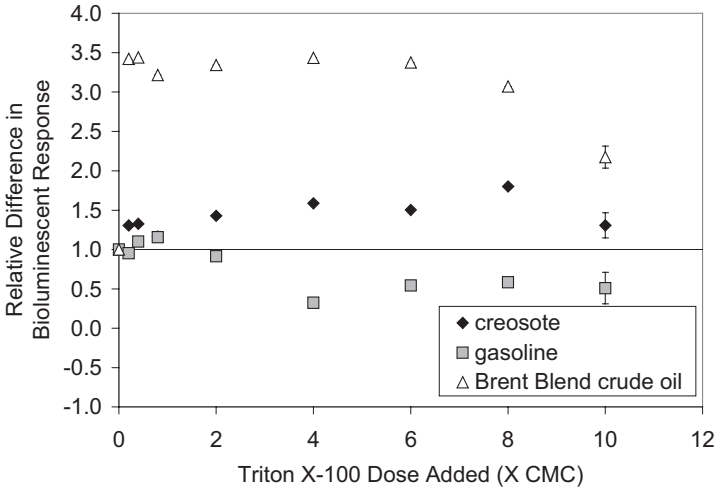

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Figure 7. Change in bioluminescent response of *Pp*F1G4 to equilibrated solutions containing HOCs partitioned from multicomponent NAPLs as a function of surfactant concentration. The horizontal line indicates the response of *Pp*F1G4 to HOCs partitioned from multicomponent NAPLs when no surfactants were present. The error bars represent the average standard error for that data series.

properties (Helenius and Simons, 1975; Florence *et al*., 1994; Glover *et al*., 1999; Lichtenberg *et al*., 1983). The type of interaction will depend on the molecular structure of the surfactant (hydrophobicity, size, etc). Upon binding of surfactant monomers to the membrane, structural changes occur that lead to increased permeability. Once membranes have been fluidized, mass flux of micelles containing target compounds into cells increase. Surfactants are also known to increase the biodegradation of HOCs by dispersing NAPLs, leading to the formation of micro-emulsions and thus increasing contact area (Breuil and Kushner, 1980; Zhang and Miller, 1995), and facilitated transport, e.g. from a sorbent phase to the aqueous phase (Aronstein and Alexander, 1992). Brij 35, for example has been shown to accelerate dissolution and biodegradation of solid dibenzofuran by a factor of 2. It also enhanced the initial desorption of dibenzofuran from Teflon in the same manner (Garcia, 2001).

Some evidence to suggest that PpF1G4 cell membranes were permeabilized in the presence of surfactants was provided by cell growth experiments, in which the biosensor was incubated with different concentrations (2 and 10 CMC) of Triton X-100, Brij 30, and Brij 35, and either toluene or glucose as sole source of carbon, for a period of 24 hours. In separate experiments it was shown that the biosensor strain was unable to grow on any of the surfactants as sole source of carbon and energy, and that none of the surfactants contributed to the absorbance readings at 600 nm. When glucose (2.2 g/L or 0.2%) was provided as a carbon source, the presence of all three surfactants significantly enhanced the growth of the cells, yielding A_{600} readings after 24 hours of growth that were 2 to 3 times higher than when no surfactants were present (data not shown). Since glucose is a hydrophilic compound that does not partition into micelles, the increase in growth observed in the presence of surfactants is attributed to membrane permeabilization. When toluene (200 mg/L) was used as a sole carbon source, the effect of the surfactants on cell growth was minimal, with the exception of the highest concentration of Triton X-100 (10 CMC), which resulted in complete inhibition of growth (data not shown). Under these conditions, the increased flux of micellar-phase toluene through the permeabilized cell membranes likely resulted in an intra-cellular concentration of toluene that was toxic to the cells. A similar response occurred in Triton X-100 systems that had been pre-equilibrated with gasoline.

In the case of Brij 30 solutions, a striking difference was observed between the single-solute and multicomponent systems. In the former case, an inhibitory effect on bioluminescence was observed but not when complex mixtures of HOCs are used. Toxicity was probably not a factor since PpF1G4 cell numbers actually increased (A_{600} measurements taken after the bioluminescence assays), to a similar extent as when Triton X-100

or Brij 35 were present. Also, the cell growth on glucose in the presence of Brij 30 was higher than the growth attained when no surfactants were present; and, there was no inhibition of light production in Brij 30 solutions containing multiple HOCs partitioned from multicomponent NAPLs. Although, it is conceivable that the Brij 30 monomers are directly involved in the suppression of light production, either by inhibiting the bioluminescence reaction (through interaction with essential reaction components such as luciferase), or by binding to the SepR repressor in such a manner as to block transcription of the *lux* genes, these are unlikely scenarios, since there is no evidence of this occurring in Brij 30 solutions with multiple HOCs. On the other hand, surfactant-cell membrane interactions and surfactant-compound interactions are much more complex than we perceive them to be.

Finally, the value of unraveling a regulatory gene circuit in the design of biosensors and other applications cannot be overemphasized. A recent issue in Current Opinion in Biotechnology (van der Meer, 2006) dedicated to bioreporter strains is a testament of a growing interest of this enabling technology.

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REDUCTION OF CHROMIUM (VI) BY BACTERIA COLLECTION STRAINS OF DIFFERENT PHYSIOLOGICAL GROUPS

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Abstract: The ability of bacteria collection strains g.g. *Pseudomonas, Rhodococcus, Acinetobacte* and *Micrococcus* to use Cr (VI) as terminal electron acceptor during breathing has been studied. It has been shown that deferent collection stains of bacteria which was not addapted to Cr (VI) prior to the batch experiments, can breath anaerobicaly and use Cr (VI) as terminal electron acceptor without imitation of anaerobic condition. These cultures reduce Cr (VI) so effectively in aerobic condition.

Key words: chromate reduction , oxidation-reducation potential.

1. INTRODUCTION

Bacteria, which can use chromium (VI) as terminal electron acceptor during oxidation of organic compounds, has attracted attention recently. It has been known from literature that different taxonomic group bacteria cultures can reduce chromium (VI) with $Cr(OH)_3$ forming. Also biotechnologies of galvanic wastewater treatment have been constructed and practised. It has been shown[1,2] that biotechnology process allows chromium (VI) concentration to decrease in wastewater to nominal limit 0.3 mg/l. This biological process is also more economical and ecologically effective than widely known physico-chemical treatment. Both pure

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cultures and their associations are used for wastewater treatment in biotechnology.

Organic compounds of different production wastes are sources of organic nutrient and electron donors for microorganisms. Thus, agricultural sewages served as carrier and carbon source for immobilized chromate-reducing bacteria[2] . A fixed-film coculture bioreactor with glass beads has been used for simultaneous chromium (VI) reduction and phenol degradation[3]. *Pseudomonas putida* is phenol-degrading bacteria and *E.coli* is Cr(VI)-reducing bacteria in this coculture. Microorganism association are used for chromate reduction in the bioreactor with vegetable raw as carbon and energy source[4]. Immobilized on granule support Bacillus sp. cells significantly reduce chromium (VI) concentration in the bioreactor[5].

It is known that chromium (VI)-reducing bacteria strains are obligate and facultative anaerobic microorganism or aerobic ones, which can breath anaerobicaly.

We had studied the culture medium oxidation-reduction potential (ORP) of inorganic compounds with varied valence, e.g. denitrification, chromate and sulfate reduction processes operation[6]. The ORP dynamic for this process shows that chromate reduction occurs in the ORP range from 400 to 200 mV. Thus, chromate reduction takes place in aerobic process diapason. The curve of ORP reduction is completely different compared to denitrification and sulfate-reduction which pass in the ORP range from 100 to -300 mV corresponding anaerobic processes.

The purpose of our work is to study the ability of collection bacteria strains of different physiological groups to use chromium (VI) as terminal electron acceptor during breathing in aerobe condition.

2. MATERIALS AND METHODS

Collection bacteria strains of different taxonomic groups *Rhodococcus erythvopolis 741, Rhodococcus ruber* 304, *Pseudomonas putida B-139, Pseudomonas alcaligenes P-16, Acinetobacter calcoaceticus Ac-1, Serratia marcescens S-1, Micrococcus luteus M-2* were used. The bacteria are got from Ukrainian Collection of Microorganism of Dept. of Microbiological Wastewater Treatment of Institute of Colloid and Water Chemistry of National Academy of Science (Ukraine).

The biomass was grown on nutrient agar medium (Serva Sd1)) at 28^0C. The cultivation was carried out in a 100-ml Erlenmeyer flack at the same temperature. The system was not isolated from oxygen diffusion to the culture medium. Chromate reduction was carry out in aerobe condition. The optical density of inoculum was determined calorimetrically at 540 nm.

The cell density of inoculum was 0,06-0.1 or biomass density 0.25-0.35 g/l by dry wet.

The model medium for chromium (VI) reduction includes potassium chromate as a potential electron acceptor, fixed mineral constituents, microelements, peptone as electron donor and carbon source. Fixed constituents were dissolved in distilled water to obtain solution of the following composition ($g \cdot L^{-1}$): 3 - KH_2PO_4, 6 - Na_2HPO_4, 1 - NH_4Cl, 0.5 - NaCl, 0.1 - $MgSO_4 \cdot 7H_2O$, 0.1 - $CaCl_2$. Microelement solution in distilled water composes 1 mg $FeSO_4 \cdot 7H_2O$, 5 mg $MnSO_4 \cdot 2H_2O$, 1 mg $CoSO_4$, 1 mg $ZnSO_4$, 0.1 mg $CuSO_4 \cdot 6H_2O$, 0.1 mg H_3BO_3, 25 mg Na_2MoO_4, 0.1 mg $NiCl_2 \cdot 6H_2O$ per litre. 1 ml of microelement solution was diluted by solution of fixed constituent to 1 L.

The peptone (Serva) content was 2g/l. Initial potassium bichromate content was 16 Cr (VI) mg per litre.

The control was the model medium without microorganisms.

The chromate concentration was determined by calorymetry with difenilcarbosid[7].

3. RESULTS AND DISCUSSION

Biological remediation of chromate-containing wastewater is based on bacteria consumption of Cr (VI) as a terminal electron acceptor during the breathing. The capability of Cr (VI) reduction is not uncommon among Cr (VI)-resistant microorganisms. The ability of denitrifying and fermenting bacteria to reduce chromium (VI) in anaerobic and aerobic condition is shown by many researchers [3-5,8]. Studding of oxidation-reducation potential (ORP) of nutrient medium during microbial chromate reduction had show that Cr(VI) reduction passes effectively at ORP level +400 - +200 mV. This is potential diapason of aerobe processes. Standard electrode potential (E^0) of reaction: $Cr_2O_4^{2-} + 8H^+ + 3e = Cr^{3+} = 4H_2O$ is 1477 mV. The potential is higher then potential of oxygen reduction process: $O_2 + 4H^+ + 4e = 2H_2O$ (E^0=1228 mV). The reactions take place in bacterial cells during respiration. Therefore, the presents of oxygen is necessary to supply high OPR level in cultural medium Thus, reduction of Cr(VI) to Cr (III) have to take place in aerobe condition.

We have studied the ability of different taxonomic groups of collection bacteria strains use chromium (VI) as terminal electron acceptor during the cultivation of free microorganisms in mineral medium in aerobic conditions.

Figure 1 shows the dynamic of Cr (VI) concentration during cultivation in aerobic condition in bacteria cultures such as *Rhodococcus erythvopolis 741, Rhodococcus ruber* 304 and *Micrococcus luteus M-2.* Initial Cr (VI) concentration and cells density of inoculum were 14 mg/l and 0,06-0.1 respectively. As fig.1 shows chromate reduction could take place in all bacteria strains. It is shown that *Rhodococcus erythvopolis 741*and *Micrococcus luteus M-2* completely reduce Cr (VI) during 6 and 11 days of cultivation respectively. Chromate reduction passes the most effectively in these cultures. Cr (VI) concentration has been reduce level 4 mg/l for 6 days in culture *Rhodococcus ruber* 304. Then chromate reduction rate increased. The last 4 mg/L Cr(VI) *Rhodococcus rubber* reduced during next 8 days of cultivation.

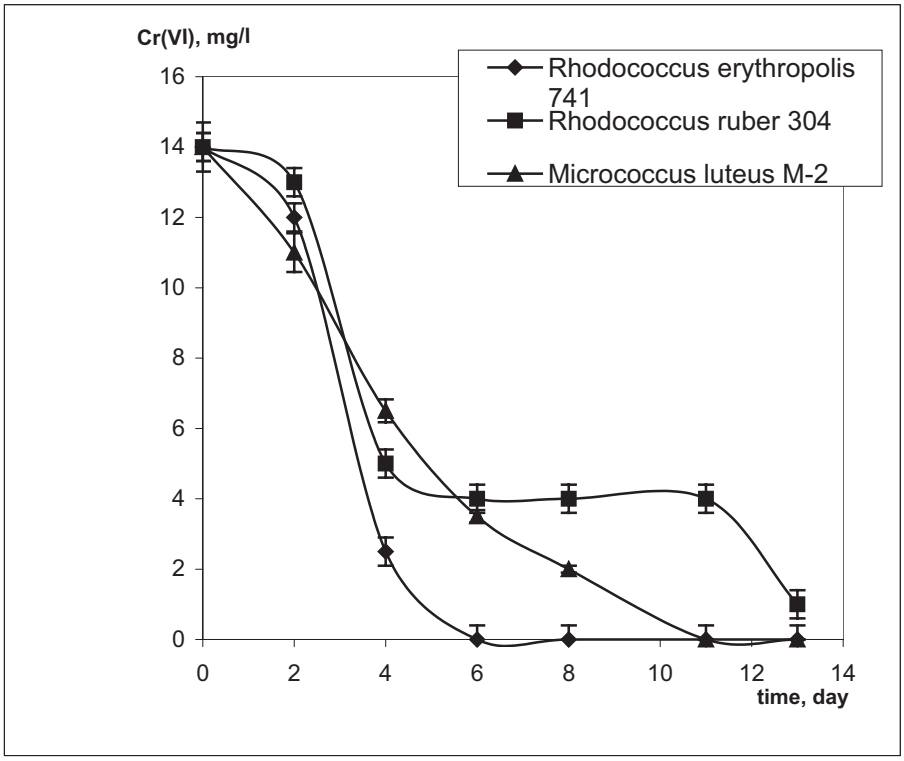

Figure 1. Dynamic of chromium (VI) concentration in cultures of bacteria.

Figure 2 shows dynamic of Cr (VI) concentration during cultivation in aerobic condition in bacteria cultures such as *Pseudomonas putida B-139, Pseudomonas alcaligenes P-16, Acinetobacter calcoaceticus Ac-1, Serratia marcescens S-1.* Initial Cr (VI) concentration and cells density of inoculum were the same. Chromate reduction also take place in all bacteria strains. Fig.2 shows that *Pseudomonas alcaligenes P-16, Serratia marcescens S-1* completely reduce Cr (VI) during 13 days of cultivation. This process comes to pass more slowly than previous process. Chromium (VI) concentration decreased twice after 4 days of bacteria cultivation. In initial stage chrome (VI) reduces sharply, but them chromate reduction rate increases.

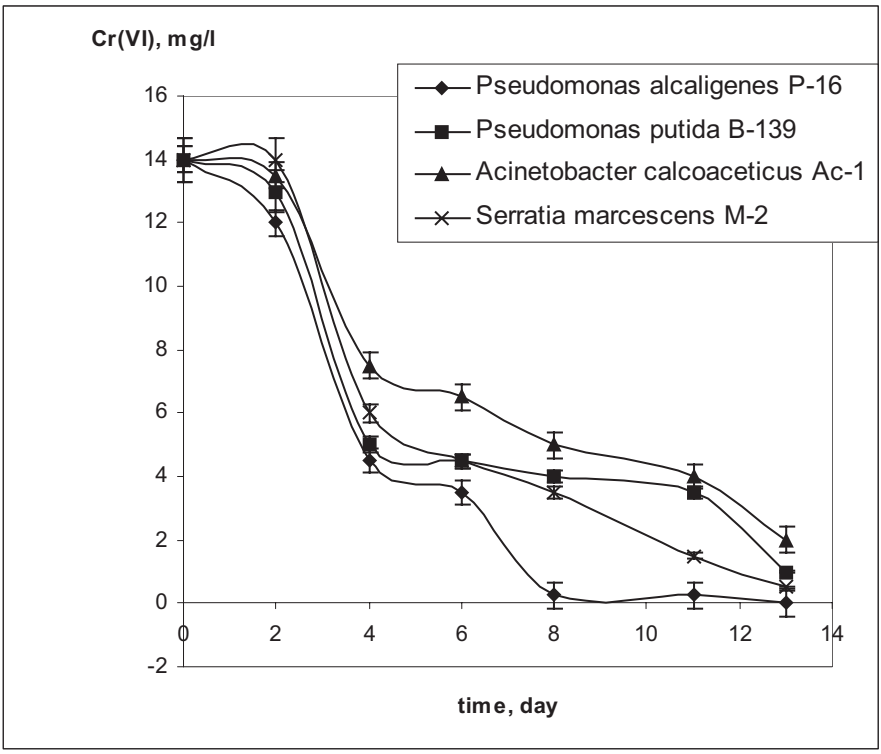

Figure 2. Dynamic of chromium (VI) concentration in culture of bacteria.

Thus, reduction rate of chromium (VI) are approximately equal in all bacteria cultures in first four days of cultivation. Cr (VI) concentration has been sharply reduce also to level 4 and 4,5 mg/l in bacteria cultures *Pseudomonas alcaligenes P-16, Acinetobacter calcoaceticus Ac-1*

respectively. Then the process rate reduces. The completely chromate reduction has been shown only after 14 day of cultivation.

4. CONCLUSIONS

The research shows that deferent collection stains of bacteria which were not adapted to Cr (VI) prior to the batch experiments, could breathe anaerobicaly and use Cr (VI) as terminal electron acceptor without anaerobic condition. These cultures reduce Cr (VI) more effectively in aerobic condition. Thus, reduction of Cr(VI) to Cr (III) have to take place in oxygen present or aerobe condition. It is the reason why standard electrode potential of chromate reduction higher then potential of oxygen reduction.

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