FOCUS ON BIOTECHNOLOGY

# Phytoremediation and Rhizoremediation

## Theoretical Background

*Edited by*
Martina Mackova, David N. Dowling and Tomas Macek

Series Editors: Marcel Hofman and Jozef Anné

PHYTOREMEDIATION RHIZOREMEDIATION

# FOCUS ON BIOTECHNOLOGY

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*Series Editors*

MARCEL HOFMAN
*Centre for Veterinary and Agrochemical Research, Tervuren, Belgium*

JOZEF ANNÉ
*Rega Institute, Catholic University of Leuven, Belgium*

*Volume Editors*

MARTINA MACKOVA
*Institute of Chemical Technology,*
*Prague, Czech Republic*

DAVID DOWLING
*Institute of Technology,*
*Carlow, Ireland*

THOMAS MACEK
*Institute of Organic Chemistry and Biochemistry,*
*Academy of Sciences of the Czech Republic,*
*Prague, Czech Republic*

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Edited by

MARTINA MACKOVA
*Institute of Chemical Technology,*
*Prague, Czech Republic*

DAVID DOWLING
*Institute of Technology,*
*Carlow, Ireland*

and

THOMAS MACEK
*Institute of Organic Chemistry and Biochemistry,*
*Academy of Sciences of the Czech Republic,*
*Prague, Czech Republic*

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**TABLE OF CONTENTS**

# INTRODUCTION

**JOHN FLETCHER**
*University of Oklahoma, Department of Botany, Norman, USA*

To appreciate the history of phytoremediation and its importance, it is helpful to start with a brief review of common remediation strategies used around the world to treat soil contaminated with toxic metals and/or organic chemicals. Three widely used strategies are: 1) immobilization or retention of toxicants within a confined area (i.e. the soil at the site of their release or in contaminated soil placed in a landfill, 2) removal of contaminants from the soil, 3) destruction of organic pollutants by chemical, physical, or biological means. These strategies either individually or in combination with each other have been routinely implemented by the remediation industry to successfully treat contaminated soil. Unfortunately, implementations have often required extensive earth moving, expensive equipment, and costly construction; all features that have aroused public resistance on occasion and have sometimes been tagged, rightly or wrongly, as more threatening to the environment than the contaminants themselves. In any event, public concern for the implementation features listed above have been instrumental in keeping pressure on the remediation industry to develop more cost effective and friendly methods, including bioremediation.

Bioremediation started over 50 years ago with research examining the fate of pesticides in agricultural soils. In view of the wide range of catabolic reactions mediated by bacterial enzymes, it is not surprising that from the beginning bioremediation research focused on bacteria. The capacity for bacteria to degrade xenobiotics was so impressive that other living organisms were virtually ignored for 30 years. As a result bioremediation became thought of as degradation of organic contaminants by bacteria even though the bio prefix suggested involvement of all life forms. Early investigators in plant remediation work were confronted with an attitude held by some persons that if remediation of a contaminant could not be achieved by bacteria with their diversified array of catabolic enzymes it sure couldn't be achieved with plants. This attitude is perhaps why investigators striving to call attention to the unique remediation features of plants felt obliged to establish a separate remediation field, phytoremediation, and include several subdivisions (i.e. phytoextraction, rhizoremediation, etc.).

Unique remediation features possessed by plants are easily illustrated by returning to the 3 common remediation strategies listed earlier to treat contaminated soil: 1) immobilization, 2) removal, and 3) destruction. Partial immobilization of water soluble contaminants is brought about by plant transpiration (soil water taken up, transported, and evaporated from leaf surfaces) since the process removes soil water that would



*M. Mackova et al. (eds.), Phytoremediation Rhizoremediation,* 1–3.

otherwise cause contaminant leaching and movement. Removal of toxic metals from contaminated soil occurs when inorganic ions are taken up by plant roots and translocated through the stem to aboveground plant parts. Regarding contaminant destruction, plants, because of their autotrophic nature, were rarely examined for catabolic properties until phytoremediation emerged and studies conducted then with nonphotosynthetic tissue culture cells and axenic roots clearly showed that plant enzymes degrade some organic pollutants. The use of plants to foster the degradation or organic soil contaminants has been further advanced by studies showing that soil microflora under the chemical influence of plant roots (rhizosphere zone) can be important in xenobiotic metabolism. The catabolic activity within the rhizosphere has been attributed to both bacteria and fungi whose presence and enzymatic expression are believed to be modulated by organic chemicals released from both living and dead roots. Since both root physiology and biosynthetic pathways vary considerably among plant species it is anticipated that rhizoremediation properties will also vary among plants. The most useful species for rhizoremediation may be previously unexplored species with no commercial importance prior to their use in rhizoremediation. Both the direct and indirect degradation of soil contaminants can potentially occur at the lowest depth of root penetration, a special feature of plant remediation. Thus, through the efforts of a relatively small group of scientists working around the world over the last 20 years, phytoremediation has become a well established, multifaceted technology capitalizing on three plant properties: transpiration, ion uptake, and metabolism with the later having both direct and indirect influences.

As phytoremediation technology has evolved it has become increasingly apparent that no single plant species excels in all three plant remediation properties, nor does any single species show maximum uptake of all toxic metals or foster degradation of all organic contaminants. Therefore, successful treatment of soils with mixed waste requires a combination of plant species with appropriate remediation properties, and also the inclusion of plant species hosting rhizosphere communities (bacteria and fungi) active against specific contaminants that are present. Thus, a major contribution that has emerged from the field of phytoremediation is the biosystems approach to soil remediation where the joint actions of several different organisms functioning in unison or in sequence are used to treat contaminated soil. The concept of a plant driven remediation system may be foreign in some remediation circles but it is certainly consistent with the fundamental principals of terrestrial ecology where the central role of plants as the primary producers and greatest users of water is well established.

Accepting the central role played by plants in biosystem remediation, raises many unanswered questions on how to assemble and manage the most effective biosystem. What are the best plant species to use since the physiology, biochemistry, and rhizosphere of very few of the thousands of native species have been studied? How does the microflora of a dead root and its degradative properties compare with that of a living root? Are genetically altered organisms necessary to degrade some soil contaminants? Do some plant roots release surfactants? Can plant species that move deep groundwater

to surface root zones be capitalized on in rhizoremediation? Should plants be introduced as single annual crops or as perennial communities? Should plant succession be encouraged? As such questions are addressed and new remediation technology emerges it is very likely that phytoremediation employing ecologically and physiologically sound biosystems will be accepted as a necessary and first step in successful ecological restoration of contaminated habitats.

# THE CHEMICAL ECOLOGY OF POLLUTANT BIODEGRADATION

***Bioremediation and phytoremediation from mechanistic and ecological perspectives***

**ANDREW C. SINGER**
*Centre for Ecology & Hydrology–Oxford, Mansfield Rd, Oxford OX1 3SR, United Kingdom, E-mail: acsi@ceh.ac.uk*

## 1. Introduction

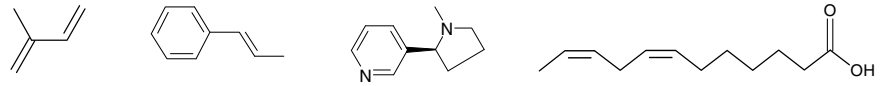
As the yachtswoman Dame Ellen MacArthur returned to the south coast of Britain in early 2005 after a record 71-day solo circumnavigation of the globe on a trimaran, she noted pointedly, "It's funny when you smell the land and you have not smelled it for two months". MacArthur's comment reflects the multitude of odours originating from *terra firma* and highlights an important and underappreciated feature of our world – a dizzying abundance and diversity of chemicals surround us and in some subtle, as well as some very direct ways, dictate the actions and reactions of all life.

Among the numerous sources of chemicals in our environment, molecules of plant origin are arguably the most abundant and best characterised. This chapter aims to highlight the ecological functions of plant-derived chemicals and discuss their roles in both multi-trophic interactions and (pollutant-degrading) enzyme evolution. Evidence to support these positions has largely been generated in the past decade and will be reviewed in the later part of the chapter.

Rhizodeposition, the release of carbon compounds from living plant roots into the surrounding soil, is dominated by low molecular mass solutes such as sugars, amino acids and organic acids. There are numerous studies which aim to understand the regulation and ecological significance of rhizodeposition, for which the reader is directed to three excellent reviews [1-3]. Although rhizodeposition plays a central role in establishing and sustaining a soil system, this chapter will focus on a class of compounds, secondary plant metabolites (SPMe), that are nearly four-orders of magnitude more diverse than the typical rhizodeposits. Over 100 000 low-molecular-mass SPMe have been described with an estimated 400 000 yet to be discovered [4]. Many of these SPMe contain one of the following chemical structural backbones: isoprene, phenylpropene, alkaloid or fatty acid/polyketide (Figure 1) [5].



*M. Mackova et al. (eds.), Phytoremediation Rhizoremediation,* 5–21.




Isoprene Phenylpropene Alkaloid Fatty acid/polyketide

*Figure 1. Typical skeletal backbones for the majority of secondary plant metabolites.*

Although referred to as "secondary metabolites", implying a function of only secondary importance to the plant, SPMe fulfill a range of vital functions: (1) antimicrobial activity; (2) insect and microbial attraction; (3) insect and microbial deterrent; (4) plant-plant signal; (5) stress response; and (6) germination and growth inhibition [6].

Volatile low-molecular mass SPMe, consisting of a range of functional groups (hydrocarbons, alcohols, aldehydes, ketones, ethers and esters), are integral in how plants interact with their environment. Volatile emissions from flowers and fruits, for example, provide clues to animals, pollinators and seed disseminators, while those from vegetative tissues contribute to plant defence systems by repelling microorganisms and animals or attracting herbivore predators, thereby protecting the plant through tritrophic interactions [7].

## 1.1. THE NATURE OF THE PROBLEM

The Twenty-Fourth Report by the Royal Commission on Environmental Pollution stated that there are between 30 000 and 100 000 chemicals on the market [8]. Every year, approaching 2000 novel xenobiotic chemicals are added to this list, the vast majority of which have not been tested for even the most basic indications of environmental hazard. It is now recognised that this policy has been responsible for a number of environmental catastrophes such as: (1) reproduction failures in songbirds resulting from the organochlorine pesticide 4,4'-(2,2,2-trichloroethane$^{-1}$,1-diyl)bis(chlorobenzene) (DDT) which was highlighted by Rachel Carson's landmark book, *Silent Spring* in 1962 [9]; (2) bioaccumulation of the organochlorine polychlorinated biphenyl (PCB) and reproduction failures at all levels of the food web from fish to eagles and humans [10]; and (3) depletion of the ozone layer induced by the release of chlorofluorocarbons (CFC) [11].

DDT, the chemical for which Carson is most noted for highlighting, was banned in the United States at the end of 1972, eight years after her untimely death from cancer. Although the DDT ban spread to many temperate countries, few tropical countries acceded to the ban, largely due to the pesticide's efficacy to control the spread of malaria and other insect-borne diseases. DDT has been shown to dissipate much more rapidly in tropical than temperate soils [12]. The mechanism for the latter is partly attributed to increased temperature-mediated volatility, but more importantly increased microbial biodegradation. The mechanisms underpinning chemical persistence in the environment are complex but are thought to be heavily influenced by the rarity of the chemical's structure and substituents.

My contention is that the chemical ecology of a site should also be considered as an important variable in determining a chemical's persistence. In this chapter, the term chemical ecology is used as defined by the International Society of Chemical Ecology, "the chemical mechanisms which help control intra- and interspecific interactions among living beings". Owing to key differences in the local chemical ecology, a recalcitrant molecule in one locale might be readily biodegraded in another. In part, the local flora provides the evolutionary mechanism for the development and modification of SPMe-degrading enzymes in the metagenome, which, it is argued, is fortuitously responsible for the presence of pollutant-degrading enzymes, *a priori* the chemicals synthesis by chemists.

## 1.2. TRITROPHIC TRINITY

Although it has been widely proposed that pollutant-degrading enzymes evolved from isozymes in response to industrial production and environmental release of xenobiotics, the *a priori* existence of readily mutable pollutant-degrading isozymes remains largely absent from the literature [13-16]. This chapter will contribute to the dialogue on the source and developmental mechanism of these pollutant-degrading enzymes.

The evolution of plants and their natural enemies was, arguably, responsible for generating much of the Earth's biological diversity [17]. A corollary was proposed [18], stating that the synergistic and antagonistic relationships between plants, micro-organisms, and insects (P-M-I) are responsible for the diversity of SPMe. A second corollary then suggested that the P-M-I tritrophic interactions serve as one of the main driving forces of pollutant-degrading enzyme evolution [19].

### *1.2.1 Theories on the evolution of pollutant-degrading isozymes*

In "The Fractal Geometry of Nature", Mandelbrot highlights the fractal structure of many natural systems [20]. In this chapter, catabolic enzymatic systems are proposed to conform to a fractal architecture. Elucidation of the organisation and evolution of catabolic systems will aid in the investigation into the origins of pollutant-degrading enzymes.

The classic example of a fractal structure can be found in the form of a tree (Figure 2). The long tree trunk provides the foundation from which a repeating series of shorter branches are serially connected. At the metaphorical "leaves" of the tree lie molecules which necessitate an individualised enzymatic step before moving into more central metabolic pathways located at the base of the tree (e.g. citrate cycle, glycolysis). Their location in the periphery of the tree can be attributed to their relatively unusual chemical structure, substituent, or both. Whereas chemicals metabolised in well connected, more centralised locales of the tree (nearer the "trunk"), consist of relatively more common chemical structures and substituents [21]. Elaborating upon Firn and Jones (2000), it is proposed that substrate specificity of biodegradative enzymes is proportional to the distance of the enzymatic reaction from the "leaves", i.e. enzymes with a low substrate specificity are located in the "leaves" while enzymes with a high substrate specificity are typical of central metabolic enzymatic reactions (i.e. "trunk"). This metabolic architecture can be helpful in developing a mechanistic understanding of the evolution of

pollutant-degrading enzymes. For example, in the event a microorganism encounters a novel molecule, it might perish if the molecule is toxic, as are many SPMe, or a mutant enzyme might emerge from the population, enabling the molecule's detoxification or

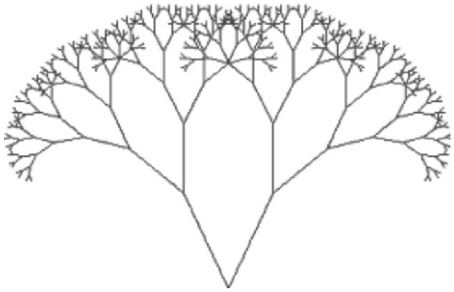


*Figure 2. Fractal structure of catabolic enzymatic system within microorganisms. Novel xenobiotic and natural chemical structures are catabolised in the outer branches, funnelling metabolites to more central, substrate specific enzymatic steps.*

metabolism. The mutant enzyme would develop in the "leaves" as it is the location of enzymes which are responsible for interacting with the environment (e.g. detoxification, communication, assimilation). Moreover, as the novel chemical is metabolised it may continue down the "tree" into central metabolic pathways, or it may persist without any further metabolism [14, 22]. Persistent metabolites would be further evidence of enzymatic activity in the "leaves", as catabolism of molecules with relatively novel structures or substituents: (1) might produce equally novel metabolites requiring additional modification to indigenous enzymes for further metabolism; (2) require low substrate specific enzymes for primary and secondary catabolism, which is a characteristic of enzymes residing in the "leaves".

Networked databases, such as MetaRouter and the University of Minnesota Biocatalysis/Biodegradation Database, provide a useful framework to visualise pollutant catabolic pathways (http://pdg.cnb.uam.es/MetaRouter/index.html) [23], UM-BBD; http://umbbd.ahc.umn.edu/) [24] and discern their fractal, interwoven structure. Integrating this information into a "suprametabolism" network (i.e. incorporating all pathways), will enable predictions to be made on the fate of both current and future environmental pollutants [22, 24].

## 2. Chemical ecology of pollutant degradation

In the early 1990's, researchers began to theorise about the "natural substrate" of pollutant-degrading enzymes. Among the first pollutants to be scrutinised were PCBs. Higson [25] and Furukawa [26] postulated that lignin may be the natural substrate for the PCB-degrading enzyme. *Rhodococcus erythropolis* TA421, a microorganism isolated from a wood-feeding termite ecosystem, was shown to degrade the recalcitrant

pollutant PCB [27, 28]. The association of TA421 with a wood-feeding termite provided the researchers with an opportunity to link lignin-degrading ability with the capacity to catabolise PCBs. Maeda *et al.,* confirmed the presence of three PCB-degrading genes (*bph*C) in TA421, each with a different, yet narrow, substrate specificity [27], which might correlate with the three monomers of lignin (Figure 3). This, however, has yet to be tested.

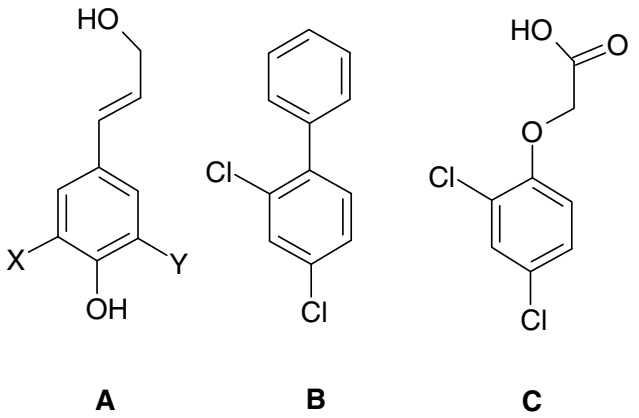



*Figure 3. Structure similarity between: (A) Lignin monomer structure, where (1) X = Y = H (p-coumaryl alcohol), (2) X = OMe; Y = H (coniferyl alcohol), (3) X = Y = OMe (sinapyl alcohol) [29]; (B) 2,4-dichlorobiphenyl, PCB congener; and (C) 2-4-dichlorophenoxyacetate.*

Among the first studies that specifically investigated the link between plant-derived chemicals and pollutant remediation was that by Donnelly *et al*., The authors demonstrated that a range of flavonoids could support the growth of PCB-degrading microorganisms [30]. The best growth substrate and concentration were determined for each of three PCB-degrading microorganisms: *Ralstonia eutrophus* strain H850; *Burkholderia cepacia* LB400; and *Corynebacterium* sp. MB1. Each bacterium was subjected to a congener depletion assay [31], which was designed to show in a 24-hour period the extent of congener degradation after growth on a particular flavanoid. Naringin proved the best growth substrate for H850 and supported its greatest metabolic activity on PCBs. Myricetin induced the greatest PCB degradation by LB400, which catabolised 16 of the 19 congeners tested. Strain MB1 degraded thirteen PCB congeners in the presence of coumarin in excess of the biphenyl controls.

The researchers suggested that fine plant roots may ultimately serve as a "naturally occurring injection system," capable of dispensing phenolic plant-derived compounds into the rhizosphere. They advocated the use of these exudates as a means to support the growth of PCB-degrading microorganisms. A major step in the development of the chemical ecology of pollution was provided by Focht (1995) who proposed that plant terpenes, rather than biphenyl [32], might be the natural substrates for PCB catabolising enzymes. Subsequent studies by Hernandez *et al.,* showed that soils enriched with

orange peel, ivy leaves, pine needles or eucalyptus leaves resulted in $10^5$ times more biphenyl (unchlorinated PCB) utilizers ($10^8$ $g^{-1}$) than their unsupplemented control ($10^3$ $g^{-1}$), which suggested that terpenes found in these plants might be natural substrates for biphenyl-utilizing bacteria. Notably, complete disappearance of Aroclor 1242 was observed in soils amended with orange peel, ivy leaves, pine needles and eucalyptus leaves [33]. The authors examined the efficacy of terpene-degrading isolates to biotransform Aroclor 1242 in broth. Three bacteria isolated from the experimental soil with the capacity to utilize cymene as a sole carbon source exhibited enhanced (20-80%) transformation of Aroclor 1242, in comparison with glucose-grown cultures. Four of five limonene-utilising isolates exhibited elevated (43-83%) Aroclor 1242 transformation compared with the controls. In conclusion, the authors speculated that biphenyl may provide soil microorganisms with a relatively labile source of carbon, which is rapidly utilized by fast-growing soil microorganisms (copiotrophs). They argued that slow-growing microorganisms (oligotrophs), which rely on low concentrations and slowly delivered secondary plant metabolites, might be more effective in degrading PCBs [33]. Evidence for the interaction between pollutant degradation and the availability of labile carbon sources is highlighted in several papers referenced in this chapter.

In a similar vein, Dzanto and Woolston, demonstrated removal of 10, 21 and 24% PCB (Aroclor 1248) from soils supplemented with pine needles, biphenyl and orange peel, respectively, compared with control soil, although the differences were not deemed mathematically significant ($P > 0.05$) [34]. The authors suggested that further promotion might be achieved by biostimulating the rhizosphere with specific inducing substrates for the target pollutant. This approach has since been demonstrated successfully in two classic studies by Narasimhan *et al.* [35] and Kupier *et al.* [36, 37], as discussed elsewhere [19].

The first methodical screening and isolation of active inducing compounds in plants was achieved by Gilbert and Crowley. The authors examined a number of plant extracts (spearmint, pennyroyal, basil, barley, green bean, dill, avocado litter and garden compost) to determine if any stimulated the degradation of 4-4'-dichlorobiphenyl by a known PCB-degrading bacterium, *Arthrobacter* sp. strain B1B [38]. Spearmint extract resulted in approximately 33% of the metabolites produced by the known PCB-inducing compound, biphenyl. Subsequent analysis of this extract identified carvone as the principal component responsible for catabolism induction. Ten terpenoids of similar structure to carvone were assayed for their ability to induce PCB (50 mg $l^{-1}$) degradation: *p*-cymene, isoprene, (S)-(+)-carvone, (R)-(–)-carvone, (S)-(–)-limonene, (R)-(+)-limonene, carvacrol, cumene, trans-cinnamic acid and thymol. These plant-derived compounds are commonly found in dill and caraway seed, spearmint, pine needles, citrus, juniper, oregano, thyme and numerous other aromatic plants. With the exception of cumene, trans-cinnamic acid and thymol, all terpenes enhanced 4-4'-dichlorobiphenyl metabolism, while *p*-cymene and isoprene accelerated catabolism in comparison with biphenyl ($P < 0.05$). The workers highlighted that not only was PCB degradation induced by nonaromatic compounds, but also among the most effective was isoprene, which lacks a ring structure. It was proposed that the relatively high antimicrobial activities of terpenes might induce a P450-like detoxification and fortuitous degradation of the PCBs. Cytochrome P450 enzymes are a large family of

enzymes that have been shown to oxidize terpenes, such as camphor (P450cam), as well as pollutants, such as polycyclic aromatic hydrocarbons (PAHs; e.g. naphthalene and pyrene [39]), chlorinated phenols [40], and biphenyls [41].

*p*-Cymene is among the more frequently investigated SPMe in pollutant-degradation studies, and is arguably among the more effective. It is a natural aromatic hydrocarbon that occurs in the oils of over 100 gymnospermic and angiospermic plants, including eucalyptus, cumin, thymine, cypress, coriander, sage, star anise and cinnamon [42, 43]. Its efficacy might stem from: (1) its structural similarity to many pollutants (e.g. toluene, xylene, ethylbenzene, biphenyl, chlorobenzene); and (2) a common evolutionary origin of the genes encoding the catabolic pathways [43].

Encouraged by carvone induction of PCB degradation by *Arthrobacter sp.* strain B1B, Park *et al.,* demonstrated expression of the *bph*C gene (2,3-dihydroxybipheyl 1,2-dioxygenase) in the PCB degrader *Ralstonia eutrophus* H850 following induction by (R)-(–)-carvone (50 mg $l^{-1}$) [44]. The researchers concluded that carvone might induce a different degradative pathway, potentially generating different congener specificity to that of biphenyl-induced cells. Jung *et al.,* examined the efficacy of carvone or limonene to induce the *bph*C gene of *R. eutrophus* H850 in soil. Although biphenyl was capable of inducing the *bph*C gene up to 4 days after addition to the soil, neither carvone nor limonene were able to maintain the induction [45]. The authors concluded that the presence of potential inducing compounds *in situ* does not necessarily ensure that induction will occur, and that a greater understanding of induction is needed before field implementation [45].

Using a similar approach to Jung *et al.*, Oh *et al.,* examined the ability of terpene to prolong the survival of a known PCB-degrading bacterium, *Pseudomonas pseudoalcaligenes* KF707, in soil [46]. The addition of 50 mg $l^{-1}$ *p*-cymene or 50 mg $l^{-1}$ α-terpinene increased KF707 survival by 10- to 100-fold compared with biphenyl-supplemented and control mesocosms. *Rhodococcus* sp. strain T104, a PCB-degrading bacterium, was shown to catabolise biphenyl as well as the SPMe limonene, cymene, pinene and abietic acid as sole sources of carbon. Limonene was capable of inducing the biphenyl degradation pathway. The bacterium contains three genes, T1, T3 and T5, which potentially code for aromatic-degrading compounds. T1 was induced by limonene and cymene, and to a much lower extent, biphenyl. Notably, glucose exerted a similar degree of induction to that of limonene. Cymene was the strongest inducer of T3, while limonene and cymene induced T5 more strongly than biphenyl and glucose [47-49]. Kim *et al.,* further demonstrated that T104 is responsible for three distinct catabolic pathways for phenol, biphenyl and limonene the last of which can induce both the upper and lower pathways for biphenyl degradation [49]. Therefore, the authors concluded that microorganisms might harbour several mechanisms for degrading structurally similar compounds [49].

Rhodococci play an important role in the carbon cycle due to their ability to degrade many semi-recalcitrant organic compounds. One of the three linear plasmids from a well-studied PCB-degrading bacterium, *Rhodococcus* sp. strain RHA1, was sequenced to elucidate the number, structure and regulation of the open reading frames [50]. The smallest of the linear plasmids is divided into three clusters, one of which contains limonene degradation genes, which are potentially responsible for its ability to grow on

limonene, as well as carveol and carvone as sole sources of carbon. Interestingly, the plasmid contains three cytochrome P450-encoding genes. Earlier, the bacterium had been shown to possess multiple isozymes (three *bph*-type ring-hydroxylating dioxygenases and seven *bph*-type ring cleavage enzymes) for PCB degradation. The identification of aromatic- and terpene-degrading genes as well as cytochrome P450 on the same plasmid suggests that the bacterium employs both broad and narrow substrate range enzymes to: (a) make maximum utilization of available carbon sources, particularly those deemed recalcitrant by specialised bacteria; and (b) detoxify compounds that may associate with, otherwise, labile carbon sources. Thus, RHA1 is an excellent model microorganism to examine the link between SPMe and pollutant degradation.

Through the use of a chromosomally-encoded lacZ reporter, Master and Mohn gained insight into the differential induction of *bph*A, the large subunit of the biphenyl dioxygenase, in two PCB-degrading bacteria, *Pseudomonas* sp. strain Cam-1 and *Burkhoderia xenovorans* LB400 [51]. The latter exhibited constitutive expression of *bph*A in the presence of twelve different inducers, including many plant-derived compounds such as pinene, limonene, cymene, cumene, carvone and salicylate. Due to its constitutive PCB-degrading capacity, however, the authors suggested a cautious interpretation of the efficacy of the inducing compounds. In contrast, the biphenyl-induced strain Cam-1 demonstrated a *bph*A activity six times greater than the basal level in cells at 30°C in the presence of pyruvate, indicating the need for induction prior to bioaugmentation of PCB-contaminated soil. Of the twelve SPMe examined, only salicylate induced Cam-1 *bph*A activity to levels greater than basal levels recorded for pyruvate-exposed cells.

Tandlich *et al.,* used carvone and limonene to stimulate biodegradation of Delor 103 (a commercial mixture of PCBs) by *Pseudomonas stutzeri*. An expansion of PCB congener removal was achieved after supplementation with 10 mg $l^{-1}$ carvone compared to glucose-grown control cells [52]. It is interesting to note that the spectrum of congeners degraded decreased with the addition of 20 mg $l^{-1}$ carvone, which suggested that terpene induction may be compound- and concentration-specific. Limonene and glycerol-cultured cells increased the range of congeners degraded as well as the total PCB catabolised compared to the controls. Increased congener depletions were recorded with elevated limonene concentrations from 10 to 20 mg $l^{-1}$, while a decline in PCB degradation resulted with the co-addition of biphenyl and carvone or limonene. Specifically, 90% of a tri-ortho-substituted PCB congener was removed by biphenyl-induced cells while no removal was observed in the presence of carvone. Furthermore, biodegradation in the presence of glycerol or xylose, with carvone or limonene addition, increased the suite of congeners degraded.

Nishio *et al.,* demonstrated the broad substrate specificity for *p*-cymene monooxygenase (CMO) found in the soil microorganism *Pseudomonas putida* F1 (PpF1). The bacterium can grow on *p*-cymene as a sole carbon and energy source by employing a different degradative pathway compared with cultivation on the structurally similar pollutant, toluene. CMO was shown to actively biotransform 4-ethyltoluene, styrene, *m*- and *p*-xylene, 4-chlorostyrene, 4-(methylthio)toluene, 3-chlorotoluene, 4-chlorotoluene, 4-fluorotoluene and 4-nitrotoluene [53]. Interestingly, the highest

biotransformation rate was found not with cymene but with 4-chlorostyrene, which shares the same chemical substructure with flavones such as anthocyanidin and isoflavone as well as the lignin monomer *p*-coumaryl alcohol (Figure 4; [53]).

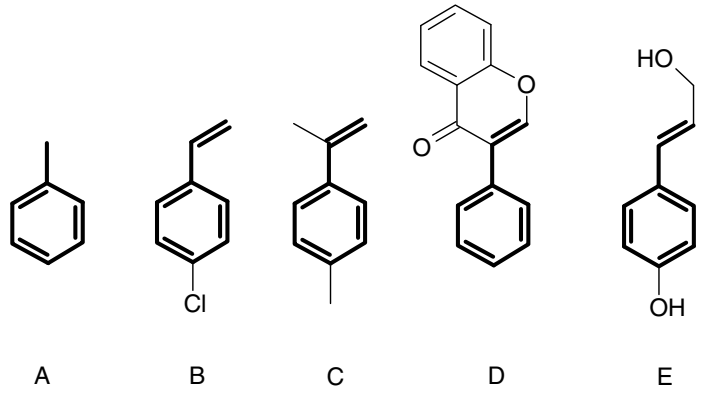



*Figure 4. Structural similarities are bolded between: (A) toluene (pollutant); (B) 4-chlorostyrene (pollutant); (C) p-cymene (SPMe); (D) isoflavone (SPMe); and (E) p-coumaryl alcohol – lignin monomer.*

Qui *et al.,* assessed the influence of the addition of two flavonoids, morin and flavone, on benz[*a*]pyrene (B[*a*]P) degradation in rhizosphere soil [54]. The soils were exposed to 0, 0.1, 1, 10, 100 µmoles of the flavonoids for 60 days. Both morin and flavone-supplemented soils recorded decreased mineralization of $^{14}$C-B[*a*]P with flavonoid concentrations as low as 10 µmoles. Flavone-supplemented soils lowered B[*a*]P bioavailability as monitored by decreased recovery from serial extractions with hexane, water and ethyl acetate. The researchers suggested that morin might have either inhibited the enzyme system responsible for B[*a*]P degradation or was preferentially used as a carbon source by the native B[*a*]*P*-degrading population. Due to a decrease in its extractability, flavone might have stimulated B[*a*]P transformation only and not mineralization, and so resulted in the sequestration, sorption or humification of the metabolite. The authors supported the need for an understanding of the complicated and potentially confounding effects of root exudation, secondary plant metabolite selection, and the specific soil system on the rate and extent of pollutant degradation in soils treated by phytoremediation [54].

Recent studies of the consumption of atmospheric methane in forest soils has indicated that monoterpenes can inhibit methanotrophy (40-100%), with (–)-α-pinene the most effective [55]. The degree of inhibition was found to be species and monoterpene specific in mono-cultures (*Methylosinus trichosporium* OB3b*)*, for unsaturated, cyclic hydrocarbon forms such as (–)-α-pinene, (S)-(–)-limonene, (R)-(+)-limonene and χ-terpinene) [56]. Amaral and Knowles applied an aqueous extract of two depths of forest soil to examine if natural substrates inhibit methanotrophy [55]. They observed a concentration-dependent and transient inhibition after the addition of 0-5 cm

depth soil extracts, whereas extracts from deeper soil (5-12 cm) proved non-inhibitory. Consistent with the current literature, monoterpene depositions from plant leaves might accumulate within the upper soil horizon and result in methanotrophy inhibition. Owing to its global implications, this system provides an interesting and environmentally important model to study the chemical ecology of terpene- and pollutant-degrading genes.

## 2.1. STRUCTURAL- AND STEREO-ISOMERS

Many of the environmental pollutants controlled under international agreements, such as the United Nations Economic Commission for Europe Persistent Organic Pollutants Protocol (U.N.E.C.E. P.O.P.s Protocol) and the United Nations Environment Programme PoPs Convention, are mixtures of structural- and stereoisomers (e.g. aldrin, chlordane, dieldrin, DDT, heptachlor, hexabromobiphenyl, hexachlorocyclohexane, PCBs, dioxin). Detailed investigation of differential biological activity on, and biodegradation of, these complex isomeric mixtures of PoPs, universally demonstrates highly variable activities and persistence [57]. Isomers are molecules with the same chemical formula. Structural-isomers have different bonding patterns whereas stereo-isomers have identical bonding patterns but differ only in the geometric position of the bond. Hence, it is misleading to discuss the efficacy of a remediation approach when addressing structural- or stereo-isomeric mixtures without acknowledging the potential for differential isomeric activity. Similarly, when investigating the recalcitrance of inducing pollutant degradation with SPMe, one must be cautious of the differential effects of structural- and stereo-isomers. Two studies are presented here as evidence of the differential effects of structural- and stereo-isomers in both the pollutant and the secondary plant metabolite.

Strong evidence for the induction of alternative PCB catabolic pathways using SPMe within many of the well-known PCB-degrading bacteria was demonstrated by Singer *et al.* [58] through the use of stereoselective degradation. Five PCB-degrading bacteria, *Ralstonia eutrophus* H850, *Burkholderia xenovorans* LB400 ([59]), *Rhodococcus globerulus* P6, *Rhodococcus* sp. strain ACS and *Arthrobacter* sp. strain B1B were assessed for their ability to differentially degrade four atropoisomeric PCBs (one tetrachlorobiphenyl and three pentachlorobiphneyls). Catabolism was assessed for each bacterium after growth on tryptic soy broth and in the presence of biphenyl, (S)-(+)-carvone or *p*-cymene. Stereoselectivity varied with respect to strain, congener and co-substrate. The authors concluded that the inducing compounds might facilitate alternative PCB-degradation pathways within the bacterium, thereby accounting for the observed stereoselective degradation pattern. The stereoselective degradative pattern for each enzyme can exist owing to the enzyme and the chemicals' chirality. Hence, changes in metabolic pathway might be detectable through the use of chiral chemical substrates as they might be differentially degraded by each enzymatic system.

## 2.2. RHIZOSPHERE ECOLOGY

Yu *et al.,* reported the recovery, by three to four orders of magnitude, of more resin acid degraders (tricyclic terpenoids originating from softwood trees) in hydrocarbon-contaminated soils than in pristine Arctic tundra soil [60]. Notably, the soil samples were collected thousands of kilometres from the nearest source of resin acids (conifer forest) and contained no native resin acids. The bacteria isolated in the study, *Pseudomonas* and *Sphingomonas*, are hydrocarbons degraders, which suggested that their ability to mineralise resin acid and xenobiotics may not be purely coincidental. The results from Yu *et al.*, were particularly interesting in light of a publication by Button who discovered that over 10% of the bacteria in a litre of seawater near Seward, Alaska (similar Arctic region to that studied by Yu *et al.,*), catabolised terpenes [61]. The author postulated that very heavy precipitation on the conifer forest of the Pacific Northwest carries the canopy drip and guttation fluid into the surface water and, ultimately, the sea. However, due to the Alaska Coastal Current, the dissolved terpenes are carried into the estuaries upstream, thereby sustaining a terpene-based food web [61]. The distribution of large quantities of SPMe in the Arctic region may provide the elusive mechanism Yu *et al.* [60] sought for the presence of resin acid (and hydrocarbon) degraders.

### *2.2.1 Induction by plant phenolics and root recycling*

It has been proposed that fine plant root recycling can provide the stimulus needed to sustain pollutant-degrading microorganisms in the rhizosphere [62, 63]. The researchers demonstrated that a majority of fine roots (<1 mm diameter) from mulberry (*Morus* sp.) die at the end of a 6-month growing season. Flavones, such as morusin, morusinol and kuwanon C, contribute to approximately 4% of the fine root biomass (dry weight) after a full growing season. The authors have demonstrated that a wide range of flavones sustain the growth of the PCB-degrading bacterium *Burkholderia xenovorans* LB400 and concluded that a continual supply in the rhizosphere, through fine root recycling, might facilitate the structure and function of the microbial populations facilitating degradation of otherwise recalcitrant pollutants [30, 62]. The biphenyl dioxygenase of *Pseudomonas pseudoalcaligenes* KF707, a well studied PCB-degrading microorganism, has also been shown to catalyze both flavone and 5,7-dihydroxyflavone [64], and as discussed earlier, was shown to exhibit protracted survival in soil supplemented with *p*-cymene or α-terpinene [46].

The fine root recycling hypothesis was evaluated by Parrish *et al.,* who, following application of an herbicide to kill the roots of fescue (*Festuca arundinacea* Schreb.) and yellow sweet clover (*Melilotus officinalis* Lam.), assessed the rate and extent of PAH degradation in the plant rhizospheres. Although they demonstrated differences in the extent of PAH removal by the two species, there was no enhancement of PAH removal due to “induced root death” [65].

Addressing a similar question, Shaw and Burns demonstrated that *Trifolium pratense* exudates and a supplement of roots grown in non-sterile soil, increased the maximum 2,4-dichlorophenoxyacetic acid (2,4-D) degradation rate and decreased the lag time to the maximal 2,4-D degradation rate [66]. Notably, both these promotions also resulted

with supplementation of autoclaved roots. Conversely, gnotobiotic hydroponic and sand-grown roots did not increase the rate of 2,4-D degradation, which suggested that the stimulatory component was both a function of the plant and cultivation medium. The authors also found evidence that unfractionated legume rhizodeposits enhanced 2,4-D mineralization. The implication was that flavonoids, as major signalling components of the rhizobia-legume symbiosis [1], might select for microorganisms capable of detoxifying and utilising the flavanoid signals or their metabolites [66]. For example, cinnamic acid is one of the possible metabolites of flavanoid degradation and has been shown to induce *TfdA*, the gene responsible for the first step of 2,4-D catabolism [66]. 2,4-D is structurally analogous to *p*-coumaryl alcohol, a lignin monomer, which has been proposed to be a natural inducer of PCB degradation (Figures 3 and 4) [26].

### *2.2.2 Salicylate*

Akin to *p*-cymene, salicylate is another SPMe that has been studied extensively, not only for its efficacy to stimulate pollutant degradation but also in relation to its role as a plant-plant signalling compound [18]. Salicylate has been shown to induce biphenyl, xylene and toluene degradation in *Pseudomonas paucimobilis* Q1 [67] and PAH degradation in *P. saccharophila* P15 and *P. putida* 17484 and PpG1 [68-70]. Filonov *et al.,* demonstrated preferential expression of the *ortho*-pathway for catechol cleavage (a metabolite of PAHs and salicylate), as well as the presence of silent genes for the *meta*-catechol cleavage pathway. Recognition of this alternative pathway extends the range of substrates utilized although at a potential cost of cell death if the microorganism is exposed to particular halogenated isomers [71].

A considerable body of results has amassed that demonstrates the funnelling of PAH metabolites through one of two intermediate pathways, salicylate and phthalate [72]. However, the discovery of naphthalene-, phenanthrene-, anthracene-, chrysene-, fluorine-, pyrene-degrading bacteria, which do not grow on salicylate or phthalic acid, suggests that a variety of pathways and inducers exist for the degradation of PAHs [72-74].

### *2.2.3 Pollutant-degrading pathway repression*

Rentz *et al.,* examined the effect of hybrid willow (*Salix alba* × *matsudana*) root exudates on the phenanthrene-degrading activity of *P. putida* 17484. Although salicylate was expected to increase phenanthrene degradation, it was repressed by approximately 21% of its maximum [70]. The researchers concluded that the prevalence of alternative carbon sources in the rhizosphere exerted catabolite repression [3]. However, in this and a previous study with *Pseudomonas fluorescens* HK44, it was suggested that increased numbers of total heterotrophs and pollutant-degrading bacteria, as well as increased metabolic activity, can, potentially, compensate for catabolite repression [70, 75]. Global carbon source regulation was implicated by a decline in phenanthrene degradation in cells exposed to 2.0 mM acetate, lactate, pyruvate, glucose and glutamate. The amino acids aspartic acid and glutamate, quantified as up to 3.9% of the total organic carbon of willow root exudates, might have contributed to the repression. In previous studies, it has been demonstrated that the availability of amino acids in the

concentration range of 0.001 to 0.1% can suppress the *Pseudomonas*-derived *DmpR*-Po $\sigma^{54}$-dependent regulatory system, and so delay expression of the (methyl)phenol catabolic enzyme. The authors emphasised that the appropriate transcriptional response to specific signals in their environment are contingent on the physiological status of the cell [76,77]. Notably, the $\sigma^{54}$ promoter for the toluene/xylene catabolic TOL plasmid has also been shown to be growth-phase regulated in rich media [78]. Hence, the efficacy of SPMe induction will likely be dependent on the availability of carbon sources (e.g. amino acids) and the growth stage of the catabolic microorganism (e.g. stationary phase). Coordinated expression of pollutant-degrading genes upon entry into stationary phase was also demonstrated by Denef *et al.*, in the PCB-degrading bacterium *B. xenovorans* LB400 [79]. Rentz *et al.,* were careful to note that root-derived substrate repression is likely to vary among different microbial strains and plant species [70]. Yoshitomi and Shann confirmed this in a study that involved continuous application of corn (*Zea mays* L.) root exudates to pyrene contaminated soil for 90 days. The researchers observed enhanced pyrene mineralization in root-exudate supplemented as compared with controls [80].

## 3. The tortoise and the hare: Exponential silencing

Repression of a microbial catabolic gene in log-phase growth on nutrient rich medium is termed exponential silencing [81]. This phenomenon has been studied in only a few microorganisms (*Pseudomonas putida* pWWO [82], *Acinetobacter sp*. ADP1 [83] and *Burkholderia xenovorans* [79]). The insights gained from understanding this process have immediate implications towards rhizostimulation and the chemical ecology of pollutant remediation.

Exponential silencing might suggest that copiotrophic rhizosphere-competent bacteria will preferentially exploit labile carbon substrates (e.g. pyruvate, malate, citrate, succinate) before degrading less labile molecules, such as pollutants (e.g. toluene, xylene, biphenyl). However, on entering stationary phase, the copiotroph experiences a general stress response, which up-regulates $\sigma^{54}$-dependent promoters and activates enzymes (e.g. monooxygenases, dioxygenases) with broad substrate specificity thus enabling utilization of semi-recalcitrant, lower energy-yielding carbon sources, which in some cases, might (fortuitously) be a pollutant. Conversely, oligotrophs, which arguably rely more on lower energy-yielding carbon sources might thereby avoid exponential silencing and thereby carry out a significant proportion of what is termed “natural attenuation”. In this way, the tortoise (oligotroph) could provide more extensive pollutant removal than the hare (copiotrophs). If validated, laboratory studies demonstrating the efficacy of copiotroph-mediated pollutant attenuation might be *in vitro* anomalies, unrepresentative of complex soil microbial systems.

This chapter has highlighted the value of consolidating interdisciplinary knowledge to generate new hypotheses for pollutant degradation. Due to the increasing literature base in all fields of science, it is now possible (and necessary) to initiate interdisciplinary collaboration between microbiology, ecology, biochemistry, botany and entomology, to resolve this complex problem.

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# DENDROREMEDIATION: THE USE OF TREES IN CLEANING UP POLLUTED SOILS

**TAMAS KOMIVES AND GABOR GULLNER**
*Plant Protection Institute of the Hungarian Academy of Sciences, Herman Otto ut 15, 1022 Budapest, Hungary, FAX +36-1-4877555, E-mail: tkom@nki.hu*

## 1. Introduction

Forests have provided shelter and habitat for our ancestors for many millennia. During historical times, man grew trees for a number of uses, including energy, furniture, building material, production of paper, fruit and rubber, etc. Recently, trees were introduced for use in dendroremediation, i.e. to depollute contaminated soils. The word dendroremediation comes from the Ancient Greek *dendron* meaning "tree" and Latin *remediare* meaning "reuse" [1-3]. Dendroremediation is an emerging phytoremediation [4-7] technology for cleaning up environment contaminated with organic or inorganic pollutants by using living trees to remove, sequester, or chemically decompose the pollutant [1, 2].

From the point of view of dendroremediation a tree may be considered as a solar driven pump-and-treat system, which may contain a contaminant plume and prevent the spread of contamination by reducing the movement of contaminated water and the erosional transport of contaminated soil. The efficiency dendroremediation has been proven in cleaning up soils polluted with crude oil, explosives, landfill leachates, metals, pesticides, polycyclic aromatic hydrocarbons, and solvents [1-7].

Trees are woody plants characterized with a large biomass, a permanent central self-supporting stem, a stable root system, and a long lifespan. Trees are highly efficient competitors for light, nutrients, and water and tend dominate the vegetation wherever conditions are favourable for plant growth. Since trees are exposed to highly variable biotic and abiotic stresses during their long lifespan they had evolved mechanisms to cope with them. For example, formation of wood can be viewed as an adaptive mechanism that enables trees to secure a dominant position in ecosystems. Wood has many functions that may be important for efficient dendroremediation, e.g. water and nutrient transport and storage of organic compounds and gases [8].

Dendroremediation considers the tree with its physical and biological environment including the soil and the associated microflora [2]. Tree roots are know to produce and release organic chemicals and create a rhizosphere zone more amenable to the microbes that degrade the contaminant. Root exudates such as organic acids and ketones may



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promote microbial growth, as may the increase in soil organic matter caused by the roots. Microorganisms fostered by trees in their root zone may contribute significantly to the success of dendroremediation by enhancing the availability of the pollutant for uptake by the plant root system, as well as by degrading some organic pollutants [9, 10].

Much is expected from a plant to be successful in dendroremediation. For efficient uptake of the pollutant a large and deep penetrating root system and a high transpiration rate is important. Large biomass producing, fast-growing, stress-tolerant trees are preferred that are characterised with low nutrient and soil-quality requirement and are capable to survive in a hostile environment and tolerate the phytotoxic effects of the pollutants. In addition, feasible reproduction, propagation and production of the trees are also highly important. Recently, the genera *Salix* (willows and osiers) and *Populus* (i.e. poplars, including aspens and cottonwoods) have emerged as the most efficient systems for dendroremediation [1-7]. Very importantly, the power of poplar as a model system among tree species has been dramatically enhanced by the recent sequencing of *P. trichocarpa* (black cottonwood) [11].

## 2. Uptake and translocation of the pollutants in trees

Although the binding of pollutant molecules to soil particles can be irreversible, usually desorption occurs: pollutants may move with the soil solution and ultimately reach the groundwater. Efficacy of dendroremediation strongly depends on the bioavailability of the pollutant. Bioavailability is determined by the physical and chemical properties of the pollutant, as well as those of the soil. Uptake of aqueous solutions of inorganic and organic pollutants and their translocation within tree tissues are usually passive processes, regulated by the water transport into the cells. Alternatively, they may also be mediated by membrane-bound transporter systems [6]. Thus, uptake and translocation of a pollutant in trees depends on the pollutant's concentration in the soil solution, its efficiency to enter the root system, and the rate of transpiration in the tree. Trees are known to take up large amounts of water lost from the leaf surface in the transpiration stream. For example, mature poplar trees can transpire 200–1000 liters of water per day [12].

### 2.1. UPTAKE AND TRANSLOCATION OF INORGANIC POLLUTANTS

In soils metal ions are usually strongly bound to soil particles. To improve the bioavailability of metal micronutrients trees have evolved several strategies [6], e.g. producing and secreting metal-chelating chemicals which, by chelation, mobilize iron, copper and zinc, as well as exuding protons in order to change the pH of the soil in the root zone, thereby solubilising the soil-bound metal ions [13]. The physiological and biochemical mechanisms that explain differences in metal mobility in trees are not well understood [8]. Since in trees metals are transported through the xylem their mobility towards the shoots may be strongly retarded by the high cation exchange capacity of the xylem cell walls. As a result, anionic metal-chelate complexes are more efficiently transported in the transpiration stream. Thus, in the practice of dendroremediation, uptake and accumulation of metals in aerial tissues of plants can be enhanced through

the application synthetic and/or natural chelating amendments, such as EDTA and citric acid to the soil [14, 15].

### 2.2. UPTAKE AND TRANSLOCATION OF ORGANIC POLLUTANTS

Physicochemical and structural properties determine the uptake of organic chemicals by plant roots from the soil [16]. Aqueous solutions of moderately hydrophobic organic chemicals (characterized with an octanol-water partition coefficient [log $K_{ow}$] of 1.0-3.5), such as low molecular weight aliphatics and aromatics, and chlorinated solvents dissolved in water are readily taken up by roots of trees and translocated to the aerial parts of the plant. Uptake of hydrophilic (log $K_{ow} < 1.0$) and strongly hydrophobic (log $K_{ow} > 3.5$) compounds is much slower and they may be practically unavailable for uptake because of their strong bonding to soil particles or to the roots of the tree. It is interesting to note that efficiencies of uptake of organic pesticides into the crop plant barley [17] and organic pollutants into poplars [16] are closely correlated. Although bioavailability of organic contaminants is typically low when compared to water-soluble inorganics, much less is known about the roles of amendments in the dendroremediation of soils polluted with organic compounds. Thus, contamination by benzene and its alkyl-derivatives (toluene, ethylbenzene, and xylenes) seems to be ideally suited for dendroremediation. However, removal of these aromatics from soil is possible only by increasing their apparent water-solubility. A new approach takes advantage of the ability of cyclodextrins to increase the elution of organic compounds from soils. Cyclodextrins have dual solubilising potency: they may act as surfactants as well as complexing agents that form inclusion complexes with hydrophobic compounds [18].

## 3. Biotransformation of pollutants in trees

Plant tissues are capable of transforming pollutants by a wide variety of chemical/biochemical metabolic reactions. Rate of metabolism of a pollutant is the main factor in determining sensitivity/tolerance between plant species and has been found to play an important role in the development of stress-resistant plants. Biotransformation reactions of xenobiotics are generally referred to as *Phases I* and *II*, where *Phase I* includes oxidation of xenobiotics and *Phase II* deals with the conjugation of *Phase I* products.

In trees, the oxidative metabolism in the *Phase I* system is usually mediated by cytochrome P-450-containing mixed function oxygenases (CYP, E.C.1.14.-.-) [19, 20]. These enzymes support the oxidative, peroxidative and reductive metabolism of both endogenous and xenobiotic substrates. They comprise a superfamily of heme-thiolate proteins present in every class of organism, including Archaea, and in humans they are responsible for 70-80% of all *Phase I* dependent metabolism of clinically used drugs [21]. In plants there are a surprisingly high number of CYP genes: 246 in *Arabidopsis* (representing approximately 1% of the plant's gene complement) compared with less than a 100 in humans [22]. CYP enzymes are characterized by the high diversity of reactions that they catalyze and the high range of their chemically divergent substrates.