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Phytoremediation: PAHs, Anilines, Phenols

Phytoremediation of Polyaromatic Hydrocarbons, Anilines and Phenols

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Abstract. Phytoremediation technologies based on the combined action of plants and the microbial communities that they support within the rhizosphere hold promise in the remediation of land and waterways contaminated with hydrocarbons but they have not yet been adopted in large-scale remediation strategies. In this review plant and microbial degradative capacities, viewed as a continuum, have been dissected in order to identify where bottlenecks and limitations exist. Phenols, anilines and polyaromatic hydrocarbons (PAHs) were selected as the target classes of molecule for consideration, in part because of their common patterns of distribution, but also because of the urgent need to develop techniques to overcome their toxicity to human health.

Depending on the chemical and physical properties of the pollutant, the emerging picture suggests that plants will draw pollutants including PAHs into the plant rhizosphere to varying extents via the transpiration stream. Mycorrhizosphere-bacteria and -fungi may play a crucial role in establishing plants in degraded ecosystems. Within the rhizosphere, microbial degradative activities prevail in order to extract energy and carbon skeletons from the pollutants for microbial cell growth. There has been little systematic analysis of the changing dynamics of pollutant degradation within the rhizosphere; however, the importance of plants in supplying oxygen and nutrients to the rhizosphere via fine roots, and of the beneficial effect of microorganisms on plant root growth is stressed.

In addition to their role in supporting rhizospheric degradative activities, plants may possess a limited capacity to transport some of the more mobile pollutants into roots and shoots via fine roots. In those situations where uptake does occur (i.e. only limited microbial activity in the rhizosphere) there is good evidence that the pollutant may be metabolised. However, plant uptake is frequently associated with the inhibition of plant growth and an increasing tendency to oxidant stress. Pollutant tolerance seems to correlate with the ability to deposit large quantities of pollutant metabolites in the 'bound' residue fraction of plant cell walls compared to the vacuole. In this regard, particular attention is paid to the activities of peroxidases, laccases, cytochromes P450, glucosyltransferases and ABC transporters. However, despite the seemingly large diversity of these proteins, direct proof of their participation in the metabolism of industrial aromatic pollutants is surprisingly scarce and little is known about their control in the overall metabolic scheme. Little is known about the bioavailability of bound metabolites; however, there may be a need to prevent their movement into wildlife food chains. In this regard, the application to harvested plants of composting techniques based on the degradative capacity of white-rot fungi merits attention.

Keywords: Anilines; bound residues; cytochromes P450; glucosyl transferases; oxidant stress; peroxidases; phenols; phytoremediation; polyaromatic hydrocarbons; rhizosphere

Introduction

Waste products have been dumped in the environment for thousands of years assuming that the environment will adequately absorb them, but this is no longer the case (esp. with industrial compounds) and accumulating pollutants are now affecting the health of living organisms. Physical and chemical remediation techniques are commercially available (for reviews, see Bull 1992, Wilson and Jones 1993), but they are disruptive to the environment. Technologies based on plants represent an attractive alternative because they are 'clean','green', independent of an external energy supply and likely to be more publicly acceptable than the use of chemical methods. They depend on agricultural practice and are perceived to have lower costs in application. The rise in popularity of phytoremediation is borne out by recent reviews on the topic (Cunningham et al. 1996, Salt et al. 1998, Morel et al. 1999, Korte et al. 2000, Macek et al. 2000, Meagher 2000, Mejare and Bulow 2001). There is however, major concern over the time-scales associated with biological methods, both plant and microbial, for which a greater

understanding of the nature of the bottlenecks and limitations will be required if they are to be widely adopted.

In this review, information about plants, their use and biological mechanisms involved in remediating sites contaminated with anilines, phenols or the much less reactive polycyclic aromatic hydrocarbons (PAH's) is examined. Plant response in terms of metabolism, health and adaptive features are considered together with the role that plants may play in ecosystem-remediating strategies involving microorganisms.

1 Properties of Pollutants

1.1 Chemical properties

'Phenols' and 'anilines' are generic terms used to describe alcohol and amine derivatives of benzene respectively (see **Fig. 1**). Both derivatives exhibit octanol-water partition coefficients (log K_{ow}) in the range from 0.5–3.0 and are soluble in both polar and non-polar solvents. They may also ionise as a function of pH value, anilines (pK_a values = 4.6) becoming positively charged at neutral pH values and phenols, negatively charged at alkaline pH values (pK_a = 7.2–8 for nitrophenols; 8.5–9 for chlorophenols and 9.9 for phenol). These properties significantly influence their potential for plant uptake.

PAHs are comprised of two or more fused benzene rings (see **Fig. 2** for examples). They are largely insoluble in water (0.1 µg to 4.5 mg/L), but highly lipophilic, with log K_{ow} values ranging from 3 to 7. Lipophilicity and water insolubility tend to increase as a function of size along with a decrease in vapour pressure (Aihara 1992). PAHs partition preferentially into the humic fractions of soils rather than the aqueous phases. Those with log K_{ow} values of 4 or less partition readily across membranes into the lipophilic compartments of living cells and can be taken up by plant roots.

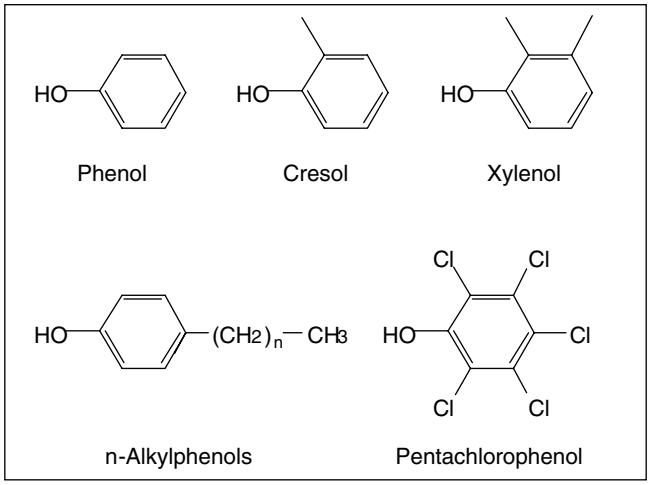

Fig 1: Structure of selected phenols

1.2 Bioavailability

Pollutant bioavailability is a major restricting factor in the bioremediation of organic pollutants. Bioavailability depends on the soil structure (content of humic substances, pH value, water content and porosity) and on properties of the pollut-

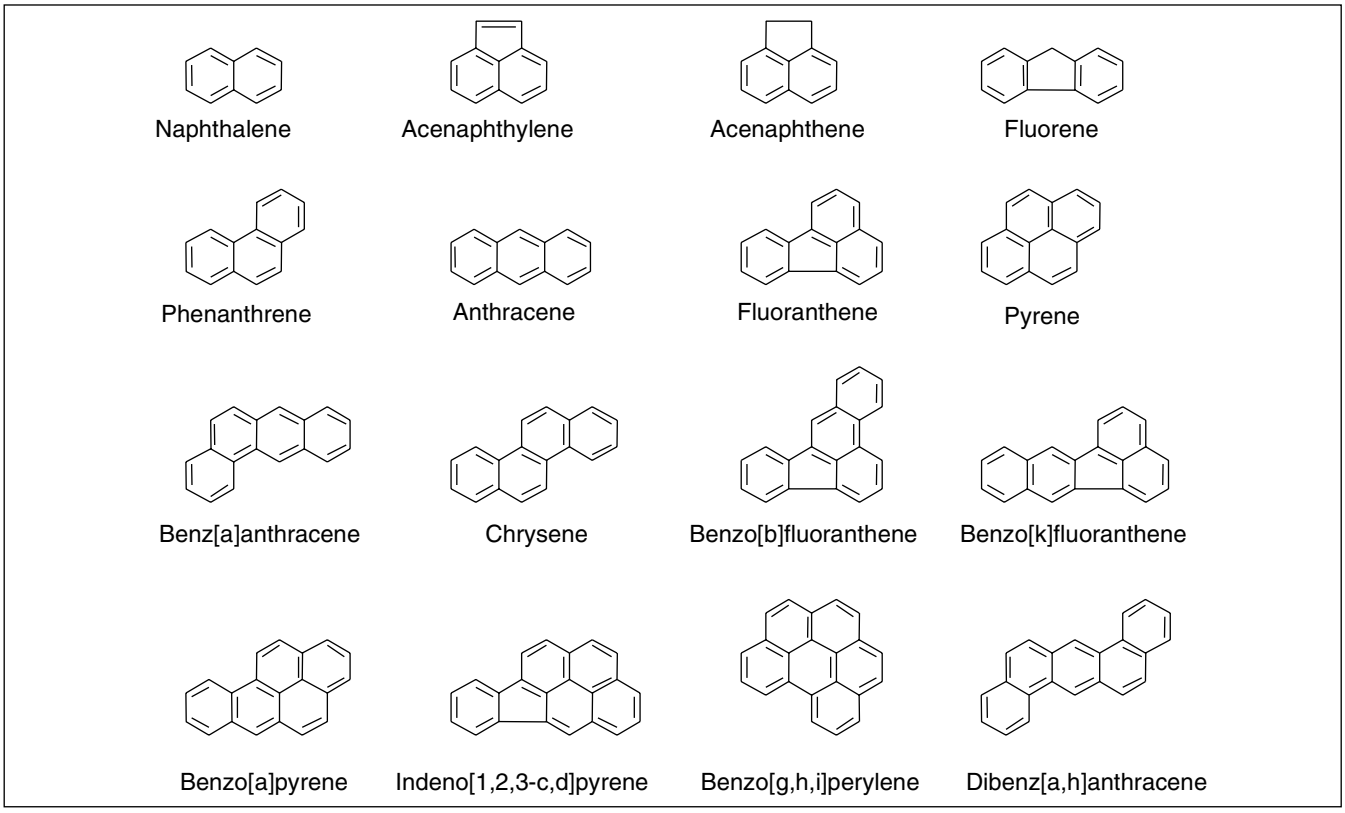

Fig 2: Molecular structure of the 16 PAHs considered as priority pollutants by the American Environmnental Protection Agency (EPA)

ant. The partition of organic pollutants between soil matrix and water is described by a constant: Kp. The estimation of this parameter is based on the interaction of pollutants with the organic matter. As illustrated by equations 1 and 2 (EPA 1996), organic matter plays a different role in sorption of ionic or non-ionic pollutants.

Equ. 1: non-ionic pollutants : $K_p = K_{oc}\, f_{oc}$

with:

K_{oc}: partition coefficient between organic matter and water

f_{oc}: soil organic matter content

Equ. 2: ionic pollutants : $K_p = (K_{oc,n}\, \Phi_n + K_{oc,i}\, (1-\Phi_n))\cdot f_{oc}$

with:

$K_{oc,n}$: partition coefficient for non-ionised form

$K_{oc,i}$: partition coefficient for ionised form

Φ_n : non-ionised fraction, depending of soil pH and pollutant pKa

Humic substances represent the main cause for the restrictions placed on bioavailability. These compounds, which represent as much as 60–80% of soil organic matter, are polydispersed polymers of aromatic and aliphatic units that are stabilised to degradation by covalent binding of their reactive sites to metal ions and clay minerals. They possess a high content of oxygen-containing functional groups including –COOH, phenolic and/or enolic –OH, alcoholic –OH and the –C=O double bonds of quinones, hydroquinones and α,β-unsaturated ketones (Stevenson 1994). They are able to bind phenolics, anilines and PAHs in a process known as ageing (Alexander 1995). They may also serve as carriers of these pollutants, increasing their solubility and motility in soil and water (Schinner and Sonnleitner 1996).

Surfactants of synthetic or biological origin have been used to enhance the apparent water solubility and bacterial degradation of organic pollutants in soils with high contents of humic substances, but little systematic attempt has been made to determine the influence of surfactants on plant uptake of organic pollutant. Non-ionic synthetic surfactants may be less well tolerated than surfactants of biological origin (Laha and Luthy 1992, Noordmann and Janssen 1995).

1.3 Toxicity and distribution

Derivatives of phenols and anilines are of major concern. For example, chlorophenols figure among the largest groups of compounds on the US Environmental Protection Agency list of priority pollutants, because of their recalcitrance to degradation; their toxicity; and their potential to partition into biota, soil and sediment. Chlorophenols are produced as by-products from the bleaching of pulp with molecular chlorine (Keith and Telliard 1979). They have also been deliberately synthesised as wood preservatives. Pentachlorophenol (PCP) is a respiratory poison with both non-carcinogenic and carcinogenic health effects and on account of its antimicrobial, herbicide and insecticide properties, it has been used as an herbicide in rice. 'Penta' contains 90% PCP, 3–10% lesser chlorinated phenols and <1% lesser chlorinated dibenzo(*p*)dioxins and furans and is found as a result of accidental spillage in the soils at many wood preservative treatment plants (Crosby 1981; Keith and Telliard 1979). PCP is a proven carcinogen in rodent studies (Waidyanatha et al. 1996) and there is some epidemiological evidence that it is carcinogenic in humans (Crosby 1981). A Public Health Goal of 0.4 ppb was set for PCP in drinking water. The U.S. Environmental Protection Agency's Maximum Contaminant Level and Maximum Contaminant Level Goal for PCP are 1 ppb and zero, respectively.

Anilines and azo-derivatives form the basis of much of the synthetic dye industry, around 10% of which is lost in industrial effluent (Vaidya and Datyre 1982). These discharges are of environmental concern because of the stability of the dyes, which have been developed to withstand extremes of light and temperature and exposure to detergents and microbes. Moreover, many azo derivatives of anilines are decomposed into potential carcinogenic amines under anaerobic conditions after discharge into the environment. For example, 4-chloroaniline (4-CA) which is both hematotoxic and genotoxic, is a known degradation product of a variety of substituted phenylurea compounds used mainly as herbicides, as intermediates in the synthesis of aniline dyes, and as accelerators and antioxidants in the rubber industry (Ellenhorn and Barceloux 1988).

There is also increasing concern over phenolic environmental pollutants with endocrine activity (Soto et al. 1991, White et al. 1994, Purdom et al. 1994, Jobling et al. 1996). Alkyl phenols, especially octylphenol and nonylphenol that are metabolites of nonionic-surfactants (alkyphenol polyethoxylates), build up in considerable amounts (up to 1g kg^{-1} dry weight) in sewage sludge (Giger 1981 1984, Sweetman 1994). The application of sludge to agricultural land and the use of the associated wastewater in irrigation may lead to uptake and metabolism of 4-nonylphenol in crop plants. An occurrence of nonylphenol and its derivatives in crop plants may result in strong impacts on food quality. Nonylphenol is also used as a wetting, emulsifying and dispersing agents in the formulation of pesticides. More recent discussions on bans or plans to introduce environmental quality standards have been triggered by findings that nonylphenols are estrogenic (Soto et al. 1991).

PAHs with log K_{ow} values above 4 are not considered to be mobile within the environment, however, those with a value less than 4 (for example, naphthalene and phenanthrene) readily enter the food chain, concentrating from primary levels to the top of the food chain because of the slowness of their degradation in biota. PAHs are formed whenever organic materials are burned – amounts of PAHs in soils coming from atmospheric fall-out have been rising steadily since the 19th century (Jones 1989, Wania and Mackay 1996). Fluoranthene, formed during the incomplete combustion of fossil fuels, is one of the most abundant PAHs with an ubiquitous environmental distribution (Wild and Jones 1995). Combustion of fuels by motor vehicles contributes a major part of PAHs in urban air (Benner and Gordon 1989, Lee and Jones 1999). PAHs are also found in high concentrations in the wood-preserving industry, which has relied heavily on creosote and anthracene oil as wood protectants (creo-

sote contains 85% by weight of PAHs) (Lim et al. 1999, Wei and Wu 1997, Yasuda and Takahashi 1998). Sewage and dredging sludges are particular sinks for heavier PAHs (Goodin and Webber 1995). Although acute toxicity is generally low, most PAHs are likely to be mutagenic, since their metabolism yields epoxy derivatives that are able to interact with nucleic acids. Fluoranthene, for example, is mutagenic (Kaden et al. 1979, Barfknecht et al. 1982, La Voi et al. 1982), cocarcinogenic for benzo(α)pyrene (Van Duuren and Goldschmidt 1976) and potentially carcinogenic (Busby et al. 1984, La Voi et al. 1994). Chronic human dermal exposure to soil containing 50–100mg kg^{-1} PAH or 0.5–5 mg kg^{-1} benzo-(α)pyrene is likely to constitute a significant hazard in terms of the potential to cause skin cancer. Long-term toxicity also implies fertility perturbations and a general increase of sensitivity to other stresses. The ICCR considers that at least 3 PAHs are probably carcinogenic (Menzie et al. 1992).

Organic pollutants are frequently found in heterogeneous streams mixed with other chemicals and distributed in a highly non-uniform manner. For example, ammoniacal liquor and coal tar are both highly polluting products of the former gas industry. Ammoniacal liquor contains phenol, ammonia, cyanide, and sulphate; and coal tar, high concentrations of PAHs and volatile aromatic and aliphatic components as well as phenolic tar acids. Spent oxide may also be present, containing 50% free sulphur and several percent cyanide. Site contamination with these pollutants may have arisen from industrial waste-disposal, particularly on smaller sites where there may not have been a demand for recovered products; and leaks and spills during storage and handling. These features pose an additional challenge in the application of a phytoremediation strategy for land reclamation.

2 Plant Uptake

2.1 Roots

Our knowledge of plant uptake of pollutants stems from an extensive literature on the phytoextraction of metal chelates, which has developed because of the very much greater experimental ease with which metals can be traced (for a review, see Mejare and Bulow 2001). These studies liken the plant to a wick in which the rate of uptake and transport of metal chelate to the shoots is dictated by a high surface area in the roots and an efficient plumbing system.

Fine roots (diameter <0.8mm) comprise most of the total root length as well as root surface area of a plant, and play the most important role in normal nutrient and water uptake (for a review, see McCully 1999). They are also the source for most of the root exudates and root cap mucilage that is secreted to the extracellular environment and are consequently important in the context of the developing rhizosphere. Fine roots appear to develop as a direct signal response to the presence of nutrients (NO_3^-) detected in the soil environment. However, under conditions of severe nutrient or water stress, root cortical tissues may become strongly lignified, limiting the development of fine roots.

Few systematic studies have been carried out with regard to the tolerance of fine roots to the exposure and uptake of organic pollutants. In general, the phytotoxic threshold depends on the plant species, development and nutritional status, nature of soil (presence of microorganisms) and type of hydrocarbon. For example, Bokern et al. (1998) found that 4-n-nonylphenol (4-NP) caused a 50% growth reduction in root cultures of *L. hartwegii* when supplied to the growth medium at a concentration of 0.1 mM, but was not as inhibitory to the growth of *L. polyphyllus* root cultures, even when supplied at 1 mM. When both root cultures were subcultivated and treated again with 4-NP, a complete growth inhibition at the lowest 4-NP test concentration (10×10^{-3} mM) was observed. These workers also observed enhanced sensitivity with the root and shoot of aseptical plants of *A. hortensis*. Growth was inhibited by more than 50% at a concentration of 50×10^{-3} mM 4-NP.

Phenolics and anilines are likely to move with the transpiration stream in the liquid phases of the soil at concentrations dependent on their relative hydrophilicity and pass passively through the root cell wall into the apoplast before loading into the xylem to be transported to shoots. Cucurbitaceae, for example, have been shown to be able to mobilise polychlorinated dibenzo-*p*-dioxins and dibenzofurans from the soil and to translocate them to the shoot after uptake through roots (Huelster et al. 1994). Negatively charged sugar acids in root cell walls may pose a barrier to charged species, whilst more lipophilic species may enter the symplast, by penetrating the lipid phases of the plasma membrane.

At concentrations above 10 mg/kg soil, there is evidence that PCP will be taken up by plants (lower concentrations are rapidly degraded by soil microorganisms) (Bellin and O´Connor 1990)). For example, rice (*Oryza sativa* L.) plants grown for 7.5 months on soil containing [^{14}C] pentachlorophenol (172 mg/kg) took up 12.9% of the applied radioactivity, which was distributed between roots (5.2%) and shoots (7.7%) (Weiss et al. 1982). Topp et al. (1986) reported significant ^{14}C uptake for barley (Hordeum vulgare) after 1 week at soil pH of 6.4 whilst Casterline et al. (1985) reported intact PCP uptake by spinach and soybean plants from an acid soil. PCP has been detected in the extracts of *Festuca arundinacea* Schreb. and *Latuca sativa* L. grown in sludge-amended soils but not in *Capsicum annuum* (Bellin and O'Connor (1990). Using hydroponically cultivated plants *Sinapis alba* and *Cyclamen persicum*, PCP absorption was found to be independent of the proton concentration of the external solution and in *Sinapis* seedlings, amounts up to 10^{-5} mol/L PCP had no poisonous effect on phloem transport (Grimm et al. 1987). However, in the alga *Selenastrum capricornutu*, PCP decreased the rate of carbon assimilation. Toxicity was due to the absorption of negatively charged PCP ions at the membrane surface. This protonophoretic action caused the decrease of membrane electrical resistance and the dissipation of hydrogen ion electrochemical potential gradients across cellular and subcellular membranes (Jayaweera et al. 1982).

All PAH congeners are readily adsorbed on the root surface but root absorption is extremely limited and highly variable depending on the species and environmental conditions (Briggs et al. 1983, Trapp et al. 1990, Trapp and Matthies 1997). PAHs are extremely water-insoluble and partition

preferentially into the humic fractions of soils rather than the aqueous phases. They pose a major problem in bioremediation (Shuttleworth and Cerniglia 1995). By contrast, partially oxidised PAHs with log K_{ow} values of 4 or less are much more soluble than the parent compound (oxidation of anthracene to phthalic acid increases the water-solubility of the pollutant from 0.07 to 7000 mg L^{-1}, whilst the oxidation of phenanthracene to diphenic acid increases the water solubility nearly 1000-fold from 1.3 to 1260 mg L^{-1}). Kulakow and co-workers (2000) compared 29 plant species for their tolerance after 180 days growth to petroleum hydrocarbons in contaminated sediments (ca. 25 mg/kg pollutant in abiotic controls). Their results also included root growth parameters such as mean root diameter and root-length density. In general, root-length density correlated positively with plant tolerance, with *Festuca arundinacea* appearing as the most tolerant species, and *Hordeum vulgare* the most sensitive. The phytotoxic threshold of PAHs is higher in soils with a high water-holding capacity. The phytotoxic threshold for crude oil and heavy fractions is about 1–1.5%w/w. Lighter more toxic PAHs such as fuel cause reduced growth at 0.6% w/w.

2.2 Leaves

Leaf wax acts as a barrier to the penetration of organic pollutants, however, in young leaves with little wax, volatile PAHs may penetrate via the leaf cuticle (Bukovac et al. 1990, Nakajima et al. 1996). Generally, the evidence suggests that the concentration of PAH in plant tissue is related to the degree of air pollution, with PAHs of low molecular weight being taken up via the vapour phase. Plant metabolism (oxidation and conjugation, see below) of air-borne PAH (pyrene) has been demonstrated in leaves of four woody plants (Nakajima et al. 1996).

3 Plant Metabolism

3.1 Pattern of pollutant deposition

Much of our knowledge of the metabolism of organic pollutants in plants has been obtained from experiments conducted using cell culture techniques. However, the validity of extrapolating data obtained with cell culture techniques to those of intact plants is still a matter of debate. It is also not known whether the nature of the association found in the non-extractable 'bound' residue fraction of the cell wall is identical in the different systems (Harms 1992). Two examples of the pattern of uptake obtained using on the one hand, cell suspension cultures and the other, wheat seedlings of the same cultivar, serve to illustrate the situation.

In the first example, wheat cell suspension cultures were spiked with PCP. Cells absorbed PCP very rapidly and formed large amounts of polar metabolites. 41% of the radiolabel was converted (via the conjugate fraction) into the non-extractable 'bound' residue fraction of the cell wall. The ^{14}C-label was bound mainly to lignin and to a high molecular weight hemicellulose fraction. PCP glycosides were predominant among the polar conjugates. In wheat seedlings, a similar overall pattern was obtained. Polar conjugates were extracted from both roots and shoots, together with PCP glycosides. In shoots and roots, by far the greater part (more than 16%) of the total radioactivity was found in the bound residues. These were fractionated into several cell wall components and yielded a pattern similar to that obtained for cell cultures.

In the second example with 4-chloroaniline, 72% of the label in cell cultures was detected in the bound residue fraction and most of the ^{14}C-label was associated with the pectin and lignin fractions of the cell wall. From wheat roots, the radioactivity of the bound residue fraction reached more than 61%, comparable to the percentage found in cell cultures.

The work of Bokern et al. (1998) with plants and root cultures exposed to ^{14}C-4-NP illustrates the general pattern observed for the uptake and transformation of phenolic compounds. They showed that mineralisation of ^{14}C-4-NP (i.e. release of $^{14}CO_2$) only occurred in the presence of microorganisms and was due entirely to microbial metabolic activities. Plants grown in nutrient media containing ^{14}C-4-NP incorporated the compound and metabolised it, but the amount taken up differed enormously depending on the plant species. Radioactivity in shoots, either in the form of ^{14}C-4-NP or of glucose and organic acid conjugated monohydroxy-4-nonylphenols indicated transport of the pollutant from the root to the shoot (Bokern et al. 1996, Bokern and Harms 1997). In aseptically grown plants the non-extractable residue constituted the major fraction showing radioactivity. Higher concentrations led to a higher uptake, not only in absolute, but also in relative amounts. By contrast, in soil, about 60% of the applied compound remained in the soil and of this, only 20–25% of the radioactivity in the soil was found to be soluble; the major part of radioactivity was associated with the bound residue in the soil.

In the case of PAHs, there is evidence for both the uptake and metabolism by plants of five-ringed PAHs such as benzo-(α)pyrene (BαPyrene) (Edwards 1983 and below); dibenz-(a,h)anthracene and perylene (Harms 1983) and fluoranthene (three rings) (Kolb and Harms 1999). In heavy metal-stressed soils there seems to be a stimulation of PAH uptake (Haas et al. 1990). However, different plants show differing abilities to metabolise these pollutants and this point is well illustrated by the data presented in **Table 1**, which shows the distribution of radioactivity obtained after the exposure of different plants in cell culture to B(α)Pyrene. In soybean cell cultures spiked with labelled B(α)Pyrene, 49.7% of the applied radioactivity was associated with polar metabolites; 16.2% of this was measured in cells, and the remaining 33.5% in the culture medium. Bound residues represented 15.6% of the applied activity. In contrast, in cells of wheat, the majority of radioactivity (48.6%) was found as unchanged B(α)Pyrene.

The binding of xenobiotics to roots is more or less a binding to the root surface or within the apparent free space of the roots. However, after application of radiolabelled compounds to cell cultures and intact plants, active uptake and in some cases, metabolism of these compounds results in large amounts of the radioactivity being associated with 'bound' or 'non-extractable residues'. These 'bound' or 'non-extractable residues' typically cannot be released from the plant matrix by extraction with

Table1: Disribution of radioactivity among different fractions obtained after the exposure of different plants in cell culture to various PAHs

	Cells			Medium		Reference
	Parent compound	Polar metabolites	Bound residues	Parent compound	Polar metabolites	
Fluoranthene						
Soybean	74.6	4.2	4.5	8.7		a
Wheat	73.0	9.1	1.7	4.7		a
Rose	16.1	49.9	1.6	27.2		a
Perylene						
Soybean	22.6	18.0	8.5	5.3	34.5	b
Wheat	73.0	6.1	3.0	7.6	0.8	b
BαPyrene						
Soybean	3.0	16.2	15.6	16.4	33.5	b
Wheat	48.6	19.5	9.0	3.1	3.2	b

a: Kolb and Harms 2000
b: Harms and Langebartels 1986

Table 2: Radioactivity released from cell wall fractions of plants treated with ^{14}C-labelled 4-chloroaniline, 3,4,-dichloroaniline, pentachlorophenol and pentachloronitrobenzene

Reagent	Material liberated	Radioactivity (%) released from cell-wall fractions of plants which received:			
		4-CA	3,4-DCA	PCP	PCNB
α-Amylase	Starch	2.3	3.4	2.8	4.3
Pronase E	Proteins	5.6	3.4	23.1	19.8
EGTA	Pectin	48.3	20.9	6.9	13.8
Dioxane/HCl (2M)	Lignin	27.4	52.1	21.5	42.5
KOH (24%)	Hemicellulose	4.8	11.5	35.5	6.0
H_2SO_4	Cellulose	1.6	0.2	1.9	1.8
	Residue	2.8	0.3	2.8	2.8

4-CA = 4-chloroaniline PCP = pentachlorophenol
3,4-DCA = 3,4-dichloroaniline PCNB = pentachloronitrobenzene

solvents, probably because of covalent associations with lignin, hemicellulose or pectin in the plant cell wall.

Bound residues can be assigned to defined cell wall fractions using a sequential fractionation procedure (Langebartels and Harms 1985). **Table 2** summarizes the distribution, using this procedure, of the radioactivity in the various cell wall components of wheat cultures after treatment with different ^{14}C-labelled chemicals (Harms 1992). The pattern of binding of the different xenobiotics depended on the plant species, and on the physical and chemical properties of the compound.

Bound residues seem to be found in those species that are most tolerant to organic pollutants (Bokern and Harms 1997) and there is increasing evidence to suggest that the bound residue fraction (the plant cell wall) is one of the most important 'detoxification' sites in plant cells (Sandermann 1994). Non-extractable residues of 12 different cell suspension cultures were characterized with respect to the main binding sites of radioactivity derived from 4-NP. All cell cultures exhibited a specific distribution of radioactivity in different fractions of bound residues. In most of the cultures (7 out of 12), lignin was the fraction to which the major part of radioactivity was associated, but in some of the cultures also the protein *(Hordeum vulgare, Chenopodium rubrum* and *Lycopersicon esculentum*) and the hemicellulose fraction (*Atriplex hortensis*) were the dominating fractions containing radioactivity. From these data it seems likely that the distribution of radioactivity in cell wall fractions is species specific.

The relative amounts of non-extractable residues of the respective cell cultures suggested that formation of non-extractable residues was associated with a higher tolerance to 4-NP. The species with the highest tolerance integrated high proportions of radioactivity to bound residues. The most sensitive species integrated only low proportions of radioactivity to the bound residue fraction. A similar phenomenon has been observed when ^{14}C-metribuzin was applied to different cultivars of soybean. The most tolerant cultivar formed high amounts of bound residues compared to the most susceptible (Dupont and Khan 1992). However, for species exhibiting EC_{50} values between 50 and 500 x 10^{-3} mM 4-NP such a relationship was less obvious. Thus, there might be additional processes influencing sensitivity or tolerance. Resistance to phytotoxicity and the formation of bound residues seem to be characteristic of plant families. Among the plant species tested, the members of the Fabaceae exhibited high capacities for residue formation and were at the same time quite tolerant to 4-NP whereas *Chenopodiaceae* species formed only limited amounts of bound residues and were shown to be relatively sensitive.

Whilst there is a differential and potentially covalent association of phenols and PAHs with cellular macromolecules (lignin, hemicellulose, protein, cellulose) depending on the chemical structure and plant species (see above), the pathways for this compartmentation are not yet known. ATP-driven ABC transporters have been identified in the vacuolar membrane that are able to sequester glutathionylated and glucosylated precursors (for review see Theodoulou 2000, Sanchez-Fernandez et al. 2001). With respect to phenolic groups the transport of the hydroxyprimisulfuron glucoside, a metabolite of the herbicide primisulfuron, was shown to occur via an ABC transporter residing in barley vacuoles (Klein et al. 1996). Importantly, an Arabidopsis member*AtMRP3* was induced at the transcriptional level by primisulfuron (Tommasini et al. 1997). It is not currently known whether the encoded protein is involved in the vacuolar compartmentation of the corresponding glucoside. However, the vacuolar location of the closely related AtMRP2 has been recently demonstrated (Liu et al. 2001). Once transported into the vacuole the conjugates may be further metabolized, stored as such, or re-imported into the cytosol (Sandermann 1994).

The whole genome of *Arabidopsis thaliana* contains about 103 members of the ABC transporter family (Sanchez-Fernandez et al. 2001). Thus, there seems to be an enormous diversity encoded in plant genomes. Their endogenous roles and substrates are mostly unknown. In addition recent work in *Arabidopsis* has identified the presence of several proton motif force-dependent MATE (multidrug and toxic compound extrusion) transporters that may be involved in detoxification as well (Brown et al. 1999, Diener et al. 2001).

The use of plants for animal or human food raises the question of bioavailability, which is generally acknowledged with respect to the soluble fraction. Pollutants bound to degradable carbohydrate complexes like pectin and cellulose fractions may be more bioavailable than those bound to covalent structures such as lignin. For non-extractable residues bioavailability is still a matter of discussion and has been rarely tested (Sandermann et al. 1990). The pattern of binding of a given chemical and its metabolites to the various cell wall components indicates that these persistent bound residues differ in their bioavailability (Sandermann et al. 1992). Knowledge of the cell wall components with which the xenobiotics are associated and the nature of the linkages will enable an estimate of the ecotoxicological risks of these chemicals.

3.2 Metabolites

Investigations (Harms et al. 1977 and Harms 1983) with benzo(α)pyrene showed that this compound was metabolized by *Chenopodium* cell cultures to quinones and other oxygenated derivatives. In further reactions these compounds were incorporated into insoluble fractions. Pentachlorophenol metabolites, dichloro, trichloro, and tetrachlorophenols, di-, tri-, and tetrachloro chloroguajacols, di-, tri-, and tetrachlorocatechols, trichloro and tetrachloro hydroquinone and chlorinated methoxybenzenes (tetrachloroveratrole and pentachloroanisole) have been identified in both rice plants (Weiss et al. (1982) and the aquatic plant *Eichhornia crassipes* (Roy and Haenninen 1992), whilst Schaefer and Sandermann (1988) identified tetrachlorocatechol as a primary metabolite of PCP in cell suspension cultures of wheat (*Triticum aestivum* L.). Similar results showing conjugation of the parent compound by O-glucosyltransferases were obtained in cell culture studies (Sandermann et al. 1984, Scheel et al. 1984, and Harms 1992). Corresponding O- as well as *N*-glucosylating activities could be purified from cell cultures as well (Sandermann et al. 1991, Harms 1992). Conjugation to a glucoside has also been described in *Lemna gibba*, when it is aseptically grown in the presence of 2,4,5-tetrachlorophenol (Sharma et al. 1997).

In the case of fluoranthene (Kolb and Harms 1999), the metabolic products of fluoranthene formed after uptake by tomato, lettuce, wheat and rose cells were conjugated with compounds such as glucose and glucuronic acid, in different proportions depending on the plant species. Formation of these conjugates is a typical detoxification mechanism in plants and has been reported for plant metabolites of 4-n-nonylphenol (Bokern et al. 1996); pentachlorophenol (Langebartels andHarms 1984, Schmitt et al. 1985, Sandermann et al. 1991) and anilines (Harms and Langebartels 1986, Winkler and Sandermann 1989, Sandermann et al. 1991). Fluoranthene metabolites were either mono-hydroxylated, or dihydroxylated and more polar dihydroxylated metabolites appeared in the shoots rather than roots (Kolb and Harms 1999). These metabolites point to the involvement of cytochromes P450 type enzymes in the detoxification pathway.

3.3 Enzymes

3.3.1 Cytochromes P450

Cytochromes P450 (P450s) are heme – thiolate monoxygenases involved in the metabolism of a wide variety of both exogenous and endogenous compounds, particularly in relation to the biosynthesis of hormones, lipids, and secondary metabolites. P450s use electrons from NADPH to activate molecular oxygen and insert a single atom of molecular oxygen into their substrates. They usually catalyse hydroxylation or epoxidation of phenolic rings, dealkylation of methoxy or amine substituents, and reductive dehalogenation (Mansuy 1998), but have never been reported to catalyse opening of aromatic rings. In P450s, the heme prosthetic group and catalytic site is completely buried in the globular portion of the protein, which leaves no part of the substrate molecule in direct contact with the solvent. As a result, P450s tend to be substrate specific and usually oxidise a restricted number of compounds in a regio- and stereoselective manner (for a recent review see Werck-Reichhart and Feyereisen 2000).

Involvement of P450 enzymes in the metabolism of polyaromatic or polychlorinated pollutants by animals is well documented (Livingstone 1998, Hassler et al. 1999). As suggested in a previous section, plants generate metabolites, which are typical P450 products, and the potential of plant P450 enzymes to metabolise exogenous compounds is clearly demonstrated by their very active role in herbicide metabolism and selectivity (Werck-Reichhart et al. 2000). How-

ever, direct proof of the participation of plant P450s in the metabolism of industrial aromatic pollutants are surprisingly scarce, and characterisation of the enzymes that are involved is still missing. Metabolism of PAHs has been reported in microsomal fractions from several plant species (Trenck and Sandermann 1980, Higashi et al. 1981, Marabini et al. 1994). Attempts to demonstrate P450 involvement in this metabolism led to contrasted results. In pea and soybean, conversion into quinones was not catalysed by P450 enzymes (Trenck and Sandermann 1980). In Jerusalem artichoke, production of mutagenic polar metabolites correlated with P450 induction in the tuber tissues (Higashi et al. 1981). In avocado pear extracts, activation of 2-aminofluorene into mutagenic compounds was at least in part dependent on added NADPH and inhibited by carbon monoxide and the P450 inhibitor 1-aminobenzotriazole (Chiapella et al. 1995, 2000). From data obtained with other inhibitors, it was concluded that activation of the promutagen was mediated by P450, peroxidase and flavin monooxygenase. More convincing is the involvement of P450 in the hydroxylation of 4-chlorobiphenyl and 2,3-dichlorobiphenyl in marine macro algae since this hydroxylation is increased in vivo by typical P450 inducers and completely inhibited upon incubation with a P450 mechanism-dependent inhibitor (Pflugmacher and Sandermann 1998). Dealkylation of methylamines is the first xenobiotic metabolising activity that was reported for purified P450 fractions. A purified P450 from avocado, which was subsequently characterised as CYP1A1, dealkylated *p*-chloro-*N*-methylaniline (O'Keefe and Leto 1988, Bozac et al. 1992). Microsomes and purified fractions from tulip bulb were shown to dealkylate both *N*-nitroso-*N*-methylaniline (NMA) and *N*-nitroso-*N*-dimethylamine (Hansikova et al. 1994 1995). NMA was further converted into denitroso- and ring-hydroxylated compounds. The same microsomal system and purified fractions were also reported to oxidise 1-phenylazo 2-hydroxynaphthalene (Sudan I) (Stiborova et al. 2000). The turnover of these reactions with purified P450 fractions seemed rather low, but was difficult to accurately estimate with reconstituted membrane systems. A clearer picture emerged recently, with the availability of recombinant enzymes. In the last years, the first P450 genes, some mediating the metabolism of natural phenolics such as cinnamic acid, have been cloned and expressed in yeast. Such recombinant enzymes have provided tools to start exploring the metabolic capacity of isolated plant P450s in an optimal membrane and redox environment. A first exploration showed that recombinant P450s could metabolise, sometimes with very high efficiency (i.e. with an efficiency comparable to that of natural substrates), exogenous compounds provided that these exogenous compounds are close structural analogues of their natural substrates (Schalk et al. 1997a, 1997b, 1998, Robineau et al. 1998). CYP73A1, a cinnamate 4-hydroxylase, for example very effectively oxidises naphthoic acid, piperonylic acid, 4-vinyl benzoic acid or thienilic acid. Metabolic efficiency however drops abruptly with structurally divergent molecules (Pierrel et al. 1994). One of the surprising disclosures of the various plant genome-sequencing projects was the extraordinary diversity of the cytochromes P450 superfamily of oxygenases. The final count of the P450 genes in *Arabidopsis thaliana* is 272 (http://www.biobase.dk/P450/), which makes of P450s the largest family of enzyme proteins in higher plants. It is now possible to build collections of recombinant plant enzymes for systematically testing the metabolism of any organic pollutant. It seems extremely likely that sets of P450 isoforms catalysing regiospecific attack of each type of environmental contaminant in different positions will be identified in the near future.

3.3.2 Peroxidases and laccases

Peroxidases are ubiquitous enzymes involved in the detoxification of H_2O_2 to water at the expense of electrons obtained from reducing substrates. In plants, they are localised mainly in cell walls and in vacuoles and are associated with the non-specific oxidative polymerisation of phenolic units in the cell wall to make lignin; with the formation of tyrosine or ferulate cross-links between plant cell wall polymers and with the deposition of aromatic residues of suberin on the cell wall (Gaspar et al. 1982, 1985, Gaspar 1986, Fry 1986, Sato et al. 1993). Plant laccases are also associated with cell wall polymerising reactions, and although physiological details are not well documented, both classes of enzyme are likely to be responsible for the formation of covalent links between hydroxylated pollutants and metabolites and plant cell wall polymers to form the 'bound residues' described above.

One of the goals of phytoremediation with respect to these enzymes is to enhance the capacity of plant cell walls to lock pollutants into bound residues, however, experiments to manipulate peroxidase levels in plants to a desired end have so far been met with limited success. The anionic peroxidase in tobacco, for example, is proposed to have a primary role in tolerance to pollution; however, it is also active towards the plant growth hormone IAA and when over expressed in transgenic plants, causes wilting and restricted growth (Lagrimini 1996).

Plant roots exude high concentrations of peroxidases and laccases into the soil, particularly in response to water stress and plant die-back (Gramss et al. 1999), and in response to chemical stress (Hirata et al. 2000). These enzymes may play an important role in polymerising reactions leading to pollutant immobilisation in soil humic matter (Zheng and Shetty 2000) and in waste- and ground-waters, rendering pollutants biologically inaccessible. Laccase uses oxygen as the electron acceptor, and possesses a highly specific binding pocket for oxygen, but the binding pocket for reducing substrates appears to be shallow and relatively non-stereospecific, which is particularly advantageous in the context of bioremediation. The governing feature of whether a compound will or will not be oxidised seems to be dictated by the redox potential differences between the reducing substrate and type I copper in the active site of the protein. This property endows laccase with the ability to oxidise a broad range of substrates provided their redox potentials are not too high (0.81 V /Saturated Calomel Electrode (SCE), Kersten et al. 1990, Xu 1996). Dec and Bollag (1990, 1994) studied the use of differently substituted phenols as substrates of laccase. The efficiency of oxidation decreased with increasing molecular weight of the

substituent and its changing position on the aromatic ring, in the order, 4>3>2. With chlorinated phenols, dehalogenation occurred as a consequence of oxidative coupling. Moreover, some pollutants that were relatively inert to enzymatic action were transformed in the presence of more reactive molecules easily oxidised by laccase. These observations have paved the way for devising bioremediation strategies in which xenobiotic substrates are incorporated into humic material and in which phenols and aromatic compounds are transformed into water-insoluble polymers.

Peroxidases in contrast to laccases, use H_2O_2 as the oxidant and the heme is the site of oxidation of the protein by H_2O_2, which becomes reduced to water following electron transfer from reducing substrates to the heme (for a review, see Dunford 1999). Peroxidases, particularly that from white-rot fungi (lignin peroxidase (LIP)) have been intensively investigated with a view to their application in bioremediation strategies. Interestingly, plant peroxidases share a striking level of structural similarity with fungal peroxidases. Iron co-ordination and most of the residues in the active site are completely conserved. The reason for their different substrate ranges (plant peroxidases make lignin; fungal peroxidases degrade it) is partly due to differences in substrate binding and accessibility to the heme. Plant peroxidases have an exposed heme edge but the heme edge in LIP is inaccessible. The entrance channel of plant peroxidases is more positive in charge. In LIP a hydroxylated Trp 171 residue may be required for electron transfer from the substrate (veratryl alcohol) to the heme, which is absent in plant peroxidases; and plant peroxidases but not LIP, possesses a ring of three peripheral Phe residues that may be important in their ability to bind aromatic substrates. The difference in their substrate range is also partly due to the strength of the axial ligand to the heme iron made by a histidine residue in the proximal cavity. In fungal peroxidases, the iron-Nε2 bond may be longer and weaker than in plant peroxidases, so that the heme becomes more electron deficient after reaction with H_2O_2 and the oxidised states of the iron are more destabilised (see Banci 1997, Smith and Veicht 1998, Dunford 1999).

The implication of these properties is that LIP will oxidise compounds with half-wave potentials ($E_{1/2}$) up to 1.49 V vs SCE (Kersten et al. 1990), considerably in excess of either laccase or plant peroxidase. Plant peroxidases can only oxidise aromatic compounds with half-wave potentials ($E_{1/2}$) up to 1.12 V vs SCE, limiting their prospects in bioremediation compared to LIP. Second, the rates of oxidation of reductants may vary considerably between different peroxidases because of differences in substrate binding properties. In this context again, LIP is of considerable interest for bioremediation purposes. The direct oxidation by LIP of many pollutants including phenolics is highly inactivating, however, with small dimethoxylated aromatic compounds such as veratryl alcohol inactivation is mitigated and the products of oxidation, radical cations, are able to act as non-specific one-electron oxidants or redox mediators (Harvey et al. 1986, 1987, Candeias and Harvey 1995). With redox mediators, the catalytic capacity of LIP can be vastly expanded to include the oxidation of large polymeric aromatic compounds such as lignin and the higher molecular weight PAHs.

3.3.3 Glucosyltransferases

Reactive groups of xenobiotics with hydroxyl or amino moieties, apart from being subject to oxidation reactions catalyse by peroxidase or laccases, may also be glycoslyated. The same is true for xenobiotics with sulphydryl moieties. Thus, the parent aglyca become more hydrophilic, usually detoxified and amenable for further modification or cellular compartmentation. UDP-glucose-dependent glucosyltransferases are the major enzymes catalysing these reactions. Several enzymes have been studied that are active towards phenols or anilines. A PCP-conjugating glucosyltransferase was identified in both soybean and wheat cell cultures. The purified soybean isoenzyme exhibited only little activity towards a few potential endogenous phenolic substrates tested (Schmitt et al. 1985, Sandermann et al. 1991). Glucosyltransferase activities conjugating the amino group of 3,4-dichloroaniline were found in both cell cultures as well (Gallandt and Balke 1995, Schmidt et al. 1995). Other activities towards xenobiotic phenols and phenolic hydroxyl groups as part of larger pesticide molecules have been described and purified. Although the majority of these glucosyltransferases is soluble in the cytosol, a membrane-bound activity has been identified (Leah et al. 1992, Gallandt and Balke 1995).

Taxonomic analyses of more than 60 lower and higher plant species revealed both a wide-spread and highly variable portfolio of glucosyltransferases acting on xenobiotics, in particular phenols and anilines (Tabata et al. 1988, Pflugmacher and Sandermann 1998b). Gallandt and Balke (1995) pointed to cultivar specific differences of soybean xenobiotic glucosyltransferase activities. However, it has to be noted that apart from detoxification, plant secondary product glycosyltransferases are involved in conjugating a plethora of small endogenous organic molecules including signalling molecules, biosynthetic precursors, or defence compounds. Thus, they are involved in many different endogenous pathways and regulatory processes.

Fig. 3 shows glucosyltransferase activities towards several potential endogenous and xenobiotic substrates that can be detected in partially purified enzyme preparations. 2,4,5-trichlorophenol, PCP, 4-nitrophenol and 3,4-dichloroaniline can be efficiently glucosylated by the glucosyltransferase isoenzyme mixture present in tobacco leaves. Interestingly, the specific activities towards these xenobiotics may exceed those of potential endogenous substrates like the cinnamic acid derivatives coniferyl alcohol, ferulic acid, *p*-coumaric acid or signalling molecules such as salicylic acid and the phytoalexin scopoletin. In addition, a 2,4,5-trichlorophenol glucosyltransferase activity was found to be slightly induced after safener or elicitor treatment in spruce and soybean cell cultures (Messner et al. 1996).

The molecular structure of a few of these plant secondary product glucosyltransferases has pinpointed an amino acid signature motif that is present in all members (Vogt and Jones 2000). Based on this motif a surprisingly high number of more than 110 members have been identified in the *Arabidopsis thaliana* genome (The Arabidopsis Genome Initiative 2000). Obviously, an enormous complexity and potential

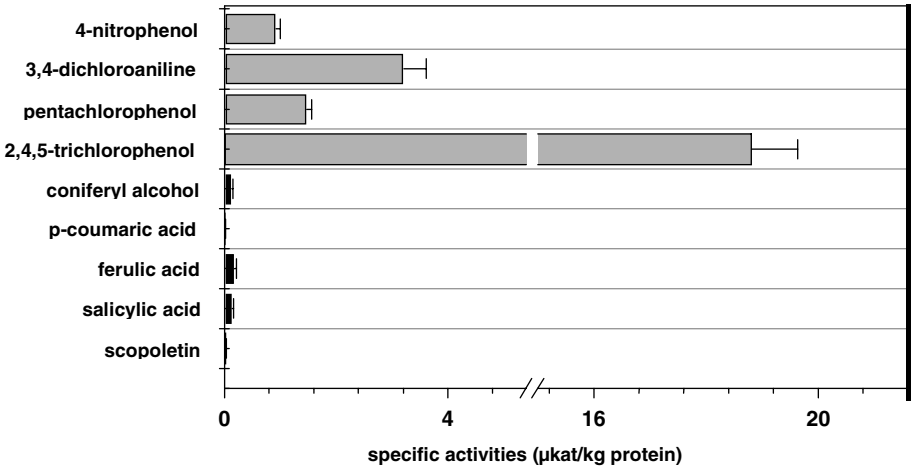

Fig. 3: Glucosyltransferase activity on endogenous (black) substrates vs. xenobiotic (grey) phenols and 3,4-dichloroaniline. A crude enzyme preparation from tobacco leaves (Samsun NN) was obtained (Sandermann et al. 1991, Pflugmacher et al. 1999). The desalted solution of the 40–80% ammonium sulfate pellet was used for enzyme assays. The O- and N-glucosyltransferase activities were assayed according to Sandermann et al. (1991)

redundancy is underlying this widespread conjugation reaction. En lieu of these huge numbers, there is only limited knowledge on the properties of individual enzymes. However, recent evidence using recombinantly expressed glucosyltransferases suggests that individual enzymes may have broad substrate specificity and exert only regioselectivity (Vogt and Jones 2000, Li et al. 2001, and references therein). In addition, experiments using glucosyltransferases expressed in *E. coli* indicate that these enzymes may possess dual functions and act on xenobiotics such as chlorophenols and anilines in addition to their potential endogenous substrates. This property may endow plants with a high flexibility and efficacy in detoxifying these compounds (B. Messner, H. Sandermann and ARS, unpublished).

3.4 Plant Stress Responses

Plant metabolic responses to xenobiotic uptake involve increases in the catalytic capacity of a range of enzymes that either directly or indirectly require reducing equivalents (NAD(P)H) for their functionality. P450s for example, require NADPH to activate molecular oxygen, and there is some evidence that H_2O_2 may be synthesised at the expense of NAD(P)H (Elstner and Heupel 1976). There is also evidence of marked changes towards a more oxidised status. Glutathione, for example, is a 'redox buffer' that maintains the sulphydryl groups of proteins in a reduced state and removes toxic peroxides formed as a consequence of normal metabolism under aerobic conditions. Its content is decreased and pronounced alterations in glutathione reductase, glutathione S-transferase and peroxidase activities have been identified in the floating aqueous plant, *Eichhornia crassipes*, under the influence of PCP in the water (Roy and Häninen 1992). In addition, some of these pollutants, PCP for example, are known to be inhibitors of oxidative phosphorylation. The drain on reducing equivalents, either directly or indirectly, will reduce the cellular capacity to reduce dioxygen to water, causing the concentration of intracellular O_2 and consequently reactive oxygen species (superoxide anion; H_2O_2) to rise and an unstable hyperoxidant state to develop. Provided reducing power can be made available to bring about a reduction of the O_2, for example, by photosynthesis, and restore the ratio of O_2: reducing equivalents, the cell may revert back to a stable state (see Hansberg and Aguirre 1990). If the cell cannot compensate the hyperoxidant state by increasing O_2 reduction it will die. Lignin formation and deposition in the cell wall can be seen as an attempt to reduce the concentration of reactive oxygen species (H_2O_2), however, cells that are heavily lignified, are nonetheless dead cells. The metabolic pathways for plant survival under conditions of oxidant stress caused by the need to detoxify xenobiotics are not well elaborated. However, increasing the ratio of O_2: reducing equivalents in fungal cells has been shown to cause gross disorganisation of cellular ultrastructure apart from at the level of mitochondria and marked increases in the activity of enzymes able to reduce H_2O_2 to water (Zacchi et al. 2000). Similar changes may also take place in those plants that appear to withstand xenobiotic phytotoxicity by increasing the quantity of 'bound' residues.

4 Plant and Microbial Interactions in Phytoremediation/ Bioremediation

4.1 Microbial degradation of pollutants

In contrast to plants, many organic pollutants are degraded and mineralised by microorganisms as a source of energy and carbon skeletons for cell protein synthesis. Microorganisms are the major agents mineralising organic pollutants in terrestrial and aquatic environments (for reviews, see Fewson 1981, Reineke and Knackmuss 1988, Hardman 1991, Commandeur and Parsons 1990, Haggblom 1992, Fetznar and Lingens 1994, Alexander 1994, Heider and Fuchs 1997, Meharg and Cairney 2000, Gadd 2001).

Micro-organisms readily mineralise phenols and anilines to CO_2 and, under aerobic conditions, ring-fission substrates are produced by the reactions of intracellular mono-or dioxygenases, which are then ring-opened by substrate-specific dioxygenases to produce products able to enter the TCA cycle. In the case of halogenated phenols, bacteria capable of dechlorination remove chlorine substituents from aromatic rings using intracellular dehalogenases. 'White-rot' basidi-

omycetous fungi, by contrast, catalyse dechlorination in the extracellular medium by ligninolytic peroxidases such as LIP (see above, and Lin et al. 1990, Valli and Gold 1991, Joshi and Gold 1993) or laccases (Roy-Arcand and Archibald 1991). Dechlorination products are mainly further oxidised and transformed to non-extractable soil-bound residues integrated into humic constituents (Lamar and Dietrich 1990, Lamar et al. 1990) or intracellularly detoxified and stabilised by being attached to glutathione with an S-glutathione transferase and then reductively dechlorinated with a glutathione S-conjugate reductase (Reddy and Gold 1999).

PAH biodegradation, as with plants, depends on the ability to introduce oxygen into the rings, which has the effect of both increasing PAH solubility and chemical reactivity (Sutherland 1992, Wilson and Jones 1993, Meulenberg et al. 1997). The two- and three-ringed structures are much more biodegradable than four, five and six-membered rings, whilst angularly-arranged ring structures (phenanthrene, benzo(α)-pyrene, pyrene) tend to be more stable than linear arrangements (anthracene, benz(α)anthracene).

Mineralisation of PAHs by bacteria is largely restricted to those structures able to penetrate the cell wall. Dioxygenases typically catalyse the introduction of two oxygen atoms into the substrate to form dioxethanes, which are then further oxidised to dihydroxy products (Butler and Mason 1997). Catechol, protocatechuic acid and gentisic acid are the usual dihydroxy products and are in turn, ring-opened to succinic, fumaric, pyruvic and acetic acids, all of which are used for energy and cell protein synthesis. Alternatively, cytochrome P450-type monooxygenases may be employed to catalyse ring epoxidation as the first step in a pathway leading to PAH detoxification *via* the formation of various conjugates (Ferris et al. 1976). These are not generally degraded (see Cerniglia 1997).

White rot fungi, in contrast to bacteria, oxidise PAH's using the extracellular oxidative enzyme system based on LIP (see above; Bumpus et al. 1985, Haemmerli et al. 1986, Hammel et al. 1986, Bogan and Lamar 1995, Bogan et al. 1996, Collins et al. 1996). The LIP-veratryl alcohol enzyme system is associated with a broad range of reactions in polymeric lignin including its depolymerisation, demethoxylation, decarboxylation, and hydroxylation as well as aromatic ring opening. Further, many of the reactions result in oxygen activation, creating oxygen radicals that perpetuate the oxidative attack (Schoemaker et al. 1985). These properties have sparked considerable interest in the potential of white-rot fungi to oxidise the higher molecular weight, large polyaromatic structures that are not degraded by bacteria. Oxidation of PAHs will increase their water-solubility and mobilisation in response to the plant transpiration stream. It will also increase the bioavailability of PAHs for degradation and mineralisation by bacteria and fungi in plant rhizospheric communities (Meulenberg et al. 1997).

Apart from white-rot fungi, there is also a broad range of ectomycorrhizal and other soil-borne fungi that have been shown to degrade lignin, humic and phenolic compounds as well as organic pollutants including PCBs and PAHs of environmental importance (Cerniglia 1997, Haselwandter et al. 1990, Gramss et al. 1999, Meharg and Cairney 2000).

Land farming techniques to encourage microbial degradative activities are increasingly being used for the treatment of former industrial land contaminated with hydrocarbon compounds as well as oil spillages (Bartha 1986, Christofi et al. 1998). Common practice involves diagnosing the problems specific to the site and then adjusting the soil conditions to accelerate natural microbial processes. A seed inoculum may be added and the polluted soil is then 'farmed'. Farming entails irrigating, tilling and adding fertilisers to the material to encourage growth of bacteria and fungi. However a number of practical problems remain. The major limiting factors include:

- A slow rate of mass transfer of the organic pollutants from the soil particles to the liquid phase to make contact with metabolically active organisms (see Field et al. 1995, Boyle et al. 1998, Head 1998, Novotny et al. 1999).
- A requirement for improved oxygen mass transfer. Organic aromatic pollutants that do not have oxygen in their molecular structure can only be degraded under aerobic conditions. Many of the enzymes involved have an absolute requirement for molecular oxygen.
- Catabolite repression may operate and needs to be overcome.
- The need to carefully control environmental conditions (nutrients, moisture levels, pH balance) for microbial activity

4.2 Biodegradation in the plant rhizosphere

Plant-based technologies may make a significant contribution to overcoming some of the limitations encountered in microbial land farming techniques. For example, degradation of phenanthrene was faster in rhizosphere soils planted with slender oat (*Avena barbata* Pott ex Link) compared with unplanted bulk soil controls and correlated with an increased number of degraders in the rhizosphere soil (Miya and Firestone 2000). The same has been reported for the degradation of several organic chemicals, including phenanthrene, in the rhizosphere of alfalfa (*Medicago sativa* L.) and alpine bluegrass (*Poa alpina* L.), suggesting the potential stimulation of bioremediation around plant roots (Nichols et al. 1997). Similarly, alfalfa growing in sandy soil promoted the bioremediation of phenol, toluene and TCE (Erickson et al. 1995) and mineralization of *p*-nitrophenol was enhanced in the rhizosphere of rice (Reddy and Sethunathan 1994). Accelerated degradation of pentachorophenol in soil is reported by using wheatgrass (*Agropyron desertorum* (Fischer ex Link) Schultes (Ferro et al. 1994). Microbial concentrations of populations important to hazardous waste bioremediation, including pseudomonads, have been found in higher abundance in the rhizosphere of poplar trees than in adjacent agricultural soils (Jordahl et al. 1997).

The importance of the plant transpiration stream in mobilising organic pollutants should not be underestimated. Even highly hydrophobic compounds such as pyrene and phenanthrene seem to be transported from areas outside the root zone towards the root *via* the plant transpiration stream (Liste and Alexander 2000a). In a recent study using pyrene, Liste and Alexander (2000b) found that in vegetated soil tested with nine different plant species, as much as 74% of

the butanol-extractable (more bioavailable) fraction of pyrene disappeared over a period of 8 weeks, compared to only 40% in non-vegetated soil. They attributed these effects to plant-stimulated microbial degradation but also suggested a role for plants in their ability to mobilise and accumulate considerable amounts of relatively hydrophobic pollutants in the rhizosphere soil from the surrounding soil via the transpiration stream. Schwab and Banks (1994) also reported on the enhanced rate of degradation of pyrene by planted (Tall fescue, Sudan grass, Switch grass and alfalfa) compared to unplanted soils. They noted that adapted microorganisms were not necessary for the increased rate of degradation that they observed in planted soils.

The ecological interactions between plants and indigenous bacteria are not completely understood. In wetlands, which have a tendency towards anaerobicity, Lin and Mendelssohn (1998) found that oil degradation rates were significantly enhanced by vegetative transplants together with fertiliser and proposed that the transplants provided more favourable microbial microhabitats through improved oxygen transport and nutrition (see also Gunther et al. 1996, Reilley et al. 1996). Chaineau et al. (2000) recorded enhanced degradation rates of saturated and aromatic hydrocarbons when maize (*Zea mays* L.) growth was exponential. These data infer a significant contribution of plant growth processes to environmental conditions in the rhizosphere, either by increasing hydrocarbon bioavailability; by producing bacterial growth stimulants in root exudates or by improving the physical properties of the soil.

A major carbon input into the rhizosphere comes from mucigel production and cell death, which is weakly correlated with plant species. Stimulants, chemotaxic compounds or siderophores may also be obtained from root exudates, which improve the growth and diversity of microorganisms around roots (Hedge and Fletcher 1996). The range of active components present in root exudates is not known. However, adding purified plant compounds to the soil can artificially simulate some of these effects. Carvone, for example, has been shown to induce the biodegradation of PCBs by a bacterial strain (Gilbert and Crowley 1997), whilst catechin and coumarin may act as co-metabolites (Salt et al. 1998). Plant root exudates also contain analogues of xenobiotics, which may reduce the lag time needed by microorganisms to adapt their metabolic pathways for pollutant degradation (Donally et al. 1994). Hedge and Fletcher (1996) quantified the release of phenolics by red mulberry (*Morus rubra* L.) roots at different growth stages within a season, and reported a continuous increase of total phenols into the soil from an early vegetative stage to leaf senescence, at which stage there was a massive release of less polar phenolics. The phenolics released to the rhizosphere may create a favourable environment for the biodegradation of the pollutants.

Several enzymes have also been found in root exudates, particularly peroxidases, laccases and dehalogenases that may contribute to the overall pattern of degradation observed in the rhizosphere (Gramss et al. 1999).

4.3 Influence of bacterial seed inoculates on plant growth

Competition for nutrients between roots and microorganisms can have an inhibitory effect on root growth. However, when they are specifically introduced as seed inocula for their potential to degrade organic pollutant, bacteria may confer beneficial effects upon plant growth and in turn enhance the potential for phytoremediation. Bokern et al. (1998) for example, showed that root growth was markedly reduced in the presence of specifically inoculated microorganisms, either a typical soil bacterium *Pseudomonas fluorescens* or an inoculum prepared from soil preincubated for 4 weeks with 4-NP. This was attributed to the rapid growth of the inoculated microorganisms and a resulting competition for nutrients between roots and microorganisms. However, addition of *Bacillus benzoevorans,* a bacterium able to degrade phenolics (i.e. to use 4-NP as carbon source), enhanced the growth of the roots in presence of 4-NP. Moreover, in experiments with ^{14}C-4-NP at a non-toxic level of 1 ppm (= 4.5 x 10^{-3} mM), root cultures treated with soil microorganisms showed an increased root growth. This was explained by the high amount of 4-NP associated with microbial activities (>30% of the added 4-NP) and the resulting lower uptake of 4-NP into the plant root. Hofflich et al. 1995 have also reported that bacterial seed inoculates increase root biomass, and length or exudate production in plants. Bacterial seed inoculants also selectively enhance the potential of the rhizospheric community to degrade certain compounds without affecting heterotrophic bacterial communities (Siciliano and Germida 1999). The microbiota associated with plant roots have been utilised as a good source of haloaromatic degrading microorganisms, such as chlorophenol and nitrophenols (Caldeira et al. 1999).

5 Practical Phytoremediation

The use of phytoremediation for the treatment of contaminated soil and effluents has been increasing in recent years. In choosing a remediation treatment, one needs to take into account

- Time scale
- Practical experience with the method and the guarantees, i.e. a performance specification that must be met
- Environmental impact of the treatment
- Relevant legislation
- Any waste streams resulting from the treatment and methods for their disposal
- Cost associated with the implementation and use of technology.

There is also a need for a clear definition of the concentration of the pollutant at the start of the treatment. For example, contaminants can be widely dispersed in the polluted material. Farming may mix the dispersed contaminants into the material and decrease the concentration of the pollutant at the start of the treatment by producing a more homogeneous mix, whilst tilling may throw the contaminated material into the air, providing the possibility that the more volatile materials are blown away.

5.1 Soils

A number of important challenges need to be considered when using plants to remediate polluted sites. Often industrial sites offer very poor habitats for plant growth, because of their poor nutritional status and soil structure and are rarely contaminated with only a single pollutant.

As a first step it is necessary to establish the correct soil conditions for successful plant growth.

The concerns are:

- Soil texture
- Soil structure
- Plant nutrients –microbial breakdown of organic matter will not be established in a new soil system and inorganic fertilisers/animal manure/legumes may be needed
- Adequate depth of clean soil for root growth may be needed.

For rhizosphere remediation to be optimised, the surface area of root-soil contact must be considered, as this is a crucial factor in the effectiveness and speed of bioremediation. Mycorrhizosphere-bacteria and -fungi have a crucial role in the establishment of the plants in degraded ecosystems. Of particular note are the activities of arbuscular mycorrhizal fungi (AMF) in stabilising plant community structure via balanced nutrient uptake and allocation in plants (for a review, see Harrison 1999). AMF colonise the roots of species in most plant families across the globe, and recent research has highlighted their ability to tolerate polluted soil conditions (Smith and Read 1997). Ectomycorrhizal fungi (ECM) have also been shown to degrade major classes of environmentally important pollutants and these fungi, similar to AMF, may indirectly influence degradation of pollutants in the rhizosphere via the 'mycorrhizosphere effect' of their mycelial systems in soil, which influence the structure and activities of soil microbial assemblages (for a review, see Meharg and Cairney 2000).

Plants will also be useful to reduce soil erosion and pollutant migration in soil or water through the production of humic materials. Phillips et al. (2000) conducted a study on bioremediation of soil contaminated with PAHs, petroleum hydrocarbons and chlorophenols, using different field box treatments involving either tillage and irrigation; amendment with nitrogen and phosphorous; or organic amendment with agricultural crop residues. Chemical analysis showed that both amendment strategies were equally effective, however, soil toxicity was mostly reduced with the organic crop residues amendment.

Ortega-Calvo and Saiz-Jimenez (1998) studied the effect of soil humic fractions, such as humic acid and clay, on the mineralisation of phenanthrene by a *P. fluorescens* strain. Additions of humic fractions and clay stimulated higher rates of mineralisation of phenanthrene, possibly by increasing the phenanthrene bioavailability by promoting the sorption of *P. fluorescens* to the humic fractions and clay.

5.2 Constructed wetlands

Constructed wetlands have been used for the removal of aromatics from water and wastewater. An artificial wastewater containing phenanthrene has been treated using an horizontal-vertical flow macrophyte-based system, planted with *Typha* spp. and *Scirpus lacustris*, achieving 99.9% overall removal (Machate et al. 1997); phenanthrene-degrading bacteria and corresponding metabolites were detected in the tanks. Aquatic plants, *Juncus fontanesii* (Gay) and *Lemna minor* L., have been used to remove phenol at concentrations varying from 8–48 mg/L, with reported efficiencies higher than microorganisms in activated sludge. However, in the case of *J. fontanesii*, an accumulation phenomenon was put forward as a possible mechanism (Oueslati et al. 1998). *Phragmites australis* Trin (common reed) has been used for constructed wetlands designed to treat effluents containing nitrophenols (Dias 1998) and waste landfill leachates (Trautmann et al. 1989, Maehlum 1995).

6 Conclusions

The vision for phytoremediation is to

- contain the further movement of organic pollutants by sequestration and accumulation
- degrade them to non-toxic by-products of plant metabolism
- support the degradative activities of microbial communities within the rhizosphere through the extracellular release of root exudates, oxygen, surfactants and enzymes
- render organic pollutants harmless to food chains by reducing their bioavailability in the extracellular environment.

Although there is good evidence that phenols, anilines or PAHs are taken up into plants, via the transpiration stream or by penetrating leaves, there is very little evidence that plants mineralise them. The more hydrophobic compounds are hydroxylated and translocated along with the more hydrophilic pollutants to other plant tissues and either volatilised, or excreted into the extracellular cell wall or into the vacuole as glucosyl or glutathione conjugates. However, there is only scarce knowledge about the factors that govern whether they will be deposited in cell walls or the vacuole. Furthermore, whilst available evidence indicates that enzymes such as P450s, peroxidases and glucosyl transferases are abundant, knowledge of their roles and metabolic capacities in pollutant detoxification processes are largely speculative based on information derived from the isolated proteins.

Research suggests that the cell wall may be one of the most important 'detoxification' sites in plant cells, since pollutants accumulate here in large amounts and their concentration increases with higher doses. Cell wall 'bound' residues are found in those species that are most tolerant to organic pollutants, but they are also characteristic of plant families. The penalty of using the cell wall as a reservoir for pollutant deposition lies, however, in an increased extent of lignification and consequently an accelerated rate of plant cell death. Allied problems are the need to harvest these plant

reservoirs of pollutant before they are returned back to the environment, and the need to prevent their movement into wildlife food chains. If pollutant-accumulating plants can be harvested, then composting techniques based on the use of white-rot fungi to mineralize these plant cell wall reservoirs of pollutant may be an attractive adjunct to a phytoremediation approach.

The capacity of plants to draw pollutants from surrounding areas via the transpiration stream into the rhizosphere where microorganisms are supported in their degradative activities by either physical or chemical factors associated with the roots is notable. Here the beneficial effects of arbuscular and ectomycorrhizal fungi in extending the rhizospheric network within soils are noteworthy. Although the degradative capacity of most microorganisms is limited to lower molecular weight pollutants, this is not the situation for white-rot fungi, which possess an extracellular oxidative enzyme system capable of degrading high molecular weight polymeric compounds and facilitating their ultimate mineralisation.

The increasingly urgent need for economic, environmentally friendly and effective remediating strategies is likely to drive forward new ways of integrating plant and microbial-based methods. In this regard, screening techniques based on *in-vitro* systems such as cell suspension and hairy root cultures seem highly promising. They lend themselves to rhizospheric studies at the same time as provide information on the phytotoxicity, metabolism and formation of bound residues of a xenobiotic compound within one experiment and in an appropriate time-scale. Furthermore, the data thus far correlate well with studies using intact plants. Basic studies of this nature are essential to make phytoremediation an effective and economically competitive technology.

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