SESSION 4D

BIOREMEDIATION OF ORGANIC AND INORGANIC CONTAMINANTS

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Soil bioremediation: bioavailability, biofilms and complexity

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INTRODUCTION

Soil microorganisms carry out many of their transformations within close-knit communities that are composed of a number of different interacting species. The collections of bacteria, fungi and other microbes are organised in the sense that there are relationships between the different species that facilitates their collective response to a variety of different and changing energy sources and impacts. These mutualistic interactions go some way to ensuring the robustness, resilience and, ultimately, continuity of the microbial community.

For much of their existence, microorganisms are located on or near soil and root surfaces rather than in freely diffusing pore water. This is a consequence of mechanisms which ensure that microbes recognise and are attracted to surfaces and, once there, resist detachment. A sessile existence is an advantage because many organic and inorganic ions and molecules are sorbed to soil surfaces creating microenvironment concentrations of energy sources and nutrients in excess of those prevailing in the aqueous phase. Thus, microbes at surfaces usually survive longer, transform substrates faster and grow more rapidly than those that are planktonic. On this basis, it is evident that the fundamental units of soil microbial activity have physical, chemical and biological dimensions and gradients measured in nanometres and micrometres. Defining the microbial processes within and between these microenvironments presents great challenges to researchers yet is fundamental to our understanding and manipulation of soil microbial activities.

Attempts to stimulate soil processes to provide economic and environmental benefit are a multitude but this article concentrates on ways to increase microbial activities in order to accelerate the degradation of potentially harmful organic pollutants. This is the topic of bioremediation. Contaminated soils account for tens of thousand of hectares of land in Europe and possibly at least one hundred times this amount throughout the world. Pollution not only reduces the value of land for agriculture and development but may also be a source of ground water contamination. This, in turn, becomes a problem for the water industry as it attempts to purify abstracted water to the high standards demanded by the health authorities, regulatory agencies and the consumer.

Some of the 'brownfield' sites are a legacy from our industrial and agricultural past when neither the agrochemical industry nor the farmer was aware of the dangers of pesticide contamination. This means that contaminated land at, for example, a herbicide manufacturing site, may contain a mixture of organic pollutants that have accumulated over decades. On the other hand, on the farm or in the orchard, comparatively low levels of pollution may arise through accidental spills, multiple applications, or simply by not following the manufacturer's instructions. In either case, a rational approach to bioremediation must be based on a detailed knowledge of such factors as: pollutant chemistry, concentration and distribution; soil physicochemical properties; the microbial composition and catabolic capabilities of the

polluted soil; and the complicated relationships between, on one hand, substrate sorption and desorption and, on the other, potential microbial response.

MICROBES AT SURFACES

When microbes are deposited at comparatively nutrient rich surfaces they proliferate and generate communities either partially or completely enclosed in a film of extracellular polysaccharides (EPS). At its most structured, the biofilm will contain a large number of interacting microbial species. The processes leading to biofilm formation and the consequences of a life at the solid-liquid interface have been well documented (Burns, 1989; Costerton *et al.*, 1995; Jass *et al.*, 2002). We know that the 3-dimensional organisation (i.e. architecture) of the microorganisms is influenced by soil surface properties (e.g. roughness, electrochemistry, hydrophobicity), soil hydrodynamics (e.g. porosity, mass transfer, flow rates), concentrations and types of nutrients and energy sources (e.g. pesticides), and, of course, the composition of the indigenous microbial population.

The most intensively investigated biofilms are aquatic and the knowledge derived from their study has found application in wastewater and drinking water treatment (Peys *et al.*, 1997). However, current research includes experimental models of soil and its inorganic and organic components (Koch *et al.*, 2001), as well as the root/soil interface known as the rhizosphere (Shaw & Burns, 2003). The 3-dimensional structure or architecture of the biofilm may be related to the carbon source (Karthikeyan *et al.*, 1999). Furthermore, the actual EPS components have been shown to have a role in the uptake and accumulation of substrates (such as diclofop) within the degradative biofilm and that microbial distribution changed from non-uniform to uniform when a more labile carbon source (tryptone soya broth) was used (Wolfaardt *et al.*, 1998).

It is important to understand the three-dimensional microbial structures associated with the natural substrata to which pesticides are attracted. This is because the bioavailability of the incoming pollutant chemical to the biofilm community will be strongly influenced by surface topology and physicochemistry as well as the spatial arrangements and properties of the resident microorganisms.

BIOAVAILABILITY

The term bioavailability has a number of definitions but, in the context of this review, it means suitability for microbial mineralisation either directly by catabolism or via co-metabolism. In addition, organics may be partially modified to produce a recalcitrant metabolite. It is tempting to provide a general definition of bioavailability such as: "that proportion of the organic that is in the aqueous phase and capable of uptake and transformation by microorganisms". However, this is simplistic as it implies that the degradation of pesticides concentrated at soil surfaces is entirely defined by sorption kinetics and aqueous phase concentrations. This is not the case.

Bioavailability of 2,4-D and simazine

The degradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) when associated with the highly adsorptive clay mineral chlorite is a good example of the complex nature of bioavailability (McGhee *et al.*, 1999). Initially, 254μg of 2,4-D was adsorbed to each gram of clay. In the non-inoculated flasks, after shaking in sterile minimal salts medium for 28 days, 114μg/g (45%) of the adsorbed 2,4-D was desorbed and 140 μg/g (55%) remained adsorbed. This means that, according to the definition of bioavailability given above, the 114μg/g in the solution phase is the maximum available for degradation. However, in flasks inoculated at day zero with a 2,4-D degrading bacterium, 148μg/g (58%) of the original 2,4-D was mineralised. Furthermore, at the end of the 28-d experiment, 55μg/g of 2,4-D remained in solution (and probably would have been degraded had the experiment been prolonged) and only 51 μg/g remained strongly adsorbed. Thus, it appears that the inoculated *Pseudomonas* species 'accessed' and degraded 34 μg/g (148-114) of the strongly adsorbed 'non-bioavailable' 2,4-D and that this may have risen to at least 189μg/g or 75.6% of the original sorbed herbicide with further incubation. Clearly the simple definition of bioavailability does not satisfy the events described here.

A second example of how microbes utilise apparently non-available herbicides comes from studies of *s*-triazines adsorbed, not to clays, but to granular activated carbon (GAC) (Feakin *et al.*, 1995; Jones *et al.*, 1998). A minimal salts medium containing GAC saturated with strongly adsorbed simazine and simazine in solution was inoculated with the triazine degrader, *Rhodococcus rhodochrous*. After 21d, 96% of the soluble simazine had been degraded but, in addition, 28% of the strongly adsorbed simazine had been degraded. Once again, it appears that a microbial species is degrading a pesticide that might be considered as non-bioavailable.

Substrate accessibility

We are now faced with the question: how do microbes access adsorbed substrates? This is important not only with regard to predicting susceptibility to bioremediation but also in determining whether sorbed or complexed pesticides are inert and do not pose any environmental threat. Possible answers have been forwarded elsewhere (Burns & Stach, 2002) and are merely summarised here. Firstly, and most obviously, as the soluble substrate is metabolised, equilibrium is maintained by the desorption of the adsorbed pesticide. Secondly, during the mineralisation of a soluble pesticide, metabolites and protons are produced which reduce the interfacial pH. As a consequence, those adsorbants and adsorbates that depend on their ionogenic properties for association may undergo a change in the distribution, intensity or even sign of their exposed functional groups. Thus, what was a strong association due to oppositely charged interactants becomes one of repulsion. At this stage the pesticides pass into the solution phase. A third explanation for increased bioavailability is provided by our knowledge of microbial surfactants. Glycolipids, lipopeptides, lipoproteins, and heteropolysaccharides will all render hydrophobic organics associated with surfaces more available by creating emulsions.

Finally, and perhaps most interestingly, many microbes produce extracellular enzymes that form complexes with soil clays and humates (Burns, 1989; Gianfreda & Bollag, 2002). This means that soil particles carry a persistent enzymatic catalytic capacity capable of transforming arriving substrates. If one considers a condition where *both* substrate and enzyme are adsorbed, then their interaction becomes density dependent. In other words, a pesticide adsorbed to saturation on a clay surface is more likely to interact with immobilized enzymes than if spatially isolated. Another scenario, particularly relevant to chronic pollution, is one in

which the pesticide is already sorbed and the indigenous microbes and their enzymes are being stimulated or new ones added. The newly generated or directly applied enzymes may interact with all or part of the xenobiotic depending upon its spatial association with the surface. Pesticides can be associated with surfaces such that rings, substituents and/or side chains are accessible to the active site of the appropriate enzyme, even though the molecule is anchored. Under these circumstances, it is possible that parts of a xenobiotic are cleaved by diffusing extracellular or mural enzymes and enter the aqueous phase or even pass directly into the adjacent producer cell leaving only a 'footprint' of the parent compound behind. Recent developments in Fourier Transform Infrared Spectroscopy and Atomic Force Microscopy have provided the potential to visualise the orientation of a substrate at a surface and to gauge the likelihood of it binding with an enzyme and the strength of that enzyme-substrate complex (Quiquampoix *et al.*, 2002).

Ageing of pollutants and soil depth

Further changes in bioavailability may occur the longer the pesticide remains in the soil. This is because 'ageing' usually results in potential substrates become physically and chemically complexed on and within soil colloids. They are bound residues. The likely outcome of ageing is that pesticides (and their metabolites) become more recalcitrant with time (Smith *et al.*, 1999; Reid *et al.*, 2000). There are numerous examples of bound residues (e.g. Kopinke *et al.*, 2001) and the acylanilide herbicides provide a good example. Propanil is degraded rapidly by microbes and, as such, the parent compound can be described as non-persistent. However, one of its metabolites, 3,4-dichloroaniline, can condense abiotically or be converted by peroxidases to form the recalcitrant genotoxin tetrachloroazobenzene as well as being copolymerised or oxidatively coupled to humic compounds to form a recalcitrant complex. It would appear that these bound residues are persistent and presumably in states that are not biochemically-or physically-accessible to microbes or their enzymes. Nonetheless, the dynamic state of bioavailability, warns against us considering bound residues as inert. Especially significant in this context is the detection of pesticides associated with humic and fulvic acid fractions that have leached through soils and into groundwater (Elimelech & Ryan, 2002). Some aspects of the complex relationships between ageing and bioavailability have been reported in a series of papers by Alexander and his colleagues (e.g. Nam & Alexander 2001).

Another consideration when assessing pesticide bioavailability (and susceptibility to bioremediation) is that soil properties change with depth (Taylor *et al.*, 2002) and that this may have a significant impact on the rates of degradation (Di *et al.*, 2001) and transport (Zehe & Fluher, 2001). This is illustrated well by the example of 2,4-D. 2,4-D was degraded much more rapidly in surface soils than in subsurface soils (Shaw & Burns, 1998). This was certainly due to the comparatively low initial numbers of 2,4-D degraders in the sub-soil but changes in soil physico-chemical properties with depth and different sorption kinetics also influenced bioavailability and degradation rates. Incidentally, another consequence of depth is that, in soils to which herbicides have been applied over a period of years, the surface layers may contain high numbers of the parent compound degraders whilst the lower horizons (which have been enriched by the breakdown products rather than the parent compound) will have higher levels of metabolite degraders. For example, in the case of 2,4-D there could be an abundance of 2,4-DCP (2,4- dichlorophenol) degraders in the sub-soil in contrast to the surface soil.

In summary, the properties of soils and the xenobiotics that pass through them, have implications for the development of rational bioremediation strategies. Soil properties change with depth (a deep horizon may be composed of a different soil to a surface horizon) as do substrate concentrations and metabolites and their bioavailabilities. Although microbes in sub-surface horizons and deep soils are far more abundant than previously thought, species and process diversity may be very different to that detected and expressed at the surface. In other words, bioremediation (whether by biostimulation or bioaugmentation) may require or target different species and different processes and require different strategies at different depths within the same soil.

MICROBIAL AND MOLECULAR POTENTIAL FOR BIOREMEDIATION

Traditional methods

One of the first questions to ask when evaluating bioremediation as a possible solution to a contaminated site is - do the competent microbes already exist in the soil? If the soil has been contaminated for a long period it is probable that some of the resident species have the capacity to transform the pollutant. In these circumstances, it is likely that, in time a process of natural attenuation will degrade at least a proportion of the contaminants. However, it is the rate of this transformation and the mobility of the parent compounds and their breakdown products that is of significance in determining the need for intervention. The identification of competent microorganisms usually involves isolating bacteria on selective media (often after further directed and prolonged enrichment using the target pollutant). Many bacteria are difficult if not impossible to isolate using conventional media and growth conditions and most pollutants are mineralised in the soil, not by a single independent species, but by a community of microorganisms. In other words, a soil may contain all the species necessary to mineralise a particular pesticide but you would never isolate and identify them using traditional methods.

Molecular site assessment

In the last few years a more thorough analysis of a soil's microbial potential has been advocated. This approach depends on 'molecular site assessment' (Sayler *et al.*, 1995). Molecular site assessment (MSA) depends on the extraction and quantification of soil DNA and mRNA. The benefit of MSA is that biases and inaccuracies associated with the incomplete isolation of non-representative microorganisms are largely overcome (Wilson *et al.*, 1999).

Extracted nucleic acids will reveal the presence or absence of catabolic potential with regard to specific pesticides, thereby permitting rational decisions about bioaugmentation and biostimulation. There are now a large number of methods for the extraction and purification of total DNA from soils (e.g. Krsek & Wellington, 1999) and all rely on treatments such as bead-milling, freeze-thawing, lysis, or a combination of these. The true value of these techniques must be based on the quantity and purity of the isolated DNA as well as its composition with regard to the completeness of the microbial sequence diversity represented (Stach *et al.*, 2001).

Cloning and sequencing of the DNA extract can be used to optimise the extraction method for the detection of specific species and/or functional genes. Fingerprint analysis of DNA extracted from soil may be conducted using Terminal-Restriction Fragment Length

Polymorphism (T-RFLP) Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE). We have evaluated recently a Single-Strand Conformation Polymorphism (SSCP) approach for assessing sequence diversity and demonstrated that a greater quantity of extracted DNA is not necessarily synonymous with greater sequence representation (Stach *et al.*, 2001).

Whatever extraction method is used, a genetic fingerprint based on total DNA may overestimate the number and variety of active species in the soil. This is because genomic DNA is derived not only from living organisms but also from dead cells and tissues and, to a limited extent, DNA bound to soil components (Stotzky, 2000). Furthermore, DNA only represents the genotypic potential of the soil to produce active xenobiotic-degrading enzymes; this is not the same as expressed biodegradative activity. What really matters is the transcription of DNA to mRNA, the prelude to production of active proteins. Therefore, mRNA-based gene detection records the activity of genes at the moment of sampling and provides more accurate markers of pesticide metabolism (Wilson *et al.*, 1999). A number of techniques have been reported for the extraction of RNA from environmental samples (Kozdroj & van Elsas, 2000). Many of these co-extract DNA and RNA and, both the presence and the activity of species/genes can be monitored from a single nucleic acid extract. Using nucleic acids extracted directly from contaminated soils promises to allow predictions about bioremediation potential as well as the monitoring of microbial changes that take place during biostimulation and bioaugmentation in the field. Examples of MSA and its application to the degradation of a broad range of xenobiotics can be found in Sayler *et al.* (1995), Meckenstock *et al.* (1998) and Wilson *et al.* (1999).

The cost and speed of MSA will be important for its widespread adoption and successful application to a range of bioremediation problems. With this in mind we have bypassed the need for cloning and sequencing by: (i) developing methodologies that provided PCR/RT-PCR ready DNA/mRNA directly from a polycyclic aromatic hydrocarbon-contaminated soil in less than one hour; (ii) constructing a culture library of phylogenetically distinct species representing the known diversity of degrading isolates; (iii) using existing primers and novel primers to amplify all currently known (naphthalene dioxygenase) genes from both Gram-positive and Gram-negative bacteria; and (iv) using single-strand conformation polymorphism (SSCP) to detect and identify diverse genes recovered directly from soil.

Table 1 summarises the MSA approach to bioremediation, the various decision points, and a number of ways in which the accelerated degradation of target compounds may be monitored and achieved.

Table 1. Molecular site assessment – a new approach to rational soil bioremediation (adapted from Burns & Stach, 2002)

Does the soil contain indigenous microbes with the potential to transform or metabolise the xenobiotic(s)?
- if yes -
Can this potential be transcribed?
- if yes -
Is the transcribed protein active?
- if yes -
Stimulate full expression: O_2, H_2O, C, N, P, K, pH, etc Improve bioavailability: surfactants, chelators, pH modification, etc
- if no -
Inoculate with one or more microbial species Stimulate and improve as before
- in both cases -
Use microbial biosensors to report on bioavailability, distribution, concentrations and progress of remediation

Biostimulation

Adopting a biostimulation strategy assumes that the appropriate species are already in the soil (whether you can isolate them or not) and that they can be encouraged to reorganise themselves and form active degradative communities. Biostimulation is appealing because it can be an inexpensive and 'low technology' solution brought about by additions of nitrogen, potassium, phosphate and organic carbon, altering the pH with lime or sulphur, aeration by tillage, and irrigation (Burns *et al.*, 1996). More complicated microbial responses may be afforded by the use of pollutant analogues (Bengtsson & Zerehouni, 2003), inducers of catabolic enzymes (Dercova *et al.*, 2003), and the stimulation of the horizontal transfer of degradative genes within the indigenous population. Changes in community structure and function during bioremediation can be monitored using molecular techniques (Zucchi *et al.*, 2003). However, it is possible that changes to the indigenous population and its activities will have only a small effect because it is the physical and chemical association of the pollutant substrates with the soil components within the soil matrix that renders them non-bioavailable (see above). Under these circumstances, additional manipulations, including the addition of surfactants (Kosaric, 2001; Cameotra & Bollag, 2003) and organic acids (White *et al.*, 2003), may prove successful.

Bioaugmentation

If the pre-existing genotype of a soil is inadequate or cannot be exploited then remediation will depend on the introduction of specialised microbes. Microbial inocula will either contain the missing biodegradative pathways or carry catabolic plasmids that can serve as donor

strains. In the former case, the microbes will need to establish themselves as part of the indigenous community and express the desired degradative property. However, establishing 'foreign' microbes in soil environments has proved difficult because new additions must be compatible with the established highly selected microbial community as well as tolerant of the prevailing chemical, physical and climatic conditions (Young & Burns, 1993). Nonetheless, introduced strains do have at least one major advantage: they can grow on an underused resource (i.e the persistant pesticide). However, the distribution of the target pollutants is likely to be heterogenous and associated with surfaces and so the inoculant will not only have to survive and proliferate but also will need to display chemotaxis (Ortego-Calvo *et al.*, 2003), access its surface located substrate, and compete with established biofilm communities. Some of the desired properties of a successful bioremediation species or a community of microorganisms are presented in Table 2.

Table 2. Properties of an ideal bioremediation inoculant species or microbial community (adapted from Burns & Stach, 2002)

- can degrade a range of target xenobiotics at both high and low concentrations
- has soil, biofilm and rhizosphere 'competence' genes
- does not disrupt beneficial microbial processes
- is resistant to heavy metal pollutants, dehydration and predation
- displays chemotaxis – locates and moves towards pesticide 'hot spots'
- is able to access bound and aged residues
- increases bioavailability by producing surfactants
- functions as a donor in horizontal transfer of catabolic genes
- can be detected and tracked once released
- serves as a reporter of pesticide concentrations and location

We have developed a continuous flow cell method (Stach & Burns, 2002) which increases the likelihood of enriching biodegraders that are able to integrate with the indigenous community, establish themselves at soil surfaces, resist shear forces and predation, and access sorbed xenobiotics. It is apparent that biofilm communities enriched in this manner are not only far more diverse than those isolated using conventionally methods but are also capable of degrading the target xenobiotic more rapidly. For example, we identified 38 bacterial and 2 fungal species in a PAH-degrading biofilm community whereas batch enrichments generated only 12 bacterial isolates. Furthermore, the biofilm community degraded naphthalene four times faster than the batch community

Introducing donor strains that can conjugate with the indigenous population is an attractive idea because this means that the often problematic prolonged survival of the inoculant is not a prerequisite for successful degradation. The transfer of 2,4-D degrading plasmids and catabolic genes from inoculants to indigenous bacteria, and the consequent acceleration in 2,4-D breakdown, has been demonstrated (Dejonghe *et al.*, 2000).

The success of both types of inoculation would be increased by using genetically modified bacteria and fungi but there is considerable opposition to this from many quarters.

CONCLUSION

It is estimated that there are as much as 360,000 hectares of contaminated land in the UK. *In situ* bioremediation has the potential to be a cost-effective and environmentally 'friendly' alternative to chemical and physical *ex situ* processes. We are on the verge of major breakthroughs in our understanding of the fundamental processes involved in soil microbiology and the long and sometimes frustrating gestation period of bioremediation science is coming to an end.

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Soil microbial response during the phytoremediation of PAH contaminated soil

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ABSTRACT

Enhanced bioremediation in planted soils is possibly a function of increased microbial activity in the rhizosphere, but few studies have monitored rhizosphere microbiology during rhizoremediation. The aim of this trial was to quantify and compare the responses of soil microbial populations during the phytoremediation of polycyclic aromatic hydrocarbons (PAHs) in a laboratory trial. All soils were contained in 1-kg pots and planted treatments consisted of a mixed ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) sward together with a rhizobial inoculum (*Rhizobium leguminosarum* bv. *trifolii*). Throughout the 180-day experimental period soil microbial biomass, rhizobial populations and populations of PAH degraders were monitored. Results will be presented that show losses of PAHs in a planted soil and the responses of the associated soil microbial populations.

INTRODUCTION

Plants may enhance microbial degradation of polyaromatic hydrocarbons (PAHs) by providing specific micro-environments for pollutant-degrading commensal (such as pseudomonads) or symbiotic (e.g. mycorrhizae, rhizobia) microorganisms. Plants may support a microflora in the rhizosphere with much greater adaptability for growth on different carbon sources (including pollutants) than non-rhizosphere microflora (Siciliano *et al.*, 1998). Furthermore, certain legumes support symbiotic rhizobial species that fix atmospheric nitrogen and improve the nutrient status of a contaminated soil.

There is mounting evidence that microbial activity in the rhizosphere may increase the degradation of persistent industrial chemicals such as PAHs (Joner *et al.*, 2001; Johnson *et al.*, in press). A recent study by Johnson *et al.* (in press) demonstrated that planting a soil with a mixed clover/ryegrass sward, together with a rhizobial inoculum, enhanced the dissipation of chrysene in a controlled experiment. It was suggested that this may have been due to the stimulation of microbial populations within the soil. The aim of this study was to test the hypothesis that the observed enhanced dissipation of PAHs in the rhizosphere was due to the stimulation of the microbial community within the soil rhizosphere. With this in mind, several microbial techniques were employed to monitor the soil microbial populations during a rhizoremediation laboratory trial.

MATERIALS AND METHODS

Chrysene ($C_{18}H_{12}$) was the model PAH compound used throughout this trial. Chrysene is a medium sized PAH molecule with a molecular weight of 228 and Log K_{ow} of 5.86. It is relatively insoluble in water and tends to be persistent in the soil environment (Mackay *et al.*, 1993).

An inoculum of an isolate of *Rhizobium leguminosarum* bv. *trifolii,* selected for it's tolerance to PAHs, was produced using a peat carrier. The inoculum and seeds of the host legume (*Trifolium repens*) were planted into soils together with ryegrass (*Lolium perenne* L.). The soils had previously been spiked with chrysene (500 mg/kg) and then aged for 4 weeks to simulate the lower bioavailability of PAHs after long-term contamination. A multifactorial greenhouse experiment including unplanted treatments and non-sterile soil, as well as soil sterilized by gamma irradiation was set-up. PAHs in spiked soils were analysed by GC-MS. Shoot- and root-biomass of plants, and the amount of root nodulation, was determined. The inoculated rhizobia were counted using a MPN-procedure as described by Chaudri *et al.* (1993). In addition, both a spray plate technique (Juhasz *et al.* 1997) and a solvent free microplate technique (Steiber *et al.* 1994) were employed to assess the use of chrysene as a sole carbon source by *R. leguminosarum* bv. *trifolii*.

Biomass

The total soil microbial biomass was assessed using a standard fumigation-extraction technique (Vance *et al.* 1987). Soil microbial biomass was assessed in all treatments at T=0, 90 and 180 days.

PAH degraders

In addition to monitoring total soil microbial biomass, a solvent free microtitre plate method was applied to determine the specific populations of PAH degraders in the pots during remediation (Steiber *et al.* 1994). The method allows a Most Probable Number estimation of PAH degraders in the soil. Chrysene was used as the model PAH in the system.

RESULTS

In sterile soil, planted treatments showed slightly higher extractable concentrations of chrysene throughout the 180-day experimental period than the comparative unplanted treatments (data not shown), although these differences were not statistically significant ($P > 0.05$). However, in non sterile soils, after 180 days the extractable concentrations of chrysene were significantly lower ($P < 0.01$) in planted treatments containing a rhizobial inoculum than planted treatments without an inoculum and unplanted treatments (Figure 1). Surprisingly, chrysene concentrations were only significantly lower than the unplanted treatments in the planted treatments that had received a rhizobial inoculum. Planted treatments with no inoculum did not show a significant reduction in chrysene concentrations at the end of the 180-day trial relative to similar unplanted treatments.

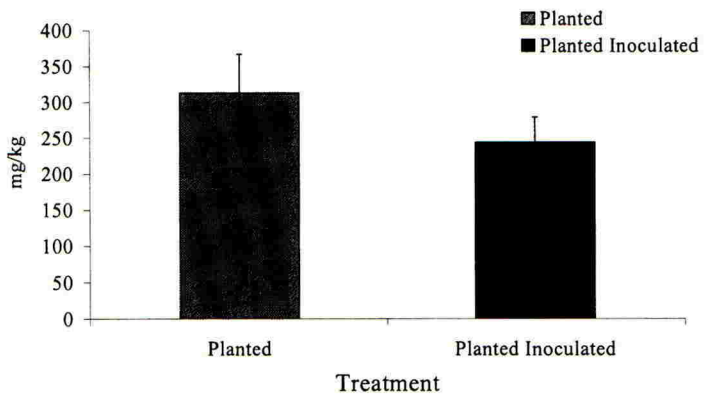

Figure 1. Total extractable chrysene concentrations at the end of the 180-day experimental period in non-sterile soils. Data are means and standard deviation.

At the end of the experimental period, the treatments that had received inocula contained significantly larger viable populations of rhizobia than those that had received no inocula. The higher rhizobial populations in the inoculated pots were reflected in the c lover nodulation. Inoculated pots had 2.2 clover nodules per g soil as opposed to 1.1 nodules per g soil in the planted treatment that had received no inoculum. Similarly both root and shoot growth were significantly greater in the inoculated pots.

In vitro degradation of PAHs by *R. leguminosarum* bv. *Trifolii*

A strain of *R. leguminosarum* bv. *trifolii* was successfully isolated from a PAH contaminated industrial soil. The spray plate technique to assess the degradation of chrysene by this strain of bacteria, revealed no zones of clearing around the rhizobial colonies. Similarly, the use of the solvent-free microtitre technique (Steiber *et al.*, 1994) also showed no colour change associated with the build up of PAH metabolites.

Soil pH and soil nitrogen status

Soil pH did not change significantly over the 180-day experimental period with a range of 6.5-7.5 in all treatments. Similarly, soil nitrogen levels (ammonium-N and nitrate-N) did not change significantly during the experiment and there were no significant differences between any of the treatments.

Soil microbial biomass

Figure 2 demonstrates that soil microbial biomass did not differ between treatments at the start of the experimental period. However, by the end of the experimental period there was a significantly greater biomass in the inoculated planted treatments than in the unplanted

treatments. No differences were observed between planted treatments and planted inoculated treatments (data not shown)

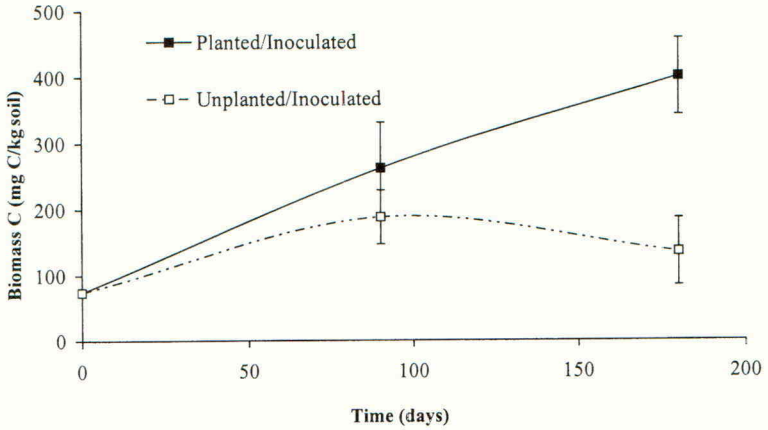

Figure 2. Changes in soil microbial biomass throughout 180-day rhizoremediation trial. Data are means and standard deviation.

PAH Degraders

The most probably number of PAH degraders was influenced by planting regime (Figure 3). Populations of microorganisms capable of degrading chrysene were greater in the planted treatments relative to the unplanted treatments. However, unlike total soil biomass, the planted treatment that had received a rhizobial inoculum had a greater number of PAH degraders than the planted treatments with no inoculum.

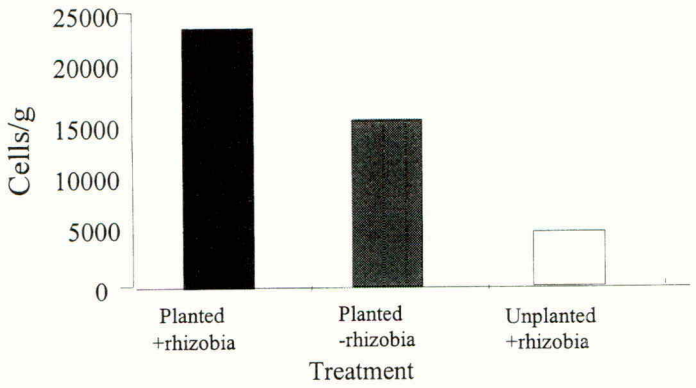

Figure 3. Most Probable Number of PAH degrading microbes at the end of the experimental period.

DISCUSSION

Both laboratory tests designed to measure the in-vitro degradation of chrysene suggest that although resistant to chrysene, the strain of *R. leguminosarum* used is apparently unable to degrade the compound as a sole carbon source. Planted sterile soils did not show significantly different concentrations of extractable chrysene with time. This suggests that the plants in this trial did not have a direct role in the remediation of chrysene. Soil borne hydrophobic organic compounds such as chrysene, with a relatively high log K_{ow}, are rarely translocated from root to shoot to a significant degree. Significantly lower concentrations of chrysene, observed in the planted treatment that had received a rhizobial inoculum (Figure 1), are therefore unlikely to be a result of direct uptake by either the clover or ryegrass or direct degradation by rhizobia.

The impact of the inoculated rhizobia is reflected in a larger shoot and, most importantly, root biomass in the inoculated pots. Microbial measurements reveal that the biomass is significantly greater in planted treatments than in unplanted treatments (Figure 2). However, there is no significant difference between the biomass of planted treatments that had received a rhizobial inoculum and those that had not (data not shown). In contrast, PAH degraders are positively influenced by the presence of a rhizobial inoculum (Figure 3). This suggests there is selective enhancement of PAH degraders within the rhizosphere which leads to enhanced chrysene loss.

Earlier studies (e.g. Siciliano *et al.*, 1998) have shown enhanced losses of PAHs in planted soils and suggested that the mechanism by which this occurs is via plant root exudates stimulating the microbial community involved in the dissipation of aromatic compounds. A recent study by Leigh *et al.* (2002) went one step further and demonstrated that seasonal fine root death releases several flavones which act as substrates for Polychlorinated biphenyl (PCB) degrading bacteria. Thus, upon death, fine roots may serve not only as injectors of bacterial substrates but also may facilitate soil aeration through the formation of air channels left after root senescence. It therefore follows that any improvement of root growth through the use of a growth stimulating rhizobial inoculum will lead to a stimulation of the growth and activity of bacteria capable of degrading chrysene.

In conclusion our results support the hypothesis that the enhanced dissipation of PAHs in the rhizosphere was due to the stimulation of the microbial community within the soil rhizosphere. However, this loss is only greater in soils that received a rhizobial inoculum. It is therefore likely that rhizobia play an important role in the rhizoremediation of high molecular weight PAHs. It would appear that microbes responsible for PAH degradation are selectively enhanced within the rhizosphere of soil that has received a rhizobial inoculum.

ACKNOWLEDGEMENTS

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The use of short rotation coppice in the bioremediation of municipal wastewater

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ABSTRACT

An EU research project designed to evaluate the use of short rotation willow coppice as a bioremediation system for municipal wastewater was set up in 1998. Three wastewater irrigation rates were compared with a mains water and non-irrigated controls and a sludge treatment. Analysis showed that significant nutrient export off the site occurred at harvest and that ground water was not generally adversely affected by the treatments

INTRODUCTION

Interest in the production of energy from short rotation willow coppice (SRC) in Northern Ireland began with the oil crises of the mid 1970s in an attempt to address escalating prices and ensure diversity of supply. Whilst both these issues are still relevant the significant drivers behind the development of alternative renewable energy supplies are environmental. In the United Kingdom context, energy from SRC is seen as a major contributor to the CO_2 abatement strategy, to achieve the 12.5% reduction in CO_2 emissions, based on 1990 levels required in the Kyoto protocol. This requirement has been reinforced in the government's white paper 'Our Energy Future' published in February 2003 (Anon, 2003) where the Royal Commission's recommendation of 60% reduction in CO_2 emissions by 2050 has been adopted as part of a low carbon economy initiative. In addition to these global objectives, local drivers are of equal importance in Northern Ireland. The predominant feature of N. I. agriculture is grass productions (85% of agricultural land producing 70% of output). Total farm incomes have shown a sustained downward trend over the past eight years with a marginal recovery in 2001, followed by a further drop in 2002. Currently, incomes stand at 50%, in real terms, of what they were in 1995. In these circumstances the provision of sustainable alternative systems is vital for the well being of the agricultural industry and the maintenance of viable rural communities. The production of energy from SRC offers a significant opportunity for agricultural diversification. However, on a purely energy basis the economics of the crop are marginal particularly in Northern Ireland. Here, because the development of the crop is at a very early stage, the pioneer producer incurs a cost penalty in comparison with areas where activity in the sector is significant, leading to economies of scale.

In this situation, to establish a viable industry which is sustainable in the long term, it would be valuable to establish a dual use for the SRC crop to provide added value. Consequently the use of SRC as a bioremediation system for wastewater was investigated, to evaluate its capacity to reduce the loading of eutrophying nutrients principally nitrogen and phosphorus

and oxygen demanding organic material (BOD) in the applied wastewater. The fate of metals e.g. copper, zinc, cadmium and lead was also of interest.

MATERIALS AND METHODS

The trial is situated adjacent to Culmore water treatment works, north of the City of Londonderry. The site is at sea level on the shores of Lough Foyle and the soil is a sandy loam with a high organic matter content. The water treatment works provides primary treatment only and the resulting effluent is pumped into the tidal reaches of the estuary. In May 1998 eighteen experimental plots (15 x 25 m) were planted with 20 cm cuttings of *Salix viminalis* 'Jorr' in double rows 0.75 m apart and with an in-row spacing of 0.65 m. The double rows were spaced at 1.25 m. This provided a planting diversity of approximately 15,000 per ha. The treatment plots were separated by buffer zones with a minimum width of 20 m. These buffer zones were planted with a mixture of *S. viminalis* 'Jorr', *S. viminalis* 'Joruun', *S. viminalis* 'Orm', *S. viminalis* 'Ulv' and *S. viminalis* x *S. schwerinii* 'Tora'. The total area planted was approximately 4.2 ha and the entire site was cut back in the winter after planting to encourage coppicing.

The plots were allocated to three replicate blocks and the six treatments below were randomly assigned within each replicate – wastewater applied at one, two and three times the calculated potential evapotranspiration rate (1 PE ww, 2 PE ww and 3 PE ww). Two control treatments were incorporated into the trial – no irrigation and irrigation with 1 PE mains water. Sewage sludge as a one-off application of 107 tonnes/ha at cut back was also included for comparison. An irrigation system was installed in the trial field, which provided 1 PE wastewater to the buffer zones through flood nozzles at 10 m centres. The treatment plots were provided with low-level sprinklers. Irrigation was delayed till July 1999 and continued to the end of October, and in 2000 and 2001 irrigation began in early May and early April respectively continuing to the end of October. The change in irrigation requirements throughout the growing season was controlled using an on-site microprocessor system, so the site did not require daily attendance. On an eight-week cycle through the year samples of superficial ground water were taken from boreholes approximately 2.0m deep, which had been established on each plot. These plastic tubes with side slits were installed centrally on each plot with the low end of the tube at or below the assumed lowest ground water table. The upper 0.5 m of the tube was sealed with bentonite clay to prevent short-circuit-flow of wastewater along the pipes. Analysis of BOD, NH_4-N, NO_3-N, PO_4-P, K, Cl, Cu, Cd, Pb and Zn were carried out on each occasion. The applied wastewater was similarly sampled and analysed.

Plant biomass was estimated each year using non-destructive measurements and in the final year (2002) a full destructive harvest was carried out. For the non-destructive estimates the diameter of each stem of ten randomly selected stools from the central area of each plot was measured at 55 cm from its base, using electronic callipers. Shoots with a range of diameter were destructively harvested outside the central recorded area and dry weight calculated. From these diameter measurements and dry weights an allometric equation relating shoot diameter to dry weight was calculated (Aronson, 1999). This equation was used to estimate the dry weight of the non-destructively harvested stems and hence the estimated biomass production in tonnes/ha. The destructive harvest carried out in December 2001 - January 2002 obtained a field fresh weight for the complete recorded plot (100 stools). Representative

samples were taken and oven dried to a constant weight (105°C for 48 hours). The resulting dry weight estimates were used to calculate total dry weight yields. This harvested biomass was also sampled for chemical analysis of N, P, K Cu Zn Pb and Cd, nitrogen by Kjeldahl and minerals by dry ash and atomic absorption.

RESULTS

Yield

Non-destructive calculations were made annually from 1999-2001 and the results are shown in Figure 1 as cumulative dry matter yields. There was an increase in dry matter production with increasing wastewater application from the calculated potential evapotranspiration rate (PE) to 3 PE. However these differences were not statistically significant. The highest yields equivalent to approximately 9 t/ha were harvested from the three PE wastewater and sludge treatments.

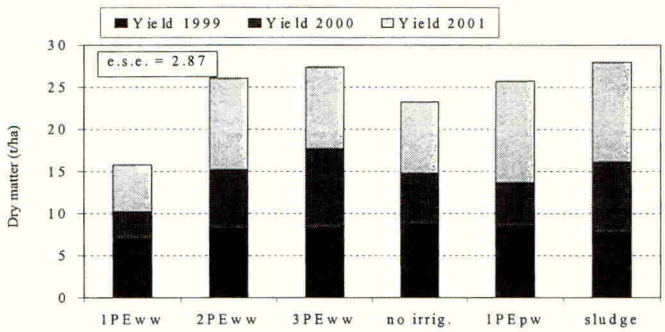

Figure 1. Calculated annual yields from coppice irrigated with watewater.

At the three-year destructive harvest the highest yields came from the 3 PE ww and sludge plots (Figure 2) and the total yields were similar to those calculated by the non-destructive technique. However yields from the 1 PE ww and control treatments produced higher actual yields than those calculated from non-destructive measurements. Similarly those from the pure water treatment and 2 PE ww produced lower yields than the calculated. In none of the treatments were these differences statistically different.

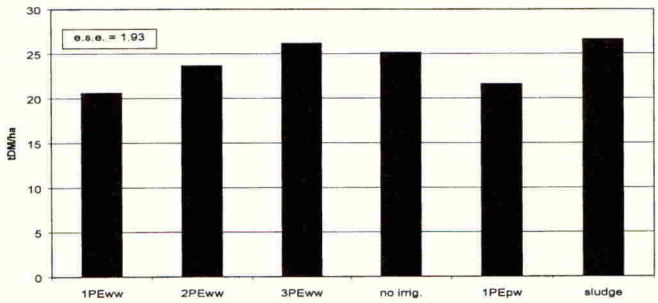

Figure 2. Cumulative yields for willow coppice irrigated with wastewater.

Inputs

Irrigation rates and precipitation at the site in 1999, 2000 and 2001 are recorded in Table 1. The plots were only irrigated during the growing season (April – October) and only when weather conditions were appropriate – application was stopped on major rain occasions.

Table 1. Wastewater irrigation rates and precipitation levels in mm/ha/yr

	Irrigation (mm/ha/yr)			Precipitation (mm/ha/yr)	Precipitation + irrigation (mm/ha/yr)		
Year	1PE	2 PE	3 PE		1 PE	2 PE	3 PE
1999	308	615	923	731	1039	1347	1655
2000	451	901	1352	766	1216	1667	2118
2001	581	1162	1742	618	1199	1779	2360

From nutrient and non-nutrient concentrations in the wastewater and using application figures in Table 1 the average total nutrient and metal application per year for the 1 PE treatment was calculated and these figures are recorded in Table 2

Table 2. Annual average application of nutrients and metals (kg/ha)

	Annual application of micronutrients and metals (kg/ha)						
	N	P	K	Cu	Zn	Pb	Cd
1PE ww	83	56	45	0.05	0.52	0.78	0.08
2PE ww	167	111	90	0.09	1.04	0.57	0.17
3PE ww	250	167	135	0.17	1.55	235	0.25

The optimum ratio by weight of N:P:K for SRC willow plantations is 100:14:72. On this basis the wastewater used at Culmore was low in K and high (x5) in P. However only 15-20% of the phosphorus applied was in the plant available form (PO_4-P).

Offtakes

The trial was harvested in winter 2001/02 and in addition to dry matter yield estimates samples were taken for chemical analyses. Determinations of N, P, K, Cu, Zn, Pb and Cd were made and together with dry matter yields, mean uptake (export) figures per hectare were calculated and recorded in Table 3. Although there was a trend towards increasing nutrient offtake with increasing wastewater application and that this, in the case of nitrogen phosphorus and zinc, this was significant, the increased offtake in no way accounted for the increased nutrient application at the higher levels of wastewater application.

Sampling of superficial ground water was undertaken at eight-week intervals throughout the year using the boreholes of approximately 2.0 M depth established on each plot. Mean analytical data for the three-year irrigation period 1999-2002 is recorded in Table 4.

Generally the 1 PE ww irrigation level resulted in minimal impact on superficial ground water with marginally elevated Cl^-, nitrogen, potassium, zinc and copper figures. Where mains water was used for irrigation the superficial ground water concentrations were generally

higher than the concentration in the applied mains water, indicating a downward transport of the constituents remaining in the soil from previous activities.

Table 3. Annual offtake of nutrients and metals in kg/ha by willow coppice

Treatment	N	P	K	Cu	Zn	Pb	Cd
1 PE WW	45.4	6.44	16.0	0.036	0.88	0.52	0.043
2 PE WW	49.9	7.71	20.1	0.040	0.84	0.62	0.054
3 PE WW	57.2	8.15	18.8	0.043	0.75	0.71	0.061
Control	47.2	7.29	17.5	0.049	0.85	0.59	0.060
1 PE PW	39.1	6.01	15.9	0.045	0.72	0.53	0.055

Table 4. Mean concentration (mg/l) of nutrients and metals in the superficial ground water under three levels of wastewater application (1, 2 & 3PE) during the sampling period from July 1999 to April 2002. Figures for wastewater (WW) and mains water (PW) are included for comparison.

Parameter	**ww**	1 PEww	2 PEww	3 PEww	**pw**	1 PEpw	Sludge	Control
pH	**6.9**	6.4	6.3	6.4	**7.1**	6.5	6.4	6.4
BOD	**106**	32	35	30	**3.6**	31	31	31
COD	**245**	171	149	196	**13**	126	119	177
N-tot	**19**	6.5	4.5	3.6	**2.7**	4.0	4.8	3.3
NH_4-N	**18**	1.6	1.6	1.6	**1.8**	1.6	1.7	1.5
NO_3-N	**0.53**	4.9	2.9	2.0	**0.92**	2.01	3.1	1.7
P-tot	**12.0**	1.3	1.3	0.89	**0.02**	1.0	1.3	1.25
PO_4-P	**2.0**	0.57	0.5	0.65	**0.0**	0.48	0.49	0.57
K	**11.0**	3.8	4.4	4.5	**1.9**	3.3	5.2	2.3
Cl^-	**215**	91	99	149	**24**	56	122	58
Cd µg/l	**0.018**	0.081	0.017	0.016	**0.0**	0.016	0.07	0.014
Pb µg/l	**0.15**	0.16	0.28	0.18	**0.22**	0.18	0.19	0.16
Zn µg/l	**120**	70	69	110	**15**	67	58	70
Cu µg/l	**15**	40	39	51	**7**	40	52	30

DISCUSSION

These results clearly indicated that biomass production in a young willow plantation has a trend towards higher productivity with increasing application of wastewater irrigation. However in none of the annually estimated yields or in the cumulative destructive yield taken in 2001 were any of the differences statistically different. This was due to large variations in the calculated yields from plots within the same treatments resulting in high standard errors. Additionally the field trial was established on a relatively fertile site so a 'lag' period would be expected before any differences in nutrient status could be expected to show as differences in recorded yield. Experience from other long running trials has indicated that nutrient reserve on a fertile site can sustain production for four three-year cycles (Dawson, 2001). The

highest yields recorded over the three years came from the 3 PE ww and from the sludge treatments and these equated to 9.3 t DM/ha/yr. This would compare favourably with yields of the first rotation willow coppice from other trials and commercial plots.

The annual uptake of nutrient in the stems and thereby removal from the system at harvest of 45.6 kg/ha N, 6.5 kg/ha P and 16.2 kg/ha K conform with figures from other nutrient investigations. In a long running nutrition trial carried out in Northern Ireland mean annual removal figures of 55-60 kg/ha N, 8.4 kg/ha P and 31.2 kg/ha K were recorded over a twelve-year period (Dawson, 2001). However, application of metals (Cu, Zn, Pb and Cd) in the wastewater exceeded plant uptake rates except in the case of zinc. No obvious correlation was found between the uptake of these heavy metals and their concentration in the applied wastewater. In the long term perspective added nutrients and heavy metals should be in balance with removal figures in the harvested biomass to avoid accumulation in the soil. The ability of the soil plant system through its capacity to fix nutrients by adsorption ion exchange and precipitation and through the action of soil micro-organisms to transform nitrogen together with the export from the system at harvest will determine the sustainability of the system. However more data over a long time frame need to be accumulated to quantify this. Generally ground water was not adversely affected by the application of wastewater or sludge although an increase in total N and NO_3-N in superficial ground water was recorded in the 1PE rate of wastewater application. This was probably due to higher de-nitrification rates in treatments with higher (2 & 3PE) hydraulic loading.

Calculation of wastewater treatment effects using a mass balance technique where total nutrient application, calculated evapotranspiration and percolation rates together with nutrient assimilation in the harvested stems produced figures for nutrient removal efficiency of 52-75% for N and 90-98% for P with a reduction in BOD of 67-74% (Hasselgren, 2003). In general the highest rates were recorded for the most heavily loaded treatments. A hydraulic loading of three times the calculated evapotranspiration rate did not influence wastewater treatment capacities. Thus managing a willow coppice system irrigated with wastewater according to water requirement and nutrient removal in the harvested stems seems possible without negative impacts as far as oxygen demanding substances and eutrophying components (nitrogen and phosphorus) are concerned.

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Perspectives for the use of cattail (*Typha spp*) in phytoremediation

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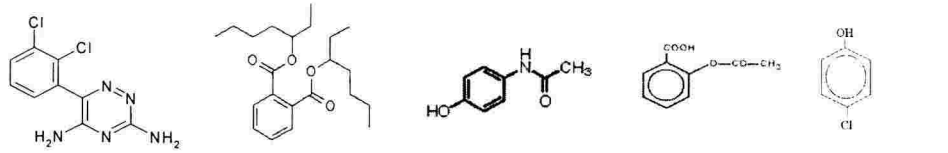
ABSTRACT

In a set of pilot experiments the ability of two species of cattail (*Typha latifolia* and *T. angustifolia*) to remove or metabolise organic xenobiotics from wastewater was tested. Rapid uptake of pharmaceuticals and xenobiotics into the rhizome tissue was observed. Under the influence of xenobiotics and medicaments, strong inductive effects on enzyme activity were observed, especially on the activity of glutathione S-transferase and peroxidase. These stimulating effects on the detoxification capacity could foster the use of *Typha* for wastewater treatment.

INTRODUCTION

Typha latifolia and *T. angustifolia* are two freshwater monocots occurring ubiquitously in Europe. They are found abundantly in creeks, swampy riverbanks, lakeshores and in drainages of agricultural areas. Cattail is often regarded a weed as it may clog agricultural drainage systems due to its intensive biomass production.

Typha has been found to be very effective in removing excess nitrogen and phosphorus from water. Besides, wastewater is often contaminated with low concentrations of anthropogenic substances such as pharmaceuticals (pain killers, antibiotics) and pesticides. Due to the strengthening of the water directive of the EU, the need to improve and maintain high quality standards for sewage treatment is of paramount importance for the future. Plant-based treatment systems may thus offer an adequate supplement to existing technologies (Schröder *et al.*, 2001; Coleman *et al.*, 2002). Some of the pollutants occurring frequently in household waste water might be chemicals like Lamotrigine (3,5-diamino-6(2,3-dichlorophenyl)-1,2,4-triazine), DEHP (Bis(2-ehthylhexyl)phthalat), Paracetamol, Aspirin or 4-Chlorophenol (Figure 1).



Lamotrigine DEHP Paracetamol Aspirin 4-Chlorophenol

Figure 1. Chemical structures of Lamotrigine, DEHP, Paracetamol, Aspirin, 4-Chlorophenol

In a set of pilot experiments we tested whether *Typha* rhizomes are able to bind these xenobiotics. In addition, activities of important detoxification enzymes like glutathione S-transferases (GST), or the peroxidases (POX), of untreated *Typha* plants and the detoxification capacity of *Typha* plants after incubation with pharmaceuticals were determined.

MATERIAL AND METHODS

Plant Material. *Typha* plants of the two species *T. angustifolia* and *T. latifolia* were harvested from ponds at the experimental farm Scheyern, Upper Bavaria, Germany. After harvest, plant roots were transferred to the lab in water filled plastic bags for the in vivo studies, and leaves were frozen in liquid nitrogen and stored at –80°C.

Experiment I. Lamotrigine (a triazine antiepilepticum, Anonymous, 1995) and DEPH (a softener for plastics) were dissolved in ethanol and diluted with water to different concentrations. One-gram pieces of a *Typha* rhizome were incubated with 5 ml of these solutions. After 1, 16 and 25 hours 100 µl aliquots were taken from the incubation vials, quenched with acetonitrile and centrifuged. 25 µl were injected to HPLC and quantified according to the method described by Castel-Branco *et al.* (2001).

Experiment II. For extraction of soluble proteins frozen leaves were homogenized and added to potassium phosphate buffer. A two-step ammonium sulphate precipitation (40% and 80% saturation) was used to preconcentrate proteins of interest. Salt was removed by gel filtration on PD10 columns. The activity of detoxification enzymes, gluthathion S-transferases (GST) and peroxidases (POX) was determined in photometric tests. Affinity chromatography via a specific GSH(S)-agarose (5 ml, Sigma) was used to separate GST from the protein mixture (Schröder *et al.*, 1997). After applying 1 ml of *Typha* protein extract to the column, 10 ml of potassium phosphate buffer (25 mM, pH 7.5) were added to remove unbound protein. After washing, bound GST was eluted with increasing concentrations of GSH (1 to 16 mM).

Experiment III. To test GST and POX detoxification capacity expanded leaves were cut and immersed with their base into Falcon flasks containing 5 ml of tap water. At the beginning of the experiment an aliquot of Paracetamol, Aspirin and 4-Chlorophenol solutions (final concentrations 33, 33 and 20 µmol per assay, respectively) was injected into the water, and leaf sections were incubated for 16 hours in daylight at room temperature. At the end of incubation, leaves were removed from the Falcon flasks, rinsed with water and dried with paper towels. The experimental procedure for the extraction and testing of detoxification enzymes is described above (Experiment II).

RESULTS AND DISCUSSION

Experiment I. The aim of this experiment was to test whether *Typha* rhizomes are able to remove xenobiotics from wastewater and bind them to plant biomass. When Lamotrigine was spiked to wastewater samples (5 ml) containing 1g fresh rhizome surface tissue, a rapid decrease of the concentration in water was observed. In wastewater without *Typha* rhizomes the concentrations of Lamotrigine and DEHP remained stable for 25 hours.

In the presence of rhizome pieces, approximately 50% of Lamotrigine had disappeared after 1 hour (Figure 2). During the next 24 hours Lamotrigine concentrations decreased to 40 % in the vials that had initially been spiked with 30 000µg. In the treatments with an initial amount of 3000 or 300 µg Lamotrigine, 80% and 98% of the Lamotrigine added had disappeared by 24 hours, respectively. This preliminary experiment elucidates that *Typha* rhizomes have a certain capacity to bind triazines. This capacity seems to be in the range of several hundred µg of the chemical per g freshweight of rhizomes.

In order to validate the uptake capacity of the tissue, the softener DEHP was used. Contrary to the results with Lamotrigine, DEHP removal from the wastewater at concentrations of 3000 µg was remarkably slower during the first 16 hours (14%, Figure 3). From 16 to 24 hours the removal rate increased strongly, leaving only 40% of the initial DEHP concentration. The lower concentration (300 µg DEHP) was totally removed within 16 hours, confirming that *Typha* rhizome surface tissues are able to remove lipophilic pollutants from water. Typically aquatic vascular plants contain a high amount of suberized cell walls and bark structures which would be candidates for the rapid binding (Mcmanus *et al.*, 1998). The chemical nature of the binding and its reversibility has to be elucidated by sophisticated analytical procedures.

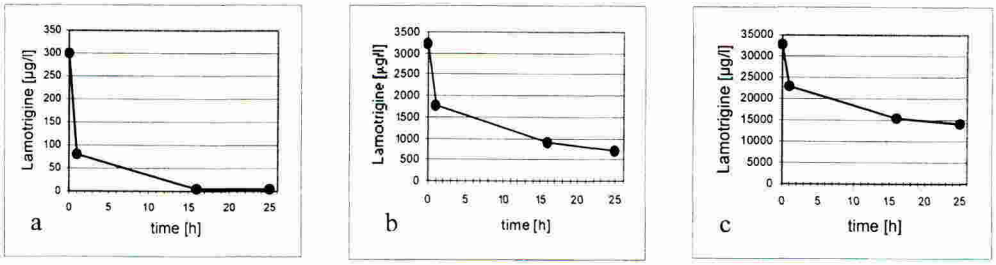


Figure 2. Time course of Lamotrigine disappearance from wastewater in the presence of *Typha* rhizome tissue. 3 different concentrations were used (a) 300 µg/l, (b) 3000 µg/l, (c) 30000 µg/l.

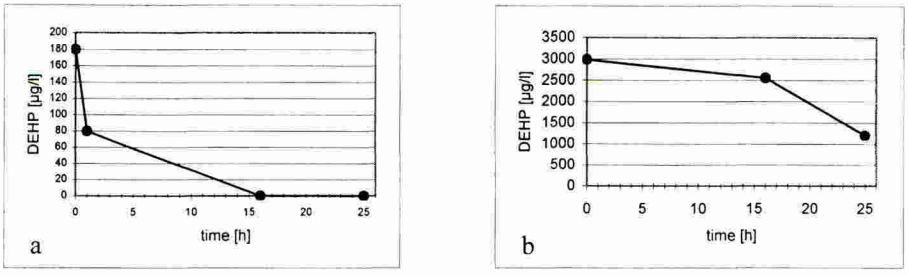


Figure 3. Time course of DEHP disappearance from wastewater in the presence of *Typha* rhizome tissue. Concentrations used were (a) 300 µg/l, (b) 3000 µg/l.

Experiment II. The goal of this experiment was (1) to demonstrate the presence of detoxification enzymes in different tissues of the plant, (2) to test enzyme activities (GST,

POX) of untreated *Typha* plants and (3) to distinguish between different GST isoforms of young, expanded and old *Typha* leaves.

(1) Untreated *Typha* rhizomes and leaves show GST activity for the conjugation of the model substrate CDNB (1-chloro-2,4-dinitrobenzene). Young and expanded leaves have highest GST activity for this substrate (Table 1). The chemically related substrate DCNB (1,2-dichloro-4-nitrobenzene) showed much lower GST activity in each of the samples (Table 1), and different distribution of activities over the tissues tested. This uneven distribution pattern of GST activities for CDNB and DCNB indicates the presence of several isoforms of GST with a different affinity to the assay substrates.

Table 1. Activity of the detoxification enzyme, glutathione S-transferase, for the conjugation of model xenobiotics in protein extracts derived from different tissues of the *Typha* plant. Measurements were done in triplicate and corrected for non-enzymatic values.

	GST actitivy [µkat/mg]	
Substrate	CDNB	DCNB
Rhizomes	5.67	0.29
Young leaves	22.02	0.35
Expanded leaves	29.10	0.91
Old leaves	7.22	1.56

Untreated *Typha* rhizomes and leaves had also considerable peroxidase (POX) activity for the model substrate guajacol (Figure 4). Different from the distribution of GST activities shown above, rhizomes and old leaves contain a high POX activity (Figure 4).

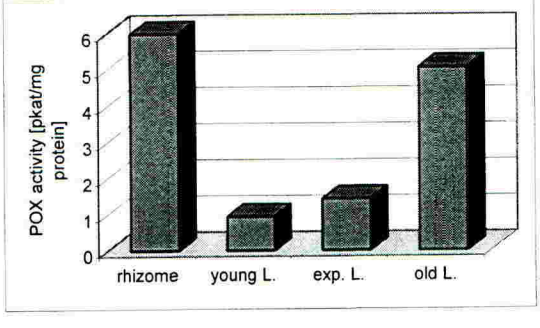


Figure 4. Activity of the detoxification enzyme, peroxidase, for the chemical conversion of guajacol in protein extracts derived from different tissues of the *Typha* plant. Measurements were done in triplicate and corrected for non-enzymatic values.

(2) When protein extracts derived from *Typha* tissues were subjected to affinity chromatography on an agarose gel containing reduced glutathione bound via the sulfur as a ligand, a single activity peak for GST active with CDNB was resolved (Figure 5). Similar patterns had also been obtained in other plant species (Schröder *et al.*, 1997). In *Typha*, expanded leaves showed highest specific GST activities with 0.160 nkat/mg protein after affinity

chromatography. Further purification by anion exchange chromatography and isoelectric focusing will yield information about GST isoforms.

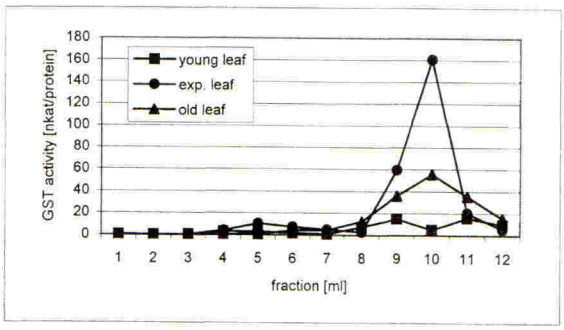


Figure 5. Specific GST activity in affinity chromatography fractions. Aliquots of *Typha* protein extracts were applied to a glutathione-S-agarose column. From fractions 1-7 columns were washed with potassium phosphate buffer to remove unbound protein, from fractions 8-12 bound GST was eluted by stepwise increasing GSH concentrations (1, 2, 4, 8, 16 mM) in the elution buffer.

Experiment III. The goal of this experiment was to identify possible inducers for the detoxification capacity of *Typha* leaves. Figure 6a shows the GST activity for the substrates CDNB and DCNB after treatment with Paracetamol, Aspirin and 4-Chlorophenol solutions. 100% activity characterizes GST activities of untreated leaves (control). Leaves treated with Paracetamol, Aspirin and 4-Chlorophenol solutions show decreased CDNB-GST activity when compared to controls. DCNB-GST activity is significantly lower than in controls after Aspirin treatment. As all enzyme extracts were purified and subjected to gel filtration prior to testing, this decrease can not be due to inhibitory effects of the chemical used for incubation. It is rather an indication for a change in the expression pattern of GST during the treatment.

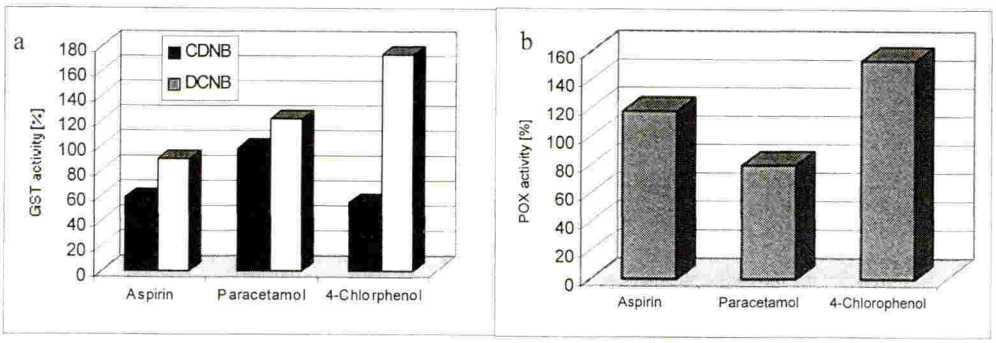


Figure 6. a: GST activity for the model substrates CDNB and DCNB in protein extracts of *Typha* leaves treated with the given pharmaceuticals for 16 hours. Bars represent GST activities above untreated controls. b: POX activity for the model substrate guajacol in protein extracts of *Typha* leaves treated in the same way. Bars represent POX activities above untreated controls. Measurements were done in triplicate and corrected for non-enzymatic values.

Figure 6b depicts the POX activity after treatment with Paracetamol, Aspirin and 4-Chlorophenol solutions as compared to controls (100%). Only leaves treated with Paracetamol exhibited a decrease of POX activity in comparison to untreated leaves.

CONCLUSIONS

In our pilot experiments we showed that *Typha* rhizomes are able to absorb lipophilic xenobiotic substances and to remove several hundred µg xenobiotic per g fresh weight of tissue from the incubation medium within a few hours. Untreated *Typha* plants contain detoxification enzymes like glutathione S-transferase and peroxidase. Under the influence of xenobiotics and medicaments, effects on enzyme activity of glutathione S-transferase and peroxidase have been observed. Detoxification enzymes in plants show high flexibility (Lamoureux and Rusness, 1989) and could thus be exploited for the removal of recalcitrant organics (Coleman *et al.*, 2002). Further studies are underway to investigate binding of xenobiotics in *Typha* tissues. It is not known whether *Typha* is able to produce bound residues or whether the compounds will be released under certain environmental conditions, implicating that there could be a risk of bioavailability of xenobiotics after degradation of plant material especially during winter, when plant material is degraded.

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