The Role of Microbial Communities in the Degradation of Polyurethane in Soil

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Lee Cosgrove

(ENYDE)

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Abstract

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Plastic waste represents a significant environmental burden in the developed world; however, an increased use of biodegradable plastics may help to reduce the environmental impact of plastic waste. Polyester polyurethane (PU) is an example of a biodegradable plastic, and an understanding of the microbial communities involved in its degradation in the environment is necessary to exploit these communities for the remediation of PU waste. The primary aims of this work were i) to investigate the microbial communities associated with the degradation of PU in the environment ii) to determine if adding nutrients to soil can enhance the degradation of buried PU and iii) to investigate whether inoculating known PU degrading fungi into soil can enhance the degradation of buried PU.

The colonisation and degradation by soil microorganisms of PU buried *in situ* in acidic and pH neutral garden soils for five months was investigated. Each soil was found to have a different native fungal soil community, and only specific subsets of these communities could colonise the surface of buried PU. Fungal communities on PU buried in the acidic soil were dominated by *Geomyces pannorum*, whilst an *Alternaria* sp dominated the fungal communities on PU in the neutral soil. PU degrading fungi were very common both in the soil and on the surface of buried PU, with approximately 40% of the fungi recovered on solid media showing putative PU degrading phenotypes. PU was severely degraded after burial in both soils, losing up to 95% of its tensile strength. Very few putative PU degrading bacteria were seen, suggesting that fungi are primarily responsible for the degradation of PU in soil environments.

Since the soil communities contained a large reservoir of putative PU degrading fungi, enhancing PU degradation by stimulating these communities with nutrients, in a process known as biostimulation, was investigated. Microcosms containing the acidic garden soil were treated with yeast extract (YE), the colloidal PU substrate Impranil, or a combination of both. PU coupons were then buried in treated soil. Impranil treatment alone had little effect on the fungal communities in the soil. Treatment with YE and a combination of YE and Impranil led to the enrichment of specific members of the soil fungal populations, with YE and Impranil co-treatment causing dramatic proliferation of *Trichosporon multisporum* and a Zygomycete species. As before, specific members of the soil fungal communities colonised the surface of buried PU. However, although

each treatment influenced the composition of PU communities, with the exception of the Zygomycete none of the enriched fungi colonised buried PU. Adding YE or both YE and Impranil to soil slightly enhanced the degradation of PU, although PU buried in untreated control soil was also severely degraded. Treatment with Impranil alone was found to retard the degradation of buried PU. Therefore, biostimulation was not a useful method for significantly enhancing the degradation of PU under these conditions in soil.

Several strains of PU degrading fungi, namely *G. pannorum*, *Penicillium inflatum*, *Nectria sp*, *Penicillium viridicatum*, *Penicillium ochrochloron*, the Zygomycete and isolates 11n and 19n were isolated from soil and from the surface of buried PU during the course of this work. The effect of inoculating wheat grains colonised by these isolates into the acidic garden soil (in a process known as bioaugmentation) on the degradation of buried PU was investigated. Although the introduction of each isolate altered the structure of fungal communities both in the soil and on surface of buried PU, only the *Nectria sp* and the unidentified Zygomycete showed significant survival in the soil, and *P. viridicatum* and the Zygomycete were the only isolates able to colonise buried PU. PU buried in control soil lost 24% of its tensile strength after one month's burial. The addition of sterile wheat to soil enhanced PU degradation, with PU losing 45% of its tensile strength. Colonising wheat with isolates 11n, *Nectria sp*, *P. viridicatum*, the Zygomycete and *P. ochrochloron* further enhanced degradation. Inoculation with *P. ochrochloron* caused the greatest amount of degradation, with PU losing 85% of its tensile strength, suggesting that bioaugmentation can be used to enhance PU degradation in soil.

In conclusion, environmental microbial communities can be used to degrade PU waste in soil. Whilst stimulating soil communities with nutrients did not significantly enhance degradation, degradation was increased by bioaugmenting soil with PU degrading fungi.

DECLARATION

No portion of this work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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THESIS ORGANISATION AND STRUCTURE.

The results chapters in this thesis are written in the style of the international peer reviewed journal *Applied and Environmental Microbiology*. These chapters have been submitted for publication within this journal as follows:

Chapter 2: Cosgrove, L.C., P.L. M^{c}Geechan, G.D. Robson, and P.S. Handley. 2006. Fungal communities associated with the degradation of polyester polyurethane in soil. *Applied and Environmental Microbiology*. Submitted

Chapter 3: Cosgrove, L.C., P.L. M^{c}Geechan, G.D. Robson, and P.S. Handley. 2006. Biostimulation of soil microbial communities and the degradation of buried polyester polyurethane. *Applied and Environmental Microbiology.* Submitted

Chapter 4: Cosgrove, L.C., P.L. M^{c}Geechan, G.D. Robson, and P.S. Handley. 2006. Degradation of polyester polyurethane (PU) buried in soil bioaugmented with PU degrading fungi. *Applied and Environmental Microbiology.* Submitted.

The chapters in this thesis therefore should be viewed as standalone works, and hence have their own figure and table numbers, as well as their own reference lists. Each chapter pertains to a related area of research, so there is some overlap and repetition in the methodology and referencing between the chapters. Results of preliminary studies that did not fall into the scope of the chapters proper are presented in the appendices.

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All practical studies and all of the written text in this thesis is the work of LC.

See, that's the thing with you plastics, you think everybody is in love with you, but in reality everyone ***hates*** *you!*

Janis Ian
Mean Girls

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ABBREVIATIONS

ARISA	automated ribosomal intergenic spacer analysis
BTEX	benzene, toluene, ethylbenzene, and xylene.
cDCE	*cis*-1,2-dichloroethene
cfu	colony forming unit
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMBL	European molecular biology laboratory
FISH	fluorescent *in situ* hybridisation
GfP	green fluorescent protein
ITS	internally transcribed spacer
LH-PCR	length heterogeneity PCR
MEA	malt extract agar
MTBE	methyl tertiary-butyl ether
OD	optical density
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCP	pentachlorophenol
PCR	polymerase chain reaction
PLFA	phospholipid fatty acid
pPVC	plasticized polyvinyl chloride
PU	polyurethane
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SEA	soil extract agar
S.E.	standard error
SSCP	single strand conformational polymorphism
TCE	trichloroethene
TGGE	temperature gradient gel electrophoresis
whc	water holding capacity
YE	yeast extract

Chapter 1:

General Introduction

General Introduction

Plastic degradation.

Synthetic polymer waste is of growing concern in the developed world. The proportion of household waste made up of plastic in the average American home has increased from 3-5% of total waste in 1969 (Eggins *et al.*, 1971) to more than 30% in 1995 (Kawai, 1995). The total amount of plastic produced per year world-wide as of 2001 stood at over 140 million tonnes (Shimao, 2001). Since most synthetic polymers are chemically and physically robust, and are therefore persistent in the environment, these figures have serious implications for the ecological impact of the growing use of such compounds (Kawai, 1995). There is therefore an obvious need for less persistent materials. Some plastic formulations have been described which have been found to be subject to degradation in the environment, and an increased use of these may go some way towards reducing the ecological burden plastics represent.

It is recognised that many factors may bring about the degradation of plastics in the environment, including abiotic processes such as photodegradation or hydrolysis (Griffin, 1980). However, the contribution of these processes to the breakdown of plastics is usually relatively small, and it would appear that in most instances the major agents of plastic degradation are microorganisms (Bentham *et al.*, 1987).

Biodegradation is the term most commonly used to describe the breakdown of compounds by microorganisms. Examples of plastics vulnerable to biodegradation include the polyhydroxyalkanoates, polycaprolactone, polylactic acid, and polyvinyl alcohol (Shimao, 2001). The work described here will focus on the polyurethanes, a group of plastics some of whose members are subject to biodegradation. These plastics are used widely in industrial and medical applications, and an understanding of the mechanisms responsible for their degradation will be required if these processes are to be encouraged for waste management applications or discouraged for product preservation.

Polyurethanes

Polyurethanes comprise a group of synthetic polymers formed by the condensation of poly- or di-isocyanates with polyols containing ester or ether bonds (Sauders & Frisch, 1964), producing chains with intramolecular urethane bonds, as illustrated in Figure 1:

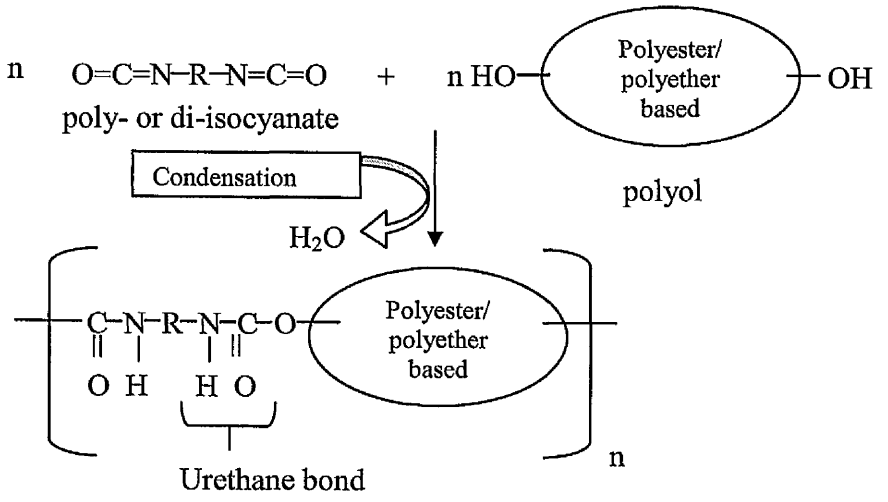


Figure 1: The production of polyurethane by the condensation of isocyanates and polyols (Nakajima-Kambe *et al.*, 1999).

Although the name of this group of compounds emphasizes the presence of the urethane bond, such bonds are usually only present in relatively small numbers within most polyurethane molecules (Shimao, 2001). Rather, ester (R'-O-$\overset{O}{\overset{\|}{C}}$-$R''$) and ether ($R'$-C-O-C-$R''$) linkages within the polyol components are the most numerous type of bond, and most experiments show that these are the bonds most frequently targeted by microorganisms during biodegradation (Nakajima-Kambe *et al.*, 1999).

Breakdown of polyurethanes by microorganisms.

PU degradation by microorganisms is thought to occur due to the action of hydrolytic enzymes produced during nutrient acquisition (Nakajima-Kambe *et al.* 1999). Hydrolytic enzymes produced by microbes to degrade biological macromolecules, which also contain ester and urethane bonds, may act upon these same types of bonds

within polyester polyurethane (PU), leading to hydrolysis of the PU backbone and resulting in degradation.

In the early work of Darby & Kaplan (1968), it was demonstrated that the susceptibility of polyurethanes to attack by fungi was strongly dependent on the composition of the plastic being assessed. Differences in both gross chemical characteristics such as whether the primary linkages were ester or ether bonds, as well as more subtle variations such as the presence of methyl side chains within these molecules, influenced the susceptibility of the plastic to microbial attack.

The degree of susceptibility to degradation was assessed by observing the amount of fungal biomass a piece of polyurethane of a given composition could support. It was found that all of the formulations containing ester bonds were capable of supporting fungal growth to varying degrees, whereas most of the ether-based polyurethanes were found to be unable to support fungal growth. The susceptibility of ester-based polyurethane, and resistance of ether-based polyurethane to microbial attack has been observed in further experiments (Dale & Squirrell, 1990), and the presence of ester bonds appears to be the most important determinant for the successful biodegradation of polyurethanes.

By analysing the breakdown products generated during the degradation of PU by microbial communities, both Kay *et al.* (1993) and Akutsu *et al.* (1998) were able to identify the ester bond within the PU as the most probable site at which the PU was hydrolysed. However, Filip (1978) obtained evidence that the urethane bonds may also act as a site for hydrolysis.

Isolation and characterisation of PU degrading organisms.

Many attempts to isolate and identify organisms with PU degrading abilities have been made, most often from soil environments. Several species of bacteria capable of degrading PU have been identified (Table 1), including *Serratia rubidaea*, *Corynebacterium spp*, and *Enterobacter agglomerans* (Kay *et al.*,1991). Few of these bacteria are capable of utilising PU as a sole carbon source, although exceptions such as *Comamonas acidovorans* (Akutsu *et al.*,1998; Allen *et al.*,1999) and *Pseudomonas chlororaphis* (Howard *et al.*,1999) have been described.

Table 1: Bacteria with PU degrading phenotypes

Bacterium	Reference
Acinetobacter calcoaceticus	El-Sayed *et al.* (1996)
Aeromonas salmonicida	Kay *et al.* (1991)
Alcaligenes denitrificans	Kay *et al.* (1991)
Arthrobacter globiformis	El-Sayed *et al.* (1996)
Comamonas acidovorans	Nakajima-Kambe *et al.* (1995) Akutsu *et al.* (1998) Allen *et al.* (1999)
Corynebacterium sp	Kay *et al.* (1991)
Enterobacter agglomerans	Kay *et al.* (1991)
Nocardia autotrophica	Pommer & Lorenz (1985)
Nocardia orientalis	Pommer & Lorenz (1985)
Methanotrix sp	Varesche *et al.* (1997)
Pseudomonas aeruginosa	Kay *et al.* (1991)
Pseudomonas cepacia	El-Sayid *et al.* (1996)
Pseudomonas chlororaphis	Ruiz *et al.* (1999) Howard *et al.* (1999)
Pseudomonas fluorescens	Vega *et al.* (1999)
Pseudomonas maltophilia	Kay *et al.* (1991)
Pseudomonas putida	El-Sayed *et al.* (1996)
Serratia rubidaea	Kay *et al.* (1991)
Streptomyces parvulus	Pommer & Lorenz (1985)
Streptomyces plicatus	Pommer & Lorenz (1985)
Streptomyces violaceoruber	Pommer & Lorenz (1985)

The majority of PU degraders, however, appear to be fungi, and examples are listed in Table 2. It is obvious that a far greater diversity of PU degraders exists amongst the fungi. Moreover, the ability to exploit the plastic as a carbon source is also much more common in this group (Pathirana & Seal, 1985; Crabbe *et al.*, 1994; Barratt *et al.* 2003), making fungi the most probable agents of PU bioremediation in the environment.

Table 2: Fungi with PU degrading phenotypes

Fungus	Reference
Acremonium spp	Stranger-Johannessen (1985)
Alternaria sp	Stranger-Johannessen (1985)
Alternaria alternata	Pommer and Lorenz (1985)
Aspergillus sp	Stranger-Johannessen (1985)
Aspergillus fischeri	Bentham *et al.* (1987)
Aspergillus flavus	Pathirana & Seal (1984) Pommer & Lorenz (1985)
Aspergillus fumigatus	Pathirana & Seal (1984)
Aspergillus niger	Filip (1979)
Apergillus ustus	Bentham *et al.* (1987) Pommer & Lorenz (1985)
Apergillus versicolor	Bentham *et al.* (1987)
Aureobasidium pullulans	Crabbe *et al.* (1994)
Chaetomium globosum	Pathirana & Seal (1984)
Cladosporium sp	Crabbe *et al.* (1994)
Cladosporium herbarum	Fillip *et al.* (1979)
Curvularia senegalensis	Crabbe *et al.* (1994)
Exophiala jeanselmei	Owen *et al.* (1996)
Fusarium sp	Pathirana & Seal (1984)
Fusarium culmorum	Bentham *et al.* (1987)
Fusarium oxysporum	Pathirana & Seal (1984) Pommer & Lorenz (1985)
Fusarium solani	Bentham *et al.* (1987) Crabbe *et al.* (1994)
Geomyces pannorum	Barratt *et al.* (2003)
Gliocladium roseum	Bentham *et al.* (1987) Pathirana & Seal (1984)
Mucor sp	Pathirana & Seal (1984)
Nectria gliocladioides	Barratt *et al.* (2003)
Nitrospora spherica	Pathirana & Seal (1984)

Paecilomyces variotti	Pathirana & Seal (1984)
Penicillium sp	Bentham *et al.* (1987) Pathirana & Seal (1984)
Penicillium chrysogenum	Bentham *et al.* (1987) Pathirana & Seal (1984)
Penicillium decumbens	Pommer & Lorenz (1985)
Penicillium funiculosum	Pommer & Lorenz (1985)
Penicillium notatum	Pathirana & Seal (1984)
Penicillium ochrochloron	Pommer & Lorenz (1985) Barratt *et al.* (2003)
Penicillium purpurogenum	Pommer & Lorenz (1985)
Penicillium rugulosum	Pommer & Lorenz (1985)
Penicillium variabile	Pommer & Lorenz (1985)
Phoma sp	Stranger-Johannessen (1985)
Phoma fimenti	Pommer & Lorenz (1985)
Rhizopus stolonifer	Wales & Sagar (1985)
Scopulariopsis brevicaulis	Pathirana & Seal (1985)
Talaromyces sp	Pommer & Lorenz (1985)
Trichoderma sp	Pathirana & Seal (1984)
Trichoderma viride	Pathirana & Seal (1984) Pommer & Lorenz (1985)
Ulocladium chartarum	Griffin (1980)

Plastic degrading microbes are most frequently isolated by adding polyurethane substrates to environmental samples in order to enrich for organisms able to degrade and use PU as a growth substrate, followed by plate culturing to recover the organisms present after enrichment. Putative PU degrading organisms are then often identified as colonies producing zones of clearance on agar plates containing the PU dispersion 'Impranil'. Nakajima-Kambe *et al.* (1995) enriched soil samples for PU degraders by serially incubating suspensions of soil samples in liquid media supplemented with blocks of PU, and Crabbe *et al.* (1994) added Impranil to soil samples prior to sampling

to achieve enrichment. Alternatively, pieces of plastic incubated in soil can serve as a substrate for the enrichment of putative plastic degraders. Barratt *et al.* (2003) and Bentham *et al.* (1987) both recovered putative PU degrading fungi from the surface of PU buried in soil for 44 and 28 days respectively.

Since putative PU degraders are often identified using liquid Impranil, degradative ability for the more common solid forms of PU must be confirmed. Nakajima-Kambe *et al.* (1995) tested Impranil degrading isolates for the ability to degrade solid PU by incubating them in liquid cultures containing PU blocks. If a reduction in the mass of the blocks occurred, degradation was assumed. Barratt (2003) inoculated dumbbell shaped solid PU pieces with putative PU degrading fungi and buried inoculated dumbbells in sterile artificial soil microcosms for four weeks, and monitored degradation by measuring changes in the tensile strength of the PU.

Most attempts to isolate PU degrading microbes have so far been focused on identifying the organisms responsible. Very little effort has been made to quantitatively determine the relative proportions of PU degraders in environmental samples. Efforts to redress this have recently been made, and Barratt *et al.* (2003) found that the predominant degraders recovered from buried PU pieces were *Geomyces pannorum*, *Nectria gliocladioides* and *Penicillium ochrochloron,* in that order of numerical dominance. However, much more work needs to be done in order to determine the dominant degraders *in situ*, as well as investigating how these organisms interact with one another *in situ* and *in vitro*.

Putative PU degrading enzymes

A number of enzymes with putative PU degrading abilities have been described. Such enzymes have been found in bacteria such as *Pseudomonas chlororaphis* (Ruiz *et al.*, 1999; Stern & Howard, 2000), *Bacillus subtilis* (Rowe & Howard, 2002), and *Comamonas acidovorans* (Akutsu *et al.*, 1998). Examples of equivalent fungal enzymes are less numerous, although Crabbe *et al.* (1994) described an extracellular, probably multi-subunit, Impranil-clearing enzyme in the fungus *Curvularia senegalensis*. Almost all of the enzymes identified so far possess esterase activities, consistent with the observations that polyester polyurethanes are generally subject to degradation, whereas polyether polyurethanes are not. The breakdown products generated during PU

degradation by microorganisms also usually indicate that hydrolysis is taking place at the ester bonds within the PU.

General esterase activities of putative PU degrading enzymes are usually established by determining whether the enzyme is capable of using p-nitrophenylacetate (Ruiz *et al.*, 1999) or fluorescein diacetate (Crabbe *et al.*, 1994) as a substrate, and specific polyester polyurethanase activity is usually inferred from the ability of the enzyme in question to break down Impranil. However, the reliability of Impranil in determining whether a particular enzyme can degrade solid PU is called into question by Nakajima-Kambe *et al.* (1997), in which the authors described two esterase enzymes secreted by *Comamonas acidovorans*. Each was capable of hydrolysing p-nitrophenolacetate, but only one was capable of degrading solid PU. Subsequent experiments showed that the solid-PU degrading enzyme possessed hydrophobic regions as an adaptation to allow adsorption to the surface of the solid PU – an adaptation found to be lacking in the enzyme that was not capable of degrading solid PU. Such adsorption was found to be a necessary step in the degradation of solid PU by this enzyme, but was not necessary for the hydrolysis of p-nitrophenolacetate (Akutsu *et al.*, 1998). This implies that degradation of soluble esterase substrates such as ρ-nitrophenolacetate, fluorescein diacetate, or Impranil can not necessarily be assumed to be indicative of potential solid PU degradative ability.

Methods for assessing solid PU degradation.

A number of methods have been devised to assess the degree to which a sample of PU has been degraded. These include:

1) Analysing the products generated during degradation. This not only allows the rate of degradation to be assessed by determining the rate of product generation, but also allows the probable mechanism of degradation to be determined by monitoring the chemical structure of the by-products of degradation. Using this approach, Akutsu *et al.* (1998) showed that the PU-degrading enzyme of *Comamonas acidovorans* degrades PU by hydrolyzing ester bonds within the plastic.

2) Cleavage of chemical bonds within the plastic usually results in changes in the mechanical characteristics of the plastic, often manifested as a reduction in tensile strength or elasticity. This reduction is proportional to the amount of cleavage, and so

the degree of degradation can be inferred from changes in these properties (Dale & Squirrell, 1990). The ease with which tensile strength can be determined means that this is the method most frequently employed to measure plastic degradation.

3) Deterioration of PU usually results in changes in the appearance of the plastic, such as cracking or discolouration. Bentham *et al.* (1987) and Dale & Squirrel (1990) used these changes to assess the degradation of PU buried in soil.

4) Biomass discrepancies between cultures supplemented with, and cultures lacking PU could be interpreted to be due to the utilisation of the plastic as a source of nutrients (Eggins *et al.*, 1971). The size of this difference could then be used as an indicator of the degree of plastic degradation. This approach cannot, however, be used to assess plastic degradation by organisms incapable of using the plastic as a nutrient source (Kay *et al.*, 1991).

The role of fungal filamentous vegetative growth in the degradation of solid substrates.

Filamentous fungi typically colonise solid growth substrates by extending hyphal filaments radially outwards from the colony centre, with growth occurring at the apical tip of each hypha. As a consequence of this mode of growth, hyphae are capable of exerting mechanical force upon their growth substrates in a manner not typically available to bacteria. Osmotically generated intracellular hydrostatic turgor pressure is applied through the extending apical tip (Bastmeyer *et al.*, 2002), allowing the growing hyphae to explore and penetrate the substrate on which they are growing. This generation of mechanical force by growing fungal hyphae has been implicated as a direct factor in causing the biodeterioration of painted art objects (Mohlenhoff *et al.*, 2001). However, these art objects were unusually fragile, and such purely mechanical degradation is unusual. Although the appressorial pegs used by phytopathogenic fungi to breach the outer surface of the host plant are capable of exerting pressures of up to 10 MPa (Deising *et al.*, 2000), the vegetative hyphae of most fungi typically generate less than 50kPa of pressure (Money, 2004). This amount of force is generally insufficient to breach uncompromised substrates. This is certainly true for the PU used in this work – forces in the range of hundreds of MPa would be required for vegetative hyphea to directly penetrate the surface of undamaged PU (Barratt *et al.*, 2003). Rather, substrate penetration by hyphae usually occurs after the integrity of the growth substrate has been

partially compromised by the action of secreted lytic enzymes. Using this strategy, fungi are able to colonise the interior of a large range of substrates. Fungi are even known to penetrate solid rock (Burford *et al.*, 2003), with organic acids serving the role played by enzymes in the penetration of biological substrates. The colonisation of the interior of PU coupons by fungal hyphae has been described previously (Barratt 2003). Colonisation of both the interior and exterior of plastic substrates provides a greater surface area over which degradation can occur. Thus, their ability to actively penetrate substrates, and their prodigious secretion of a wide variety of lytic enzymes (Aust, 1995) makes it highly probable that fungi play a major role in the degradation of solid wastes such as PU in the environment.

The use of environmental microbial communities to bioremediate manmade pollutants.

A number of factors are responsible for the removal of pollutants within the environment, including dilution, dispersion, volatilisation, precipitation, ion exchange, sorption, transformation and degradation. Collectively, these processes are referred to as natural attenuation, many of which are mediated in part by microorganisms (Röling & van Verseveld, 2002). Although many of these processes have little relevance to the removal of solid plastic waste, microbially induced degradation and mineralization plays a major role in the remediation of plastic waste in the environment (Zheng *et al.*, 2005).

Natural attenuation of pollutants by microorganisms.

The huge diversity of metabolic activity in microbial communities within the environment means that polluted environments often contain microorganisms which possess potential waste degrading phenotypes (Alexander, 1994). Under the correct conditions, these microbes may therefore bring about the bioremediation of waste. Sarkar *et al.* (2005) found that the natural microbial communities in a soil spiked with diesel fuel were capable of removing 93% of the pollutant over the course of 8 weeks. Because natural attenuation is an innate property of the microbial communities in the environment, no action other than perhaps monitoring the progress of degradation is necessary. In some instances, however, natural attenuation is not sufficient to bring

about the removal of pollutants in the environment in a timely manner. In these cases, human intervention may be required. Most commonly, this intervention takes the form of biostimulation or bioaugmentation.

Biostimulation.

Although the microbial communities within a polluted environment may contain members with the metabolic potential to degrade the pollutant, in some cases the pollutant degrades too slowly or not at all. A number of factors have been identified as contributing to these failures, some of which are described on the next page. By adding exogenous nutrients, in a process known as biostimulation, these inhibiting factors can be reduced, allowing waste remediation to occur more rapidly. Biostimulation has been used to successfully enhance the degradation of a wide range of pollutants, examples of which are listed in Table 3.

Table 3: Examples of bioremediation in which biostimulation was successfully used to enhance the degradation of pollutants by native microbial communities.

Pollutant	Nutrient	Reference
Crude oil	Inorganic fertiliser Minimal media	Delille *et al.* (2003); Mills *et al.* (2004); Röling *et al.* 2004 Stallwood *et al.* (2005)
Linear alkylbenzene sulfonate	yeast extract, peptone, glucose or tryptone soya	Sanz *et al.* (2005)
Trichloroethene	Lactate, methanol	Major *et al.* (2002)
Diesel	Maize, biosolids Sludge/Compost	Rivera-Espinoza & Dendooven (2004) Namkoong *et al.* (2002)
Pyrene	Mineral salt medium	Yu *et al.* (2005)
Uranium	Glucose, ethanol	North *et al.* (2004)
MTBE	Oxygen	Smith *et al.*(2005)
Anthracene/Hexadecane	Compost	Kästner *et al.* (1995)
Polycyclic aromatic hydrocarbons	Soil extract Inorganic N and P	Bengtsson & Zerhouni (2003) Breedveld & Sparrevik (2000)

Methods used for biostimulation.

Given below are some of the factors responsible for poor waste remediation by environmental microbial communities, as well as examples of where biostimulation has been used to overcome these inhibitory factors.

Pollutants often lack macronutrients other than carbon.

Many pollutants, especially saturated hydrocarbons such as petroleum products, and to a somewhat lesser extent plastics, have high carbon content but very low proportions of macronutrients such as nitrogen or phosphorus. Hence, although these compounds may be potentially excellent carbon sources for the microbes responsible for their degradation, they are unsuitable as growth substrates for potential bioremediatory microbes due to the absence of N and P. In these cases, the addition of exogenous N and P, most often in the form of inorganic fertiliser, has been successfully employed to enhance bioremediation of crude oil (Delille *et al.*, 2003; Röling *et al.*, 2004) and polycyclic aromatic hydrocarbons (Breedveld & Sparrevik, 2000)

Hydrolytic enzymes may not be induced by pollutants

In some cases, although microbes in the environment may possess the genes for enzymes able to degrade the pollutant in question, the pollutant itself may be incapable of inducing these enzymes. Production of these enzymes can sometimes be induced by the introduction of the correct substrate. Ogunseitan *et al.* (1991) showed that the addition of salicylate caused an upregulation of Pseudomonad *nah* genes in soil; these genes are involved in naphthalene degradation. Focht & Brunner (1985) found that polychlorinated biphenyl (PCB) levels in contaminated soil could be reduced by 75% if the PCB analogue biphenyl was added to the soil. In comparison, only 2% of PCB was removed in untreated soil. Both gelatin (Barrat, 2003) and yeast extract (Zheng, 2004) have been found to enhance the rate at which the colloidal PU dispersion Impranil is degraded in pure culture. Both of these compounds may be inducing the enzymes responsible for PU degradation, and may therefore be useful for the biostimulation of PU waste degradation.

Local redox conditions may not be amenable to waste degradation.

Unfavourable redox conditions in polluted environments can mean that the degradation of waste materials is energetically unfavourable. Under these conditions, the addition of electron donors or acceptors such as oxygen, nitrates, or ethanol can allow waste degradation to proceed. In Smith *et al.* (2005) the addition of O_2 to groundwater contaminated with the fuel additive methyl tertiary-butyl ether (MTBE) significantly enhanced the removal of MTBE by the native microbial communities. Similarly, North *et al.* (2004) used glucose and ethanol to bring about the microbially induced reduction of soluble uranium (VI) to the insoluble uranium (IV), effectively immobilising the radionucleotide. However, in some instances, the addition of electron donors/acceptors can be counterproductive. For example, Mills *et al.* (2004) found that the addition of nitrate to anoxic wetlands reduced the degradation of spilled oil compared to untreated controls, an effect the authors attributed to nitrate toxicity.

Nutrient deficient environments may inhibit degradation of pollutants.

Under nutrient poor conditions, microbial communities in the environment may be in a state of metabolic torpor. The addition of nutrients to these communities may stimulate general metabolic activity, allowing waste to degrade more readily. Yu *et al.* (2005) added a mineral salts medium to nutrient poor polycyclic aromatic hydrocarbon contaminated mangrove sediments and were able to enhance pollutant degradation.

Failures in biostimulation

Although biostimulation is generally successful in enhancing bioremediation, there have been a few reported cases in which biostimulated communities performed less well than untreated control communities. Viñas *et al.* (2005) found that adding a mineral salts medium to creosote contaminated soil led to less hydrocarbon removal compared to untreated control soil. The addition of inorganic nitrogen and phosphorus to diesel contaminated soil was found to retard the degradation of hydrocarbons in the soil compared to untreated control soil (Bento *et al.*, 2005). Furthermore, whilst in many cases biostimulation increases the rate at which a pollutant is degraded, natural

attenuation often removes a similar amount of waste material, merely taking longer to do so. Although this may be of importance in the removal of noxious wastes, PU does not present such a problem. Greater potential for plastic waste remediation may be found in the more technically difficult, but often much more effective technique of bioaugmentation.

Bioaugmentation

The success of biostimulation, as described previously, is obviously dependent on microorganisms with waste degrading phenotypes being naturally present in the community at a polluted site. In some instances, however, microorganisms with the correct waste degrading phenotypes may be completely absent in a polluted environment, or present in too low numbers. In these cases, microorganisms with waste degrading abilities may be added exogenously in a process termed bioaugmentation (van Veen *et al.,* 1997). Bioaugmentation has been used to successfully bioremediate a wide range of anthropogenic wastes, some of which can be seen in Table 4.

Methods used for bioaugmentation

In the simplest terms, bioaugmentation is a strategy for bringing about the microbial degradation of pollutants within the environment by introducing often large quantities of microorganisms with waste degrading phenotypes (Singer *et al.*, 2005). These organisms are generally isolated using enrichment and selection strategies, using the pollutant in question as the substrate. Although the majority of bioaugmentation attempts are based on these simple precepts, there are many variations in the particulars of how bioaugmentation is carried out, some of which are detailed in the following sections.

Bioaugmentation may use single strains or mixed consortia as the inoculum.

The inoculum used for bioaugmentation usually falls into two broad categories – single isolates may be used, or consortia made up of multiple organisms may be employed. A further distinction in methodology may be made when consortia are used as the

Table 4: Examples of successful bioaugmentation to enhance the degradation of manmade pollutants.

Pollutant	Inoculant used for bioaugmentation	Reference
Diesel	*Pseudomonas aeruginosa* Undefined soil consortium	Ueno *et al.* (2006) Bento *et al.* (2005)
PCB	*Arthrobacter* sp. *Ralstonia eutrophus*	Gilbert & Crowley (1998) Singer *et al.* (2000)
Chlorobenzoate	Undefined soil consortium	Gentry *et al.* (2004)
Cadmium	*Bacillus* sp.	Jézéquel *et al.* (2005)
Biological phosphorous	Undefined sludge consortium	Dabert *et al.* (2005)
PCP	Undefined soil consortium	Barbeau *et al.* (1997)
BTEX	Undefined aquifer consortium	Da Silva *et al.* (2004)
Fluorine	*Absidia cylindrospora*	Garon *et al.* (2004)
Chlorobenzene	*Pseudomonas putida*	Wenderoth *et al.* (2003)
Chlorinated solvents	*Dehalococcoides* spp	Aulenta *et al.* (2005)
Phenol	*Pseudomonas mendocina, P. fluorescens*	Heinaru *et al.* (2005)
Crude oil	*Acinetobacter baumannii* *Bacillus subtilis* *Pseudomonas aeruginosa*	Mishra *et al.* (2004) Das & Mukherjee (2006)
Metal working fluids	*Clavibacter michiganensis* *Rhodococcus erythropolis* *Methylobacterium mesophilicum* *Pseudomonas putida*	van der Gast *et al.* (2003)
Atrazine	*Clavibacter michiganese* *Pseudomonas* sp Undefined soil consortium	Newcombe & Crowley (1999) Runes *et al.* (2000)
Naphthalene	*Pseudomonas putida* *Cycloclasticus* sp.	Piskonen *et al.* (2005) Miyasaka *et al.* (2006)
Dichloroethenes	*Bacillus* sp., *Pseudomonas* sp, *Acinetobacter sp.*	Olaniran *et al.* (2006)
3-chloroaniline	*Comamonas testosteroni*	Boon *et al.* (2000)
4-chlorophenol	*Pseudomonas putida.*	McLaughlin *et al.* (2006)
Tetrachloroethene	Undefined soil/groundwater consortium Undefined aquifer consortium	Major *et al.* (2002) Takeuchi et al. (2004)
PCP	*Trametes versicolor* *Phanerochaete chrysosporium, Bjerkandera adusta, Trametes versicolor*	Walter *et al.* (2004) Leštan and Richard (2006)

inoculum. Consortia may be constructed by mixing together known isolates, as in van der Gast *et al.* (2003) where a consortium consisting of *Clavibacter michiganensis*, *Rhodococcus erythropolis*, *Methylobacterium mesophilicum*, and *Pseudomonas putida* was used to effectively remove toxic components of metal working fluids in a bioreactor. However, a more 'black-box' approach may be taken, in which the composition of the consortium is highly complex and generally unknown. Such consortia are often composed of environmental samples which have been exposed to a particular pollutant for a length of time to allow selection for microbes competent in waste degradation. No effort is generally made to isolate particular members of the communities that develop. These consortia are then introduced to a polluted site. Barbeau *et al.* (1997) were able to remove 99% of PCB in a contaminated soil containing no native PCB degraders using a consortium developed by exposing slurry of a different soil to PCB in a bioreactor. A novel use of undefined consortia can be seen in Gentry *et al.* (2004) in which two soils are described, both contaminated with two forms of chlorobenzoate. Each soil could degrade only one of the two forms of chlorobenzoate, and each soil could degrade a different form. By inoculating each soil with a sample of the other, all of the chlorobenzoate could be removed from both soils.

Repeated application of inoculum can enhance the performance of bioaugmentation

The majority of bioaugmentation strategies employ a single, often massive, input of inoculum at the onset of the treatment, with no further subsequent inoculation taking place. However, one of the primary reasons for failure of bioaugmentation efforts is poor survival of inoculum following inoculation (Singer *et al.*, 2005). In an effort to avoid this potential source of failure, the inocula for bioaugmentation may be applied repeatedly over a period of time. Gilbert & Crowley (1998) found that repeated application of an *Arthrobacter* species to a PCB contaminated soil achieved degradation rates above that seen in untreated control soil. A single application of *Arthrobacter* had no effect. Newcombe & Crowley (1999) used an irrigation network to deliver repeated doses of an atrazine degrading bacterial consortium to *in situ* soil mesocosms, resulting in the removal of 75% of atrazine in the soil over 12 weeks. Again, a single application of the same consortium did not have any effect on the levels of pollution within the soil.

Inoculum may be applied as free cells or bound to a substrate

Most frequently, the inoculum for bioaugmentation is introduced into the environment as a suspension of free cells. This is usually done for ease of use, as inoculum is typically prepared by growing the cells in liquid media to high cell densities. The liquid nature of the inoculum may also aid in evenly distributing the inoculum throughout the environment. Using free cells of the *Pseudomonas aeruginosa* strain WatG, Ueno *et al.* (2006) were able to remove 50% of total petroleum products in oil contaminated soil over the course of one week; only 15% was removed from untreated soil, mostly via abiotic routes. Alternatively, inoculum may be introduced bound to a substrate. Ullah *et al.* (2000) grew the chlorophenol-degrading fungus *Coriolus versicolor* on wheat bran, noting high levels of enzyme production during growth. Wheat bran colonized with actively growing *Coriolus versicolor* was then introduced to water contaminated with chlorophenol, resulting in over 90% of the pollutant being removed over the course of 100 minutes. A slightly different approach was taken by Leštan & Richard (1996), where fungal mycelium or spores were suspended in an alginate hydrogel which was then used as a coating for pellets of a sawdust-cornmeal-starch mixture (SCS). Doing so was found to improve the stability of the inoculum prior to use, and the SCS supported the growth and survival of the inoculum once it was introduced into pentachlorophenol (PCP) contaminated soil. This approach enabled up to 90% of PCP in contaminated soil to be removed over the course of 12 weeks.

Failures in bioaugmentation

Whereas biostimulation is generally at least somewhat successful, bioaugmentation is much more prone to failure. Some instances where biostimulation was found not to enhance bioremediation of waste can be seen in Table 5. In many cases, this failure is due to the introduced organisms being unable to survive for any significant length of time after inoculation. This is perhaps to be expected: in biostimulation, waste degrading organisms are by definition native to the environment in which waste degradation is to take place, and are therefore likely to be well adapted to the prevailing conditions.

Table 5: Examples of work in which bioaugmentation failed to enhance the degradation of manmade pollutants.

Target for degradation	Inoculant	Reference
Diesel	*Actinomycete* sp	Margesin and Schinner 1997
Metolachlor	*Streptomyces* sp	Liu *et al.* 1990
Phenanthrene	*Pseudomonas* sp	Weir *et al.* 1995
Crude oil	Undefined marine consortium	Tagger *et al.* 1983
Biological nitrogen	*Microvirgula aerodenitricans*	Bouchez *et al.* 2000
Polycyclic Aromatic Hydrocarbon	Undefined bacterial consortium	Viñas *et al.* 2005
Polycyclic Aromatic Hydrocarbons	*Rhodococcus sp* *Acinetobacter sp.* *Pseudomonas sp.*	Yu *et al.* 2005
Metal cyanide	*Rhodococcus* spp.	Baxter and Cummings 2006

The isolates used in bioaugmentation on the other hand are often initially isolated from environments with conditions that may be quite different to the environment to which they are introduced, and may not be well adapted to survival. Survival of isolates has been found to depend heavily on the selection of the correct strain for inoculation (Thompson *et al.*, 2005). In soil, factors such as pH, soil pore size and other physicochemical conditions have all been found to affect the survival of exogenously added microbes (van Veen *et al.*, 1997). Predation by protozoa has also been found to be a major cause of the loss of inoculum during bioaugmentation (Bouchez *et al.*, 2000). Poor survival of inoculum, however, does not necessarily spell failure: Boon *et al.* (2000) found that, although the 3-chloroaniline degrading bacterium *Comamonas testosteroni* fell to low levels shortly after being inoculated into a 3-chloroaniline contaminated sludge bioreactor, bioremediation of 3-chloroaniline occurred much more rapidly than in the control reactor, proving that high cell densities of the inoculum do not necessarily need to be established for successful bioaugmentation.

Bioaugmentation may also fail due to competition between the introduced strains and the native microbial communities, especially if the native microbial communities already possess significant numbers of organisms with waste degrading phenotypes

(Margesin & Schinner, 1997; Tagger *et al.,* 1983; Weir *et al.,* 1995). Under these conditions, the introduced strains possess no selective advantage over the native microorganisms, and are therefore often out-competed. In these circumstances, it may be possible to remove this competition by sterilising the target site prior to inoculation. This approach has been met with some success - sterilisation of soil contaminated with 2,4-dichlorophenol allowed a subsequently introduced strain of *Pseudomonas* to degrade 60% of the pollutant over 12 days, whereas in non-sterile soil, the amount of degradation following inoculation was not significantly different to control soil (Goldstein *et al.,* 1985). However, sterilization prior to inoculation is not always appropriate: quite apart from the fact that sterilization would be very difficult to achieve *in situ* on large scales, in some cases an active native community was found to be necessary for the introduced strains to be able to remediate the pollutant. Walter *et al.* (2004) found that if the fungus *Trametes versicolor* was inoculated into sterile soil contaminated with PCP it could not mineralize the pollutant; if the fungus was inoculated into non-sterile soil, however, PCP was rapidly removed.

Combination of biostimulation and bioaugmentation.

Although biostimulation and bioaugmentation are frequently used in a mutually exclusive manner, there is no reason why they cannot be used in conjunction, with, for example, bioaugmentation providing the metabolic potential for degradation, and biostimulation supporting the growth and activity of the inoculum. Stallwood *et al.* (2005) showed that adding both *Pseudomonas* strain ST41 and minimal media to oil contaminated Arctic soil together was more effective at bringing about biodegradation than the addition of either of those alone. Major *et al.* (2002) found that biostimulating the native communities in trichloroethene (TCE) contaminated groundwater led to rapid partial degradation of the pollutant to *cis*-1,2-dichloroethene (cDCE). However, the native communities were unable to fully mineralize the pollutant to ethane, so bioaugmentation with a consortium of bacteria able to dechlorinate cDCE was required in addition to biostimulation to fully remove TCE from contaminated groundwater. Neither biostimulation or bioaugmentation have so far been investigated as techniques for bioremediating solid plastic waste. However, the fact that these techniques have been successful in treating such a wide variety of wastes substrates suggests that they are worthy of investigation.

Molecular techniques for monitoring microbial communities

Environmental microbial communities are of indisputable importance in the degradation of manmade waste in the environment. It is clear that these microbial communities must be investigated in order to more fully understand the role that they play in waste remediation. It is now widely accepted that the classical, culture-based techniques for identifying and monitoring organisms in natural environments are insufficient for this task. It is estimated that less than 1% of bacteria (Torsvik & Ovreas, 2002; von Wintzingerode *et al.,* 1997), and fewer than 17% of the fungi (Bridge & Spooner, 2001) present in nature have been observed, either within their natural habitat or under laboratory conditions, suggesting that the vast majority of environmental microbes cannot be easily cultivated in the laboratory. This inability to culture the majority of organisms under laboratory conditions is thought to be a consequence of the fact that the majority of environmental organisms have nutritional requirements that are not satisfied by the standard media used in most laboratories. Since the majority of PU degrading organisms identified so far have been isolated from environmental samples, relying on culture based techniques is not only likely to result in many non-cultivable PU degraders being overlooked, but will also make it difficult to monitor interactions within natural environments between different PU degraders and also between PU degraders and non-degrading organisms.

There are a number of techniques for monitoring microbial communities that are not dependent upon the cultivation of microbes. These techniques instead rely on various molecular markers present in microorganisms, and can be grouped into two broad categories: nucleic acid based techniques, and non-nucleic acid based techniques.

Nucleic acid based techniques for monitoring microbial communities.

The most frequent targets of molecular techniques are the nucleic acids, in the form of DNA and RNA. Not only are these molecules present in every life form on Earth, the information encoded within them allows accurate identification of organisms by phylogenetics. A number of techniques that exploit DNA and RNA have been developed. Some of these techniques allow profiles of entire communities to be generated, whereas others make it possible to monitor specific members within a community.

Ward *et al.* (1990a) were able to use DNA to characterise microbial communities in hot springs by first generating a clone library of PCR amplified 16S rDNA sequences, followed by sequencing of members of the library to determine the identities of the microbes present. An alternative technique that makes use of nucleic acids in whole community analysis is denaturing gradient gel electrophoresis (DGGE), which is described in detail later.

Since some nucleic acid sequences are highly specific to a particular organism, or a specific group of organisms, targeting such sequences makes it possible to follow microbes possessing them even in a background of many other non-target organisms. One method of targeting such sequences is to use labelled (usually with fluorescent dyes) oligonucleotide probes, small sequences of nucleic acids that are complementary to the sequence of interest. These probes can then be used to visualize whole cells containing these sequences under a microscope, as in fluorescent in situ hybridisation (FISH). In this way, target microbes may not only be enumerated via counting, but their relationships to other microbes within structured communities (such as biofilms) can be observed (Theron & Cloete, 2000). Alternatively, these probes can be used to interrogate the DNA profiles generated using techniques such as DGGE. Teske *et al.* (1996b) used this technique to monitor the sulphate reducing bacteria populations within a stratified water column.

Organisms possessing specific sequences can also be monitored by amplifying these sequences using PCR, followed by analysis of the PCR products generated. Using this approach, Cullen & Hirsch (1998) were able to detect a transposon-containing organism artificially introduced into soil when it was present as only 10^{-7} of the total soil population.

Most of the methods described above require an initial step where DNA is extracted from the entire microbial community. This is often the Achilles heel of such techniques, as in many cases DNA is not extracted with equal efficiency from all of the organisms present. This can lead to the production of a biased picture of the community being studied. Moreover, the DNA extraction method employed can influence the community profiles generated (Krsek & Wellington, 1999). Furthermore, the various biases associated with PCR may be an additional source of error when using techniques employing the polymerase chain reaction, which are described in one of the following sections. Therefore, care must be taken when interpreting data obtained using nucleic-acid based techniques.

Non-nucleic acid based molecular techniques for monitoring microbial communities.

In order to avoid the biases associated with nucleic acid based monitoring techniques, other molecular markers may be targeted. Although these markers often lack the specificity of nucleic acids, they may still offer sufficient discrimination to achieve some level of community characterisation. Like the nucleic acid based techniques, such markers may be used to both describe entire communities, as well as allowing specific community members to be monitored.

Phospholipid fatty acid (PLFA) analysis is often used to generate general profiles of microbial communities. The exact chemical composition of the PLFA molecules in the membranes of microbial cells varies between different taxonomic groups. Therefore, by measuring the relative quantities of each type of PLFA extracted from a microbial community, the general composition of the community can be determined (Kozdroj & van Elsas, 2001). Ibekwe *et al.* (2001) used this technique to monitor changes in a soil population in response to exposure to fumigants. Unfortunately the taxonomic resolution of PLFA is quite low, and there is usually insufficient variation in these molecules to allow species-level characterisation (Kozdroj & van Elsas, 2001).

Specific organisms within a community, such as inoculants added during bioaugmentation, may be monitored in one of two ways. By using labelled antibodies raised against components of target cells, these cells can be visualised microscopically (Theron & Cloete, 2000). Alternatively, metabolites produced only by the target organisms can be used to monitor those organisms. Unfortunately, few easily distinguishable, unique metabolites exist, and it is often necessary to engineer a metabolic marker, such as green fluorescent protein (GfP) or luciferase into the organism of interest (Jansson & Prosser, 1997; Mohlenhoff *et al.*, 2001; van Elsas *et al.*, 2000). For example Mishra *et al.*(2004) were able to follow the fate of *lux* tagged *Acinetobacter baumanni* following its addition to crude oil contaminated soil, and McLaughlin *et al.* (2006) were similarly able to monitor the survival of GfP tagged *Pseudomas putida* during an attempt to bioremediate 4-chlorophenol in soil. Of course, both the generation of specific antibodies and the insertion of genetic markers require that the organism of interest be isolated and cultivated, which limits the applications of these techniques to cultivable organisms.

Because they avoid the biases inherent to nucleic acid extraction, it would be preferable to employ non-nucleic acid based molecular techniques whenever possible, such as when community characterisation based on broad taxonomic grouping is required. For

example, PLFA analysis could be used to monitor the response of a microbial community to the addition of PU. These techniques may also be of use in monitoring degraders that have already been isolated.

However, when specific information is required, such as in the identification of community members, or if the behaviour of an organism needs to be followed with a high degree of sensitivity, nucleic acid-based techniques should be employed.

The use of the polymerase chain reaction in microbial community analysis

The polymerase chain reaction is used in a variety of culture-independent techniques that employ nucleic acids as a substrate for microbial community analysis. Most frequently, PCR is used to generate large numbers of specific DNA fragments (amplicons) from a sample of DNA extracted from the microbial community of interest. These amplicons are then analysed using a variety of techniques, the results of which are used to gain knowledge of the composition of the microbial community. PCR is not, however, without its limitations and biases, which must be taken into account when using methods that employ PCR.

The strengths and limitations of the polymerase chain reaction in microbial community analysis

PCR is often employed for the extreme sensitivity it offers, which allows for the detection of very small amounts of the target sequence of interest (van Elsas *et al.*, 2000; Mohlenhoff *et al.*, 2001). By employing highly variable sequences, PCR can also be highly specific, which makes it a useful technique for monitoring the fate of specific members within a microbial community, even against a background of vastly more numerous non-target sequences (Cullen & Hirsch, 1998). Conversely, by choosing relatively highly conserved sequences as the target for PCR, or by using degenerate primers, it is possible to generate amplification products for the majority of the members of a microbial community, making PCR useful as a step in whole-community microbial analysis (Nicolaisen & Ramsing, 2002).

Although highly useful for the reasons stated, PCR is subject to a number of biases and limitations. It is highly sensitive to the random variations that are inherent to virtually

all experimental techniques (Crotty *et al.,* 1994). Insufficiently stringent reaction conditions or fortuitous sequence homology can lead to the amplification of non-target sequences, which may produce misleading data (Don *et al.,* 1991). Unequal amplification of target sequences, due to differences in gene number (Haugland *et al.,* 1999) or gene architecture (Farrelly *et al.,* 1995) may make it difficult or impossible to relate biomass to the amount of PCR product generated, hence limiting the use of PCR as a method of quantifying biomass. PCR is also highly sensitive to the presence of inhibitory compounds such as humic or fulvic acids which is especially problematic when analysing environmental samples, as such samples are often contaminated with these compounds (Steffan *et al.,* 1988).

Although the disadvantages appear to outweigh the advantages, there are many techniques for reducing the impact of the errors to which PCR is prone. For example, non-specific priming events can be reduced by employing more stringent PCR conditions, or using a 'touch-down' regimen (Don *et al.,* 1991). The addition of DNA denaturants can also reduce spurious amplification events (Varadaraj & Skinner, 1994). The presence of PCR inhibitors can be reduced by careful optimisation of the DNA extraction procedure (Krsek & Wellington, 1999; Steffan *et al.,* 1988). If every effort is therefore made to reduce sources of error, PCR has the potential to be employed to good effect.

Microbial community analysis: techniques employing PCR amplicons

Most of the techniques that employ PCR amplicons as the substrate for community analysis operate by separating such amplicons in a length- or sequence-dependent manner. Since each member of the community should, ideally, generate amplicons with different length or sequence, examining the patterns of amplicon separation makes it possible to make inferences about the composition of a microbial community. Examples of the techniques employed to separate PCR amplicons include:

i.) Temperature gradient gel electrophoresis (TGGE), is a technique whereby a temperature gradient across a polyacrylamide gel is used to separate amplicons according to sequence (Heuer *et al.,* 1997).

ii.) Single strand conformational polymorphism (SSCP). Separates amplicons according to size by running them as single stranded molecules on polyacrylamide gels. The single stranded molecules adopt secondary structures, which retards their progress

through the gel – since secondary structure is influenced by sequence, sequence in turn influences the migration behaviour of the amplicon (Giraffa & Neviani, 2001). Unfortunately, this technique is prone to producing multiple bands for any given species, making data analysis more complicated.

iii.) Length heterogeneity PCR (LH-PCR). This technique separates amplicons according to size. LH-PCR has been used to monitor fungal community changes in response to the addition of fumigants to soil (Ritchie *et al.,* 2000). LH-PCR lacks the resolution associated with sequence-based separation techniques, as length variations have fewer combinations and permutations than sequence variations.

iv.) Automated Ribosomal Intergenic Spacer Analysis (ARISA). A variation on LH-PCR, ARISA generates microbial community fingerprints based on the length of PCR amplified intergenic spacer regions in the rRNA gene complex. Fluorescently labelled PCR primers allow for the automated screening of community PCR products, reducing workload and allowing for rapid, high throughput analysis of microbial communities (Fisher & Triplett, 1999; Gleeson *et al.*, 2005).

A further technique for separating PCR amplicons exists. It offers greater resolution than TGGE (Farnleitner *et al.,* 2000), and is less prone to generating multiple bands for single species than SSCP. This technique is denaturing gradient gel electrophoresis (DGGE).

Denaturing gradient gel electrophoresis

DGGE is increasingly used for profiling and characterising microbial communities. The fundamental principle of this technique is that the melting behaviour of a specific DNA fragment is dependent upon the sequence of that fragment (Fischer & Lerman, 1983). Fragments of DNA (in the form of PCR amplicons) are applied to a modified polyacrylamide gel and electrophoresed. As the DNA fragment migrates through the gel, it is exposed to increasing concentrations of denaturant (a mixture of urea and formamide). At some point, the concentration of denaturant will cause the melting of a portion of the (double stranded) DNA molecule. Upon this transition, the electrophoretic mobility of the molecule is reduced, and migration ceases. Since the sequence of the DNA sample loaded will influence the melting behaviour, it will determine the point at which migration stops. In this manner, DNA can be separated

according to sequence. By applying samples containing multiple types of DNA fragments, such as those amplified from an environmental sample, DGGE can be used to estimate the diversity within that sample (inferred from the number of bands). These profiles can also be used to identify the members of a community, which is accomplished by the sequencing of DNA extracted from the DGGE bands, or by hybridisation with oligonucleotide probes (Muyzer & Smalla, 1998).

There are many advantages to employing DGGE as a method of microbial community analysis. These include the culture-independence of the method, which means that the biases associated with culturing are avoided (Muyzer & Smalla, 1998). DGGE is capable of detecting relatively minor members of a community, with members present at less than 0.1% of the total community visualised on a DGGE profile (van Elsas *et al.*, 2000). By selecting the appropriate primers, DGGE can be used to generate whole-community profiles or, alternatively, can be used to monitor the presence of particular members of a microbial community. DGGE is capable of resolving DNA sequences differing by only a single base pair in greater than 95% of cases (Myers *et al.*, 1985), which makes it possible to distinguish between organisms at the species and sub-species level (if sufficiently variable sequences are employed).

The sensitivity, resolution, rapidity, and culture-independence of this technique make it especially useful for the characterisation of complex communities containing members not amenable to cultivation. Consequently, DGGE has been employed most frequently in environmental studies. Since many of the potential PU degraders are environmental organisms, this technique is likely to be useful in isolating, characterising, and monitoring such organisms.

Applications of DGGE in microbial community analysis

Although DGGE is broadly defined as a technique for determining the composition of microbial communities, there are many variations in how it is used. The main uses of DGGE in community analysis are listed on the next page.

DGGE to monitor changes in microbial populations over time.

DGGE is often used to monitor changes in a particular population over time, or in response to changing conditions (either naturally or artificially induced). This usually involves comparing the banding patterns of DGGE-generated community profiles from before and after the change. An example of temporal variation can be seen in Kowalchuk *et al.* (1998) where seasonal changes in ammonia oxidising bacterial communities in a shallow lake were investigated. An example of the effect of changes in local conditions on community structure is detailed by Ishii *et al.* (2000), in which the authors describe how physicochemical changes during a composting process were mirrored by changes in the resident microbial population. Examples of artificially induced changes include the response of soil microbial communities to fumigants (Ibekwe *et al.*, 2001), heavy metals (Macnaughton *et al.*, 1999a) and crude oil (MacNaughton *et al.*, 1999b). This approach could be used to monitor the effects of the addition of PU substrates on environmental communities. This may aid in the identification of PU degraders, and may also allow the interactions between them to be investigated under natural and artificial conditions.

DGGE to compare different populations

DGGE may also be used to compare different populations that vary in either spatial location or prevailing environmental conditions. By using this approach, Ferris *et al.*, (1996) were able to detect differences between hot spring bacterial mat communities growing at different temperatures. A more sophisticated method of community comparison can be found in Ovreas *et al.* (1997), where bacterial- and archeal-specific profiles were generated separately (using bacterial- and archeal-specific PCR primers) in order to compare the archeal and bacterial populations at various depths in a lake. By using the number of bands in each profile as an indicator of diversity, the authors discovered that bacterial diversity decreased with depth, whilst archeal diversity increased with depth.

DGGE to link organisms to a 'process' of interest

DGGE is often used to imply the involvement of organisms in certain processes. This usually involves generating a DGGE community profile from DNA extracted from the site at which the process of interest is taking place. The species present are then identified via band sequencing or oligonucleotide probing. Such an approach has been used to infer the involvement of certain organisms in chitin decomposition in grassland (Krsek & Wellington, 2001), in disease in marram grass roots (Kowalchuk *et al.*, 1997), and in the degradation of a variety of pollutants (Piskonen *et al.* 2005; Miyasaka *et al.*, 2006). Unfortunately, this approach only implies correlation between the presence of an organism and a particular process. Further observations are required to establish causality. For example, Rolleke *et al.* (1996) found that some of the organisms determined by DGGE to be present on degraded artworks also possessed phenotypes (such as hyphal growth and the ability to metabolise paint components) that could theoretically contribute to the degradation observed.

DGGE may aid in designing culture conditions for isolating microbes from the environment.

By allowing the identities of the members of a given microbial community to be determined (e.g. via band sequencing), DGGE makes it possible to predict the phenotypes of these microbes (assuming these, or similar microbes have been previously characterised). By utilising this kind of information Teske *et al.* (1996a) were able to devise the necessary enrichment protocols to obtain pure monocultures from a bacterial co-culture.

DGGE in monitoring the persistence of specific members of microbial populations

It is sometimes desirable to follow the fate of specific microorganisms following their introduction into a heterogeneous microbial population, for example during bioaugmentation. By monitoring the presence and intensity of the band representing this organism within the community profile over time, its persistence within the community

can be assessed. Gomes *et al.* (2005) were able to use DGGE to monitor the fate of *Pseudomonas putida* following its inoculation into naphthalene contaminated soil: *Pseudomonas putida*, which was able to degrade and use naphthalene as a carbon source became firmly established as a dominant member of the soil community following its introduction. This technique cannot, however, be used in excessively complex communities, as such communities produce a continuous smear in DGGE. Nor can it be used on communities containing resident organisms with band migration behaviour identical to that of the test organism, as occurred in van Elsas *et al.* (2000) in which the authors attempted to follow the fate of *Trichoderma harzianum* following its introduction into soil, but were unable to distinguish *Trichoderma harzianum* from another native member of the soil community with the same DGGE migration behaviour.

Choice of template for DGGE

When generating community profiles via DGGE, the choice of nucleic acid template used to generate the DGGE fragments can have a strong influence on the profile produced. Templates may be broadly characterized into two main categories: they may be grouped on the basis of the nature of the nucleic acid of which they are composed, that is DNA or RNA. Alternatively, they may be grouped according to their sequence: sequences within rRNA genes or sequences within metabolic genes are those most commonly targeted.

Each template type will generate a profile that will reflect a different aspect of a community, and so the choice of template should reflect the aims of the experiment being carried out.

The influence of nucleic acid choice when generating community profiles.

When comparing DGGE profiles generated from community DNA or RNA, differences in the banding patterns produced from each type of template are often observed. The amount of target DNA in an organism is relatively constant, whilst the amount of a specific RNA fragment is dependent upon the rate of its production and degradation. Hence, DNA-generated profiles are thought to be a reflection of the

relative abundance (in terms of biomass) of organisms within the community, whereas RNA profiles are thought to represent levels of transcription, and hence the metabolic activity of community members (Muyzer & Smalla, 1998). Support for this hypothesis can be found in Wawer *et al.* (1997). The intensity of bands (in this case run on an agarose gel rather than DGGE) generated from RNA transcripts of the [NiFe] hydrogenase gene were found to be greatest when the RNA was extracted from actively growing cells. Conversely, the intensity of bands generated directly from the gene itself (i.e. from DNA) was found to be dependent on the amount of biomass present.

The implications of this can be seen in Teske *et al.* (1996b), in which microbial community profiles were generated from samples obtained from different depths in a fjord water column. DGGE fragments were generated from a section of the 16S rRNA molecule and also from the same sequence in its corresponding gene. A number of bands in the DNA-derived profile were not detected in the RNA-derived profile and vice-versa, implying that numerical dominance and metabolic activity are not necessarily equivalent.

The influence of sequence choice when generating community profiles.

Although, theoretically, any nucleotide sequence may serve as a target for DGGE, in the vast majority of cases sequences within genes are used. These sequences may be divided into two main groups – those within metabolic, protein-encoding genes and those within ribosomal RNA genes

Ribosomal RNA genes

The sequences most frequently targeted in DGGE are those belonging to the ribosomal RNA genes. Most often, sequences within the small subunit RNAs are employed, although the large subunit RNAs (Mohlenhoff *et al.*, 2001) and the ITS regions (Scheinert *et al.*, 1996) within the rRNA gene complex have also been used. These genes contain both highly conserved and moderately variable regions (Giraffa & Neviani, 2001). This has allowed the design of primers capable of amplifying DNA from a wide range of species via binding to the conserved regions, whilst still allowing separation of these sequences via the less conserved regions. A further advantage of

using the rRNA genes is that in most cases phylogeny is assigned based on ribosomal RNA gene sequence. Hence, sequences retrieved from bands in a DGGE profile generated from rDNA can be used to interrogate phylogenetic databases, and are therefore useful for determining the identity of community members.

When ribosomal RNA molecules themselves are used as the template for DGGE (as opposed to the genes encoding them), the profiles generated can be used as a general measure of transcriptional activity (Teske *et al.*, 1996b). However, these profiles cannot be used to monitor particular metabolic activities.

Metabolic genes

Although useful for generating profiles that represent most of the members of a microbial community, ribosomal RNA genes are often too highly conserved to allow profiles of a specific subset in a mixed microbial community to be generated. In these cases, the less highly conserved metabolic genes can be employed. By using metabolic gene sequences, communities can be profiled based on phenotype rather than phylotype. For example, Nicolaisen & Ramsing (2002) targeted a sequence within the ammonia monooxygenase gene in order to generate a profile of the ammonia oxidising bacteria (AOB) population within a soil community. It was found that these sequences targeted ammonia oxidisers with much greater fidelity than did allegedly AOB-specific rDNA sequences.

By using mRNA transcripts of metabolic genes as a template for DGGE, particular metabolic activities can be monitored. For example, in Wawer *et al.* (1997) the authors detected changing levels of expression of the [NiFe] hydrogenase gene by members of a methanogenic bioreactor community upon incubation in a lactate medium. Since lactate is known to induce this gene, the results demonstrated that such DGGE profiles are capable of reflecting levels of gene transcription.

The ability of metabolic gene sequences to generate community profiles based on phenotype could theoretically be employed as a means of identifying possible PU degrading microbes. Unfortunately, such an approach would require a conserved sequence motif common to polyurethanase enzymes. Although sequences within the active sites of specific PU-degrading enzymes have been described (Stern & Howard, 2000), motifs common to all, or even the majority of polyurethanases have yet to be discovered.

Thesis aims and objectives

The primary aim of this work was to investigate the microbial communities in soil that are involved in the degradation of buried PU. Furthermore, the viability of using biostimulation and bioaugmentation to enhance the degradation of buried PU, with a possible long term aim of developing more efficient plastic waste remediation strategies was investigated. The following specific objectives were therefore identified:

Fungal communities associated with the degradation of polyester polyurethane in soil

1) To compare the fungal communities in two different types of soil to determine if soil type influenced the structure of the native soil fungal communities
2) To compare the fungal communities growing on the surface of PU buried in two different soils to determine if soil type influences the microbial communities that developed on the surface of buried PU
3) To identify the fungi colonising the surface of PU
4) To investigate if soil type influences the extent of degradation of buried PU

The effect of biostimulation on soil microbial communities and on the degradation of buried PU.

1) To investigate how the addition of yeast extract and/or Impranil to soil affected the structure of fungal communities in the soil and on the surface of PU subsequently buried in treated soil
2) To identify fungal isolates enriched after the addition of the PU-based substrate Impranil to soil.
3) To determine if the addition of yeast extract and/or Impranil to soil enhances the degradation of buried PU

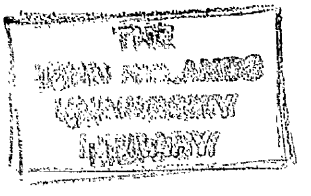
Degradation of PU buried in soil bioaugmented with PU degrading fungi

1) To determine what effect the addition of large quantities of wheat colonised by PU degrading fungal isolates to soil has on the structure of fungal communities within the soil

2) To monitor the survival of PU degrading fungal isolates following their inoculation into soil

3) To determine if PU buried in soil inoculated with PU degrading isolates was successfully colonised by these isolates after one month's burial

4) To investigate whether PU buried in soil inoculated with PU degraders was significantly more degraded than PU buried in uninoculated control soil



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Chapter 2:

Fungal communities associated with the degradation of polyester polyurethane in soil

Abstract

Understanding the role of microorganisms in the biodegradation of plastic waste in the environment is critical in managing and developing effective waste remediation strategies. In this work, *in situ* soil fungal communities involved in the biodegradation of polyester polyurethane (PU) coupons were investigated. PU coupons were buried in two different sandy loam soils; one was acidic (pH 5.5) with a low organic carbon content and the other was more neutral (pH 6.7) with a higher organic carbon content. After 5 months burial the fungal communities on the surface of the PU were compared with the native soil communities using culture-based and molecular techniques. Putative PU degrading fungi were very common in both soils, as <45% of the fungal colonies could clear the colloidal PU dispersion Impranil on solid medium. Denaturing gradient gel electrophoresis (DGGE) showed that fungal communities on the surface of PU were less diverse than in the soil, and only a small number of species in the PU communities were detectable in the soil, indicating that only a subset of the soil fungal communities colonised the PU. Soil type influenced the composition of the PU fungal communities and *Geomyces pannorum* and an *Alternaria sp.* were the dominant species on the PU from the acidic and neutral soils respectively. PU was highly susceptible to degradation in both soils, losing up to 95% tensile strength after five months burial. Very few PU degrading bacteria were detected on the plastic and it is concluded that fungi were primarily responsible for PU degradation.

Introduction

The fate of plastics in the environment is growing in importance as the worldwide production of synthetic polymers continues to rise, resulting in an increased environmental burden through the generation of plastic waste. More than 140 million tonnes of plastic were produced worldwide in 2001 (Shimao, 2001) and the proportion of household waste made up of plastic in the average American home increased from 3-5% of total waste in 1969 (Eggins *et al.*, 1971) to more than 30% in 1995 (Kawai, 1995a). The majority of plastic formulations are both physically and chemically robust, characteristics that have serious implications for waste management (Bouwer, 1992). However, several families of plastics can undergo degradation in the environment, and an understanding of how this degradation occurs may aid in the development of strategies to exploit these processes for waste management purposes.

Although it is recognised that many factors contribute to the degradation of plastics in the environment, microorganisms are generally considered to be responsible for the majority of the degradation that occurs (Bentham *et al.*, 1987). Abiotic factors such as photodegradation or hydrolysis usually play a much more minor role in plastic degradation (Griffin, 1980).

Biodegradation is the term most commonly used to describe the breakdown of compounds by microorganisms. Examples of plastics vulnerable to biodegradation include the polyhydroxyalkanoates, polycaprolactone, polylactic acid, and polyvinyl alcohol (Sabev *et al.*, 2006a; Shimao, 2001). This work investigates the degradation of the biodegradable synthetic polymer polyester polyurethane (PU) in soil. PU is used in a variety of industrial and commercial applications, including insulating foams, fibres, and synthetic leather and rubber goods. The presence of ester and urethane linkages in the backbones of these compounds makes them susceptible to hydrolysis by enzymes secreted by microorganisms during nutrient acquisition (Nakajima-Kambe *et al.*, 1999). Cleavage of intramolecular bonds within the PU leads to the weakening of the plastic, with the tensile strength of the material inversely proportional to the extent of degradation (Dale & Squirrel, 1990). The breakdown products of PU degradation may act as a carbon source for a variety of microbes (Akutsu *et al.*, 1998; Kawai, 1995b; Pathirana & Seal, 1985), allowing PU to serve as a growth substrate on which organisms may proliferate.

Many PU degrading fungi (Barratt *et al.*, 2003; Bentham *et al.*, 1987; Crabbe *et al.*, 1994; Sabev *et al.*, 2006b) and bacteria (Akutsu *et al.* 1998; Howard *et al.*, 1999; Kay *et al.*, 1991) have been isolated from a variety of environments, indicating that there are potential reservoirs of PU degrading organisms widespread in the environment. However, most of these studies have focused on the isolation of potential PU degrading organisms using laboratory-based enrichment and screening strategies. Little attention has been given to the ecology of PU colonisation and degradation *in situ* in the soil. It is not yet known which fungi predominate in PU degradation under different environmental conditions and whether the fungal strains influence the final amount of biodegradation.

In this study, fungal communities that developed on the surface of PU buried *in situ* in two different soils for five months were investigated using culture-based and molecular techniques. The colonisation and degradation of PU in both soils were compared and the dominant organisms on the PU surface were identified by sequencing the ITS region of the fungal rRNA gene complex.

Materials and methods

Fabrication of PU coupons

PU pellets (Elastogran, U.K.) were pressed at 180°C using an electric press (Bradley & Turton Ltd., Kidderminster, UK) into sheets with a thickness of 1.5mm. Rectangular coupons of PU measuring 6.25 x 4.2 x 0.15 cm were cut with a scalpel, giving a total surface area available for colonisation of 55.5 cm^2.

In situ burial of PU coupons in soil

PU coupons were surface sterilised via immersion in 70% (v/v) ethanol. Coupons were then buried in two types of garden soil with a sandy loam texture, at two separate locations, both in South Manchester, UK. Coupons were buried in a vertical position approximately 5cm apart, such that the tops of the coupons were approximately 6 cm below the surface. Six PU coupons were buried in each soil type. Coupons remained buried for the five-month period January to May 2003.

Recovery of biomass from the surface of buried PU

Biomass was recovered from PU coupons after 5 months burial in the two soils in order to analyse the fungal communities growing on the surface. Loosely adhered soil particles were first removed by agitating the PU coupons in sterile phosphate buffered saline (PBS) (Sambrook & Maniatis, 1989) for 5 min. Coupons were then submerged in 20 ml sterile PBS, and the biomass was scraped from both sides of the PU into the PBS using a sterile scalpel blade. An aliquot of 1 ml of this biomass suspension was used to determine microbial viable counts. The remainder was centrifuged at 3000g for 30 min at 4°C, the supernatant was discarded, and the biomass was used for DNA extraction and DGGE analysis.

Tensile strength determination of buried PU

The tensile strength of PU coupons after burial in soil for five months was determined to assess the extent of degradation. Once microbial biomass had been removed from the surface of the PU, coupons were cut into strips measuring 4.5 x 0.5 x 0.15 cm. Replicate strips (n=15) were stretched at a rate of 200 mm min^{-1} and the tensile strength determined using an Instron 4301 (Instron Ltd, Swindon UK). Unburied PU strips were used as a control.

Fungal viable counts

Viable counts of fungi in the soil and on the surface of buried PU were determined on solid agar media. Samples of soil in which the PU was buried, and samples of biomass recovered from the surface of buried PU, were serially diluted in PBS and spread onto soil extract agar plates (SEA) (Alef & Nannipieri, 1995) and Impranil agar plates (Crabbe *et al.*, 1994). Colonies were counted after 5-7 d incubation at 25°C. Total fungal viable counts were enumerated on SEA, whilst putative PU degrading fungi were enumerated as colonies producing zones of clearance on Impranil agar. Both media included 50 μg ml^{-1} of chloramphenicol to inhibit bacterial growth. The number of Impranil degrading fungi was then calculated as a percentage of the total number of colonies recovered. Bacterial populations were analysed in the same way, with media containing 50μg ml^{-1} nystatin to inhibit fungal growth.

DNA extraction

The FastDNA SpinKit for Soil (Q-Biogene, California U.S.A) was used to extract total DNA from 0.4 g soil samples, or 0.5 g samples of biomass (wet weight) recovered from the surface of buried PU. To remove all traces of PCR inhibitory compounds, 20 μl of extracted DNA was run for ca. 15 min on a 1.0% (w/v) agarose/TAE gel. Bands of genomic DNA were then excised, and DNA was recovered using the Nucleospin Extract II gel extraction kit (Machery-Nagel, Düren Germany).

PCR amplification of fungal community DNA

PCR was employed to generate the necessary DNA fragments for fungal community Denaturing Gradient Gel Electrophoresis (DGGE) analysis. PCR DNA template consisted of approximately 50 ng per reaction of extracted DNA. Biomix Red PCR master mix (Bioline, London U.K) was employed in all reactions. Primers were present in each reaction at a concentration of 1 μM. Fungal DGGE fragments were generated using the universal fungi-specific GM2/JB206c primer set (GM2: 5'-CTGCGTTCTTCA TCGAT-3', JB206c: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGG GGGAAGTAAAAGTCGTAACAAGG-3', Brookman *et al.*, 2000), which amplifies the ITS1 region found in the fungal rDNA gene complex. The PCR regime employed was as follows: 94°C initial denaturation for 5 min; 20 'touchdown' cycles of 94°C for 30 s, annealing for 30s at 59 to 49°C with annealing temperature being reduced by 0.5°C per cycle, extension at 72°C for 45 s; 30 cycles at 95°C for 30 s, annealing at 49°C for 30 s and extension at 72°C for 45 s; 1 final extension at 72°C for 5 min.

DGGE analysis of fungal communities in the soil and on the surface of buried PU

The composition of the fungal communities in the soil and on the surface of buried PU was compared using DGGE (Muyzer *et al.*, 1993). The D-Code universal mutation detection system (Biorad, Herts U.K.) was employed. Gels measured 16 cm x 16 cm x 1 mm and contained 10% (v/v) acrylamide in 1 x TAE (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA). A gel with a denaturant gradient of 25-55% parallel to the direction of DNA migration was used. For all gels, approximately 500 μg of PCR product was used per lane; gels were run in 1 X TAE buffer at a constant temperature of 60°C for 16.5 h at 42 volts. After electrophoresis was complete, gels were stained with SybrGold (Molecular Probes, Netherlands) for 45 min and photographed under ultraviolet light. Gels were analysed using the Phoretix 1D software package (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) to create densitometric representations of the banding patterns in each DGGE lane. All densitometry profiles were normalised against the background.

Identification of fungal isolates with putative PU degrading activity.

Putative PU degrading fungal isolates were recovered from the surface of soil buried PU. They were detected by their ability to produce zones of clearance on Impranil plates and then grown in malt extract broth (MEB) (Oxoid, UK) until sufficient biomass was generated. Genomic DNA was extracted using the method described by Anderson *et al.* (1996). The ITS1-5.8S-ITS2 region of the fungal rDNA gene complex was then PCR amplified using the universal fungi-specific ITS1/ITS4 primer set (ITS1: 5'-TCCGT AGGTGAACCTGCGG-3, ITS4: 5'-TCCTCCGCTTATTGATATGC-3', White *et al.*, 1990) using the Expand High Fidelity PCR System (Roche, Mannheim, Germany). The PCR regime was as follows: 94°C for 3 min; 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min; a final extension at 72°C for 5 min. PCR products were then sequenced using in-house facilities. Sequences were used to interrogate the EMBL database using the blastn algorithm.

Identification of fungi on the surface of PU via cloning and sequencing of ITS-1 DGGE products

In order to identify fungi on the surface of buried PU in a culture-independent manner, ITS-1 DGGE fragments generated from DNA extracted from fungal communities on the surface of buried PU were cloned into the pGEM-Teasy plasmid (Promega, UK) and transformed into *E.coli* strain JM109 as per manufacturer's instructions. Individual clones were screened using colony PCR to reamplify the ITS 1 fragments contained within them using the DGGE PCR regimen described above. These fragments were then run on a DGGE as described previously alongside whole PU community DGGE products. Clones producing bands that co-migrated to the same position as bands within the PU community profiles were then selected for sequencing. These sequences were then used to interrogate the EMBL fungal database as described previously.

Results

Soil chemical analysis

The two soils employed in this work were analysed for pH and for phosphorus, potassium, magnesium and organic carbon content (analysis carried out by Adas Laboratories, Wolverhampton, UK), and the results are shown in Table 1. The soils could be clearly distinguished by colour and texture with soil 1 being black and having a coarse texture, whilst soil 2 was much paler, with a fine, sandy consistency. Both were characterised as sandy loams. Soil 1 was more neutral with a pH of 6.7 and contained more organic carbon; this soil was designated 'neutral'. Soil 2 was more acidic with a pH of 5.5, and contained 45% less organic carbon; this soil was designated 'acidic'. Each soil had differing levels of phosphorus, potassium and magnesium.

Table 1: Chemical analysis of the soils in which polyester polyurethane coupons were buried.

	pH	Organic carbon (% w/w)	Phosphorus (mg/L)	Potassium (mg/L)	Magnesium (mg/L)
Soil 1 ('neutral')	6.7	7.85	37	171	244
Soil 2 ('acidic')	5.5	4.34	141	243	119

Total viable and putative PU degrading fungi recovered from soil and from the surface of buried PU.

The numbers of viable fungi and the prevalence of putative PU degrading fungi in the two soils and on the surface of PU coupons buried for five months were determined. Total viable counts were determined on SEA, whilst putative PU degrading fungi were enumerated as colonies producing zones of clearance on Impranil agar (Table 2). There was no significant difference in the numbers of viable fungi in the two soils ($p<0.05$), with either 6.3×10^5 (neutral soil) or 5.5×10^5 (acidic soil) colony forming units (cfus)

present per gram of soil. However, 5.5 fold more fungal cfus were recovered from the surface of PU buried in the acidic soil (5.5×10^3) compared to PU buried in the neutral soil (9.9×10^2). In the acidic soil there was no significant difference ($p>0.05$) between the percent of Impranil degrading fungi on the plastic (41.2%) compared with the soil itself (37.4%), whereas in the neutral soil there was a significant increase ($p<0.05$) in the percent of Impranil degrading fungi on the PU (58.5%) compared to the soil (45.1%), indicating that enrichment had occurred for Impranil degraders during growth on buried PU in the neutral soil. While the percentages of Impranil degrading fungi were greatest in both neutral soil and plastic buried in neutral soil, the greatest numbers of Impranil degraders were recovered from the surface of PU buried in the acidic soil (2.3×10^3 cm^{-2} of PU from acidic soil compared to 5.7×10^2 cm^{-2} on PU buried the neutral soil).

In conclusion, the ability to degrade colloidal PU was a very common property amongst the fungi in all of the communities investigated, ranging from 37.4% of the fungi in the native acidic soil communities to 58.5% in the communities growing on the surface of PU buried in neutral soil. Impranil clearing fungi were enriched for on the surface of PU buried in neutral soil but this was not the case for PU buried in acidic soil. However, PU buried in acidic soil could support a greater absolute number of both Impranil degrading and non-Impranil degrading fungi than in the neutral soil.

Table 2: Total viable numbers of fungi, and the percentage viable fungi able to degrade Impranil, in soil and on the surface of buried PU. In all cases n=3.

	Fungi in soil			Fungi on the surface of buried PU		
Soil Type	Viable Fungi (cfu g^{-1})	Impranil degrading fungi (cfu g^{-1})	% of colonies clearing Impranil	Viable Fungi (cfu $sq.cm^{-1}$)	Impranil degrading fungi (cfu $sq.cm^{-1}$)	% of colonies clearing Impranil
Neutral	6.3×10^5	2.9×10^5	45.1	9.9×10^2	5.7×10^2	58.5
Acidic	5.5×10^5	2.0×10^5	37.4	5.5×10^3	2.3×10^3	41.2

Community analysis using DGGE

Sequence dependent separation of PCR-amplified ITS1 rDNA using DGGE was used to analyse and compare the species composition of fungal communities in each type of soil (the native soil communities) and communities colonising the surface of the PU after 5 months of burial (Fig. 1). These profiles were subjected to densitometric analysis, where peaks represent bands, peak height is proportional to band intensity and pixel position on the y-axis denotes vertical position in the profile, with pixel position zero correlating with the top of the lane (Figure 2 a-d). This was done to aid in the comparison of band positions in each lane for each sample. The arrows indicate peaks that are present in both profiles being compared.

The different band migration behaviours seen when comparing DGGE profiles revealed clear differences in the species composition of all of the communities investigated (Figs 1 and 2a-d). At least 30 visible peaks were present in each native soil community profile (Fig 2a), indicating a considerable diversity of fungi in these consortia. Only 5 peaks were seen in both types of soil profile where both peaks exactly co-incided (Fig. 2a, arrows), but there was only one strong peak in both soil profiles (Fig 2a asterisk). Both native soil communities were therefore distinct in the fungal species present, with the majority of the detectable fungal species unique to each soil type. Replicate soil samples from both soils gave highly reproducible DGGE profiles (data not shown), indicating that there was spatial homogeneity in the fungal communities in these soils.

When the fungal communities on the surface of the plastic were compared to those in the soil (Figs 2b & 2c), fewer peaks were visible in profiles of plastic communities ($\leq$20) compared to soil communities ($\leq$30), indicating a lower diversity of fungi on the surface of the buried PU. Furthermore, many of the bands from the PU community profiles were not detectable in the corresponding native soil community DGGEs, with $\leq$5 bands in either of the PU-associated community profiles also visible in their repective soil profiles (Figs 2b and 2c, arrows). Thus, specific members of the native soil fungal communities were selected for during growth on buried plastic, with many of these fungi being minor members of the native soil communities.

The type of soil in which the PU was buried influenced the composition of the communities that developed on the surface of the PU coupons. A comparison of the

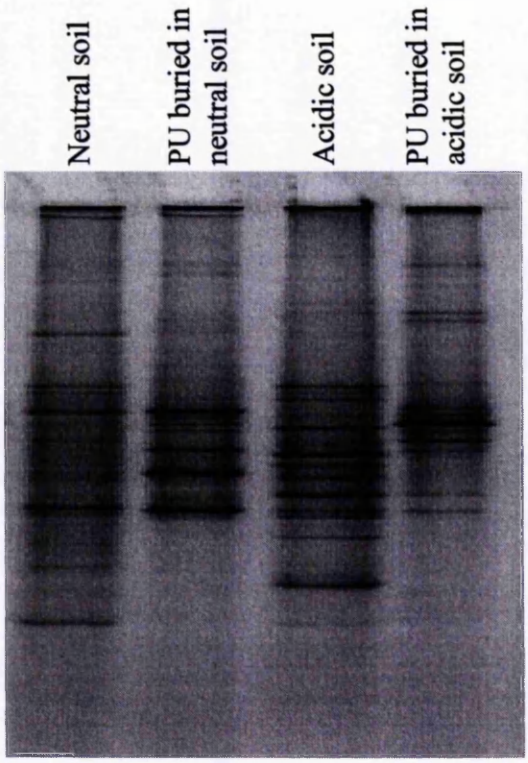

Figure 1: Comparison of DGGE profiles of soil fungal communities and fungal communities growing on the surface of soil buried PU.

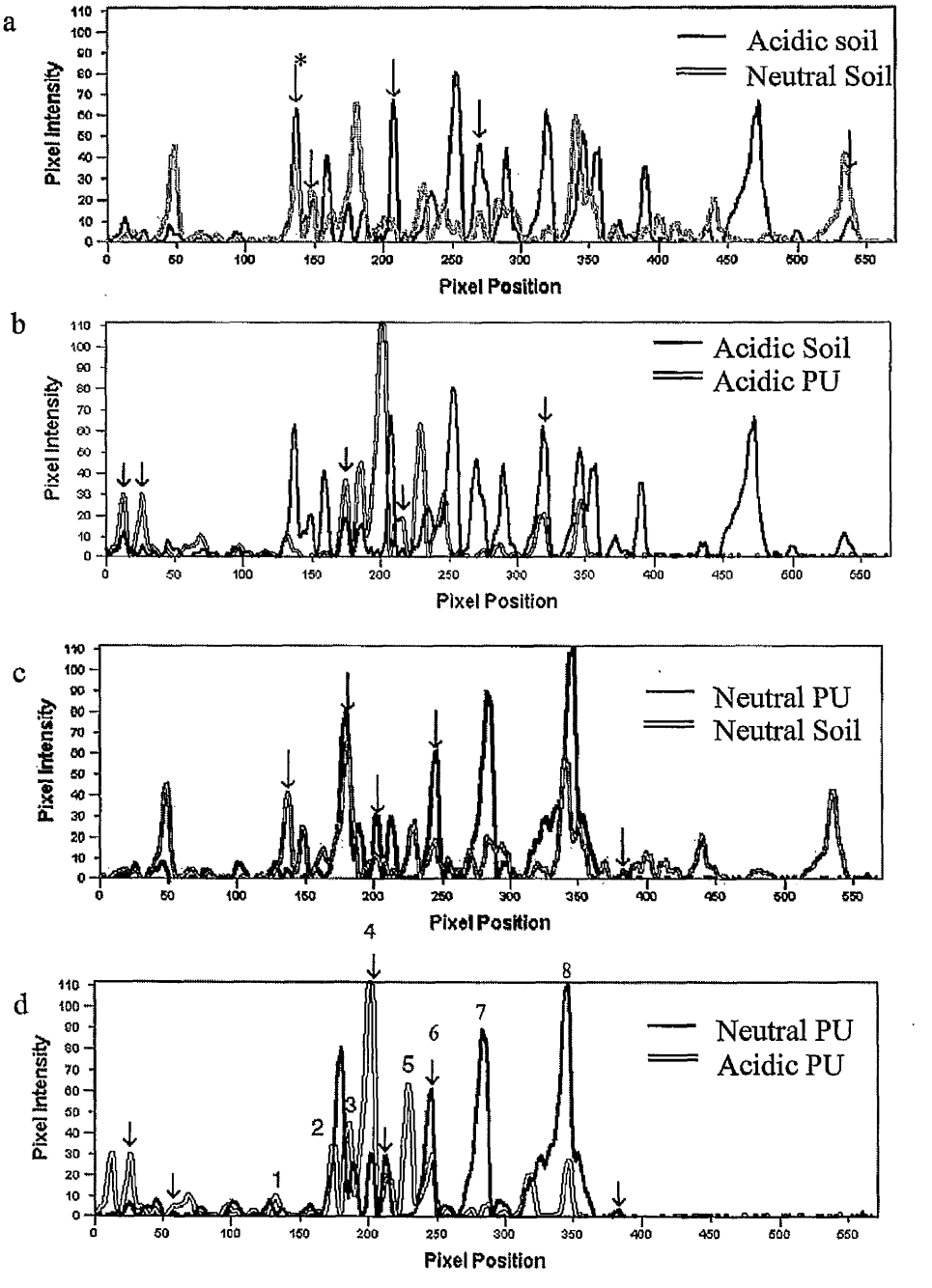

Figure 2: Densitometric comparisons of DGGE profiles of fungal communities in neutral and acidic soil and on the surface of buried PU. Each peak represents a band in the DGGE profiles. Arrows indicate peaks which occur in both of the communities compared. Compared were communities from (a) neutral soil and acidic soil, (b) acidic soil and acidic plastic (c) neutral soil and neutral plastic and (d) neutral plastic and acidic plastic. The numbers in (d) represent DGGE fragments that were subsequently cloned into *E. coli*, sequenced and identified. The asterisk in (a) indicates a peak that was strong in both soil community DGGE profiles.

DGGE profiles of fungal comunities recovered from the surface of buried PU in both soils (Fig 2d) demonstrated that only 7 peaks (indicated by arrows), from a total of ~20, were present in both PU community profiles.

Identification and DGGE analysis of isolates recovered from the surface of soil-buried PU

In order to identify cultivable fungi colonising the surface of buried PU, the most frequently occurring unique colony morphotypes were isolated from the SEA plates used to enumerate the viable fungi on the surface of buried PU. Isolates were sub-cultured onto Impranil agar to determine putative PU degrading ability, and their identities were determined via sequencing of the ITS1-5.8s-ITS2 rRNA gene complex. Isolates were recovered from PU buried in either acidic or neutral soil (Table 3).

In total, 9 distinct colony morphotypes were recovered, five from the PU buried in acidic soil and four from PU buried in neutral soil. The two most dominant fungi recovered from the PU buried in acidic soil were identified as *Geomyces pannorum* and a *Nectria sp BC11*. Fungi present in lower numbers on PU buried in acidic soil were *Cylindrocladiella parva, Penicillium inflatum,* and *Plectosphaerella cucumerin.* Of these, *G. pannorum, Nectria sp* and *P. inflatum* were able to clear Impranil. The most dominant fungal species recovered from PU buried in neutral soil was identified as an *Alternaria sp.*; also, *Penicillium venetum, Neonectria ramulariae* and *Penicillium viridicatum* were present in considerably lower numbers. Of these, the *Alternaria sp., N. ramulariae* and *P. viridicatum* were able to clear Impranil.

Each isolate was also subjected to DGGE, and its band position was compared to the DGGE profiles of the fungal communities growing on the surface of the buried PU (Fig. 3). All of the pure-culture isolates produced a single intense band after DGGE of their amplified ITS-1 sequence. Secondary bands were also visible, although these were much fainter indicating that no significant heterogeneities existed between ITS1 copies within a single organism.

Of the ten colony morphotypes recovered, *N. ramulariae, Nectria sp. BC11, Alternaria sp. 18-2* and *G. pannorum* migrated to the same position as bands seen in the PU community profiles (indicated by arrows in Fig 3). *G. pannorum* and *Nectria sp BC11* were the dominant cultivable fungi recovered from the PU buried in acidic soil (Table 3) and these isolates produced bands that co-migrated with the most intense

Table 3: Fungi isolated from the surface of neutral and acidic soil buried PU coupons. Isolates were identified via ITS1-5.8s-ITS sequence homology. GenBank accession numbers for each sequence are given in parentheses.

Species of isolate	Soil type in which PU was buried	Observed frequency of morphotype on SEA plates †	% homolgy	Impranil clearance‡
Plectosphaerella cucumerina (DQ779781)	Acidic	+	99.8	-
Penicillium inflatum (DQ779783)	Acidic	+	99.7	+
Nectria sp. BC11 (DQ779785)	Acidic	++	100	+
Cylindrocladiella parva (DQ779786)	Acidic	+	99.6	-
Geomyces pannorum (DQ779788)	Acidic	+++	99.2	+
Penicillium viridicatum (DQ779779)	Neutral	+	99.11	+
Neonectria ramulariae (DQ779780)	Neutral	+	99.1	+
Penicillium venetum (DQ779784)	Neutral	+	100	-
Alternaria sp.18-2 (DQ779787)	Neutral	+++	98.8	+

†Subjective measure of the frequency with which each morphotype was observed on the SEA plates: + = less than 5% of the colonies had this morphology; ++ = 10-20% of the colonies present have these morphologies; +++ = the dominant morphotype, representing >80% of colonies present on the plates.
‡ Ability of each isolate to produce clear zones on Impranil agar.

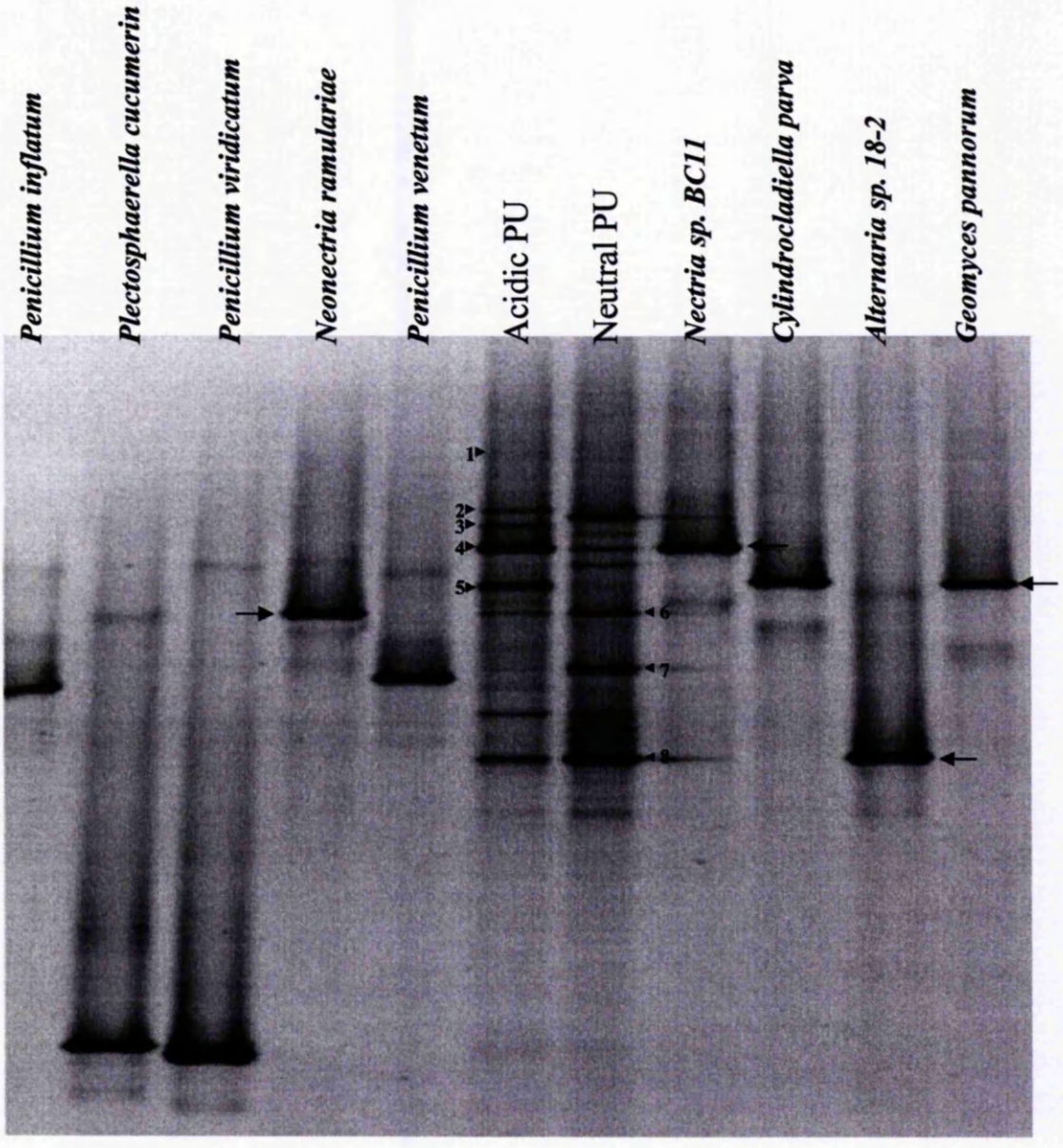

Figure 3: Comparison of DGGE bands produced by fungi isolated from the surface of buried PU with bands produced by fungal communities growing on the surface of PU buried in neutral and acidic soil. Isolate DGGE bands indicated by arrows migrate to the same position as bands within the DGGE profiles of fungal communities colonising the surface of PU. Numbered bands were cloned into *E. coli*, sequenced and identified. The identities of these bands can be found in Table 4.

bands in the DGGE profile of the fungal community on PU buried in acidic soil. Similarly, *Alternaria sp. 18-2*, which was the dominant cultivable organism recovered from the surface of PU buried in neutral soil, produced a DGGE band that co-migrated to the same position as the most intense band in the DGGE profile of the fungal community on PU buried in neutral soil.

In addition, four fungi, *G. pannorum, N. ramulariae, Nectria sp. BC11 and Alternaria sp. 18-2* produced bands that were visible in the DGGE profiles of fungal communities growing on PU buried in both soil types and probably represent fungi well adapted to growth on the surface of PU. However, the bands representing these isolates differed in intensity between the two community profiles, indicating that soil type affected the abundance of these potentially well adapted isolates.

The remaining five isolates (*P. viridicatum, Pl. cucumerin, P. inflatum, P. venetum* and *C. parva*) did not co-migrate with any of the bands in either of the PU community profiles. All of these morphotypes were recovered on SEA plates in low numbers from the surface of the PU and may therefore have not been present in sufficient quantities to be detectable by DGGE. There were also numerous bands within each PU community profile that did not co-migrate with any of the isolated cultivable species, and some of these bands were amongst the most intense in their respective profiles suggesting that they represented species that could not be recovered on SEA plates.

Identifying community members via DGGE amplicons

In order to identify members of the fungal community from the surface of PU buried in both the acidic and neutral soils in a cultivation-independent manner, PCR using the DGGE primers was performed on DNA extracted from fungal communities colonising the surface of buried PU. DGGE-PCR products were cloned into *E. coli*. The cloned ITS1 sequences from over a hundred transformants were screened by DGGE. In total, 8 different ITS1 sequences that migrated to different positions on the DGGE gel were recovered, five from fungi on the surface of PU buried in the acidic soil and three from PU buried in the neutral soil. These fragments were then sequenced in order to determine their putative identities. Of the eight ITS 1 fragments cloned, four (clones 4, 5, 6 and 8; Fig. 3 & Table 4) had sequences homologous to those of *Nectria sp. BC11, G. pannorum, N. ramularia* and *Alternaria sp. 18-2*. These fragments were also found to have 100% homology with the ITS 1 fragments generated from the corresponding

isolates and migrated to the same position in the DGGE gel as these isolates (data not shown).

Of the four remaining cloned ITS1 fragments, three (Fig. 3 & Table 4 ;clones 1, 3 and 7) returned no significant matches upon database interrogation (≤ 93% homology), or were homologous to uncultured soil fungi. Furthermore, these sequences did not co-migrate with any of the cultivable isolates (data not shown), indicating that these sequences represented potentially non-cultivable members of the PU fungal community. The final clone (clone 2) was putatively identified as a *Sarcosomataceae sp.* Colonies with a morphotype consistent with this species were not isolated from the surface of PU buried in either soil type.

Table 4: Putative identities of ITS-1 DGGE fragments cloned from fungal communities recovered from the surface of PU buried in neutral and acidic soil. Clones that gave sequences identical to those of isolates recovered from the surface of soil buried PU are also indicated. GenBank accession numbers for each sequence are given in parentheses.

Clone	Present as bands in community DGGE profiles from PU buried in indicated soil	Putative identity	% homology	Recovered as isolate on SEA
1	Acidic soil	Unidentified ascomycete (DQ779777)	<90%	-
2	Acidic soil	*Sarcosomataceae sp.* (DQ779774)	100%	-
3	Acidic soil	Uncultured soil fungus (DQ779776)	99.7	-
4	Acidic and neutral soil	*Nectria sp. BC11* (DQ779773)	100%	+
5	Acidic and neutral soil	*Geomyces pannorum* (DQ779771)	100%	+
6	Acidic and neutral soil	*Neonectria ramulariae* (DQ779772)	100%	+
7	Neutral soil	Uncultured ascomycete (DQ779778)	98.1%	-
8	Acidic and neutral soil	*Alternaria sp. 18-2* (DQ779775)	100%	+

Degradation of PU buried in two soil types for five months

The degree of degradation of PU after 5 months burial was determined by measuring the tensile strength of the PU coupons. As cleavage of the PU backbone during degradation weakens the plastic, the extent of degradation is inversely proportional to tensile strength. Burial of PU in either the neutral or the acidic soil led to severe degradation after five months (Fig. 4). The tensile strength of the control PU before burial was approximately 360 N, while after burial its tensile strength had decreased approximately 15-fold. PU buried in the acidic soil was slightly more degraded (21N) than PU buried in the neutral soil (27N) ($p<0.05$).

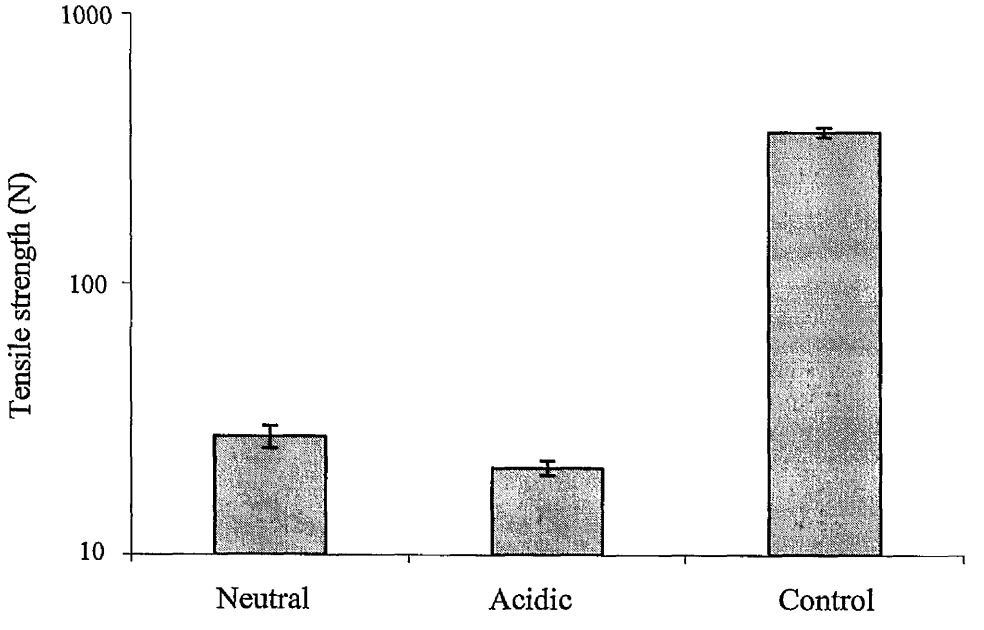

Figure 4: Loss of tensile strength of PU after burial in neutral or acidic soil, *in situ*, for five months. The tensile strength of unburied control PU is included. Error bars indicate standard error (n=15).

Discussion

This work has shown that PU degradation *in situ* is most likely carried out primarily by fungi and that the extent of degradation is very similar in two soils with different pH and different organic carbon content. PU was found to be highly susceptible to degradation in both soil types, losing up to 95% tensile strength after five months burial. PU is known to be highly susceptible to degradation in soil environments under controlled conditions, as has been noted in a number of laboratory microcosm-based studies (Barratt *et al.*, 2006; Bentham *et al.*, 1987; Dale & Squirrel, 1990). However, this is the first work to quantify the degradation of PU *in situ* in the environment. We previously reported (Barratt *et al.*, 2006) that the maximum observed loss of tensile strength after 1.5 months burial in laboratory based microcosms at 25°C was approximately 60%, compared with 95% in this study. Although PU in this work was buried for a much longer period of time, much of the burial period was during the winter and early spring months, when soil temperatures are typically low and are likely to retard degradation of PU compared to the 25°C microcosm.

Most previous studies of PU degradation have focused on bacterial degraders (Akutsu *et al.*, 1998; Howard *et al.*, 1999; Kay *et al.*, 1991), with the majority of these bacteria isolated using enrichment and screening strategies. However, there are many more reports of fungal species being isolated from the surface of PU than bacterial species (Crabbe *et al.*, 1994; Pommer & Lorenz, 1985; Sabev *et al.*, 2006b; Stranger-Johannessen, 1985) and we have previously demonstrated in a laboratory soil microcosm that fungi were the predominant PU degraders (Barratt *et al.*, 2003) with only a very small number of the bacteria recovered displaying Impranil clearance (<0.0001 %). However, this laboratory microcosm was composed of John Innes N°2 compost which is a partially sterilised and blended soil that is unlikely to reflect the microbial diversity of soils in the environment. In this current study using two different environmental soils with total bacterial counts of ca. $4x10^7$ c.f.u per gram in the neutral soil and $2x10^7$ c.f.u per gram in the acidic soil, no significant numbers of Impranil degrading bacteria were detected, confirming that fungi are the dominant PU degraders in the environment. In order to determine what proportion of cultivable fungi were putative PU degraders in environmental soils, we determined the percentage that were capable of clearing the colloidal PU suspension 'Impranil' on SEA agar. Of the

cultivable fungi from the acidic and neutral soil, 37% and 45% respectively were putative PU degraders, a proportion similar to that reported previously for a laboratory soil microcosm (Barratt *et al.*, 2003). Environmental soils therefore contain a large reservoir of fungi with the potential to degrade PU. PU contains many molecular bonds that are analogous to those found in biological macromolecules and fungi encode a broad range of secreted hydrolases making the probability of fortuitous degradation high (Nakajima-Kambe *et al.*, 1999).

The most dominant cultivable organism isolated from the surface of PU buried in the neutral soil was identified as an *Alternaria sp*, while *G. pannorum* and to a lesser extent a *Nectria sp* were the dominant cultivable fungi from the surface of PU buried in the acidic soil. *G. pannorum* has previously been found to be involved in the degradation of PU buried in John Innes compost No2 (Barratt *et al.*, 2003), and has also been implicated in the degradation of plasticized polyvinyl chloride (pPVC) buried in Bulgarian grassland soil (Sabev *et al.*, 2006b). This fungus may therefore prove to be an important agent for plastic waste remediation in the future.

Whilst the *Alternaria* spp. and *G. pannorum* were found to be capable of clearing Impranil, other isolates from the PU from both soils were found to lack this ability. Thus not all members of the community on the surface of the PU had the capacity to degrade PU. Previously we have demonstrated in longitudinal studies on the colonisation of pPVC buried in soil (Sabev *et al.*, 2006b), and pPVC exposed to the air (Webb *et al.*, 2000) that whilst early colonisers all possessed the ability to degrade the plasticizer, other fungi without this ability later appeared in the community. We suggested that breakdown products from the primary colonisers might act as carbon sources for non-degraders, which may explain the presence of non-degraders after 5 months of burial in this present study.

It is well known that culture-based techniques have a number of shortcomings when used to try and identify and quantify fungal communities from environmental samples (Bridge & Spooner, 2001; Pugh, 1969; Zak & Visser, 1996). We therefore also used DGGE as a non-culture based technique to study the composition of the PU community. DGGE has been successfully used to analyse fungal communities from a variety of environments (Bougoure & Cairney, 2005; Gomes *et al.*, 2003; Viebahn *et al.*, 2005). DGGE revealed that only a subset of the total diversity of fungal species present in

either soil were present on the surface of the PU after five months (Fig.1). Both the DGGE profiles and culture based data indicated that the two soils possessed two largely different fungal communities that therefore led to differences in the fungal species colonising the buried PU. Moreover, DGGE revealed that the community that colonised the surface of the PU was very different from the soil community it was buried in, indicating an enrichment of species capable of colonising and/or degrading PU (Fig 1). Soil conditions have been found to influence the composition of fungal communities on the surface of buried plastics. Sabev *et al.* (2006b) found that pPVC buried in forest soil supported a different range of fungi than pPVC buried in grassland soil; whilst Barratt *et al.* (2003) found that changing the water holding capacity within the same soil also altered the fungal communities on the surface of buried PU. Soil organic carbon content and pH have been found previously to influence the structure of microbial communities in soil (Drenovsky *et al.*, 2004; Garbeva *et al.*, 2004) and both of the soils used in this study were found to differ in these parameters (Table 1). The initial attachment of microorganisms to buried PU is thought to be primarily mediated by non-specific hydrophobic interactions (Bos *et al.*, 1999), and local environmental conditions are known to influence the surface hydrophobicity of both fungi (Smits *et al.*, 2003) as well as bacteria (An & Friedman, 1998). Thus, as well as containing different microbial communities, differences in the physico-chemical properties of the two soils could also influence which microbes successfully colonised the surface of the PU.

Although Impranil clearance assays revealed that a large reservoir of putative PU degrading fungi were present in both soils, very few of these fungi were seen to colonise the surface of the PU. Akutsu *et al.* (1998) found that some enzymes capable of degrading the colloidal PU dispersion Impranil were unable to degrade solid PU due to physiochemical differences between the two forms of the plastic. Therefore, fungi identified as putative PU degraders by Impranil assay may have been unable to degrade the solid PU coupons, and so may not have been able to proliferate on the surface of the buried PU by using the PU as a nutrient source. However, Impranil-based clearance assays are the only methods currently available to detect potential PU degrading abilities in both bacteria and fungi.

All of the major cultivable species isolated from the surface of buried PU produced bands that co-migrated with bands in the community DGGE profiles for PU buried in each soil. However there were several clear bands from the PU profile that were not

represented by any of the isolates recovered, indicating that important members of the PU community were not recovered through cultivation. It is estimated that only 17% of fungi in the environment are able to be grown in culture (Bridge & Spooner, 2001). When DGGE amplicons were transformed into *E.coli* and the inserts screened by DGGE, eight distinct sequences were obtained, and four of these produced sequences identical to those of fungi recovered as isolates from the PU. Three others gave sequences that did not align with any identified fungi in the EMBL database, and the remaining sequence aligned with a potentially cultivable fungus that was nevertheless not recovered as an isolate in this work. DGGE therefore proved to be a useful method for investigating both culturable and unculturable members of the fungal communities.

Although the culturable fractions of the fungal communities on the surface of the buried PU were dominated by a small number of fungal species, other less numerous fungi were recovered as isolates. However, bands corresponding to these isolates were not seen in the DGGE profiles, indicating that DGGE lacked the sensitivity to detect rarer members of the microbial communities. This insensitivity has been well documented, with common estimates of the detection threshold varying between 0.1% of the total fungal population (van Elsas *et al.*, 2000) to 5% (Muyzer *et al.*, 1993). However, such rare members of the fungal communities are unlikely to contribute significantly to the degradation of the PU.

Although sterile soil was not used in this work, PU has been shown to be for the most part resistant to abiotic degradation (Bentham *et al.*, 1987; Dale & Squirrel, 1990; Woods, 1990). The degradation of PU observed in this work can therefore be confidently attributed to the action of microorganisms in the soil.

In summary, PU was highly susceptible to degradation in soil, and culturable and nonculturable fungi were the putative PU degrading organisms. The type of soil in which PU was buried influenced the composition of the fungal communities on the surface of the PU after five months burial. However differences in soil properties did not have a very great effect on the final extent of biodegradation in this study indicating that PU will be successfully degraded in a range of soil types. Further work must be carried out to further elucidate the processes involved in PU degradation.

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Chapter 3:

Biostimulation of soil microbial communities and the degradation of buried polyester polyurethane.

Abstract

Supplying microbial communities with nutrients, in a process known as biostimulation, has often been found to enhance the ability of these communities to degrade manmade waste. In this work, soil microbial communities treated with yeast extract (YE), the colloidal polyurethane dispersion 'Impranil', or YE and Impranil together were investigated, and the degradation of polyester polyurethane (PU) coupons buried in treated soils was determined. Denaturing gradient gel electrophoresis revealed that each soil treatment led to the selection of specific members of the soil fungal communities. *Trichosporon multisporum* and an unidentified Zygomycete were strongly selected for in soil in the presence of Impranil and YE together. *Hohenbuehelia sp*, *Trichosporon gracile*, *Alternaria sp. 18-2*, *Mortierella hyaline* and *Geomyces pannorum* were also selected for to a lesser extent after Impranil treatment. Specific members of the soil populations colonised the surface of buried PU, and each treatment influenced the composition of the fungal communities that developed. Impranil agar clearance assays showed that adding both Impranil and YE was the most effective in increasing the numbers of putative PU degrading fungi both in the soil and on the surface of buried PU. Treatment with Impranil alone had no effect on the numbers of PU degrading fungi in the soil, and decreased PU degraders on the surface of PU. Although YE and YE plus Impranil treatment increased PU degradation compared to the control, this increase was minor compared to the already severe degradation that occurred in the control soil, suggesting that nutrient treatment is of limited use for PU waste remediation in soil under these conditions.

Introduction.

As the environmental burden of plastic waste increases, the need for strategies for the remediation of this waste becomes more urgent. Environmental microbial communities have proved to be a potentially important source for the remediation of a wide range of man-made waste products, usually via the hydrolysis of waste by enzymes produced during nutrient acquisition, followed by the subsequent utilisation of breakdown products as nutrient sources (Zheng *et al.* 2005). Although the metabolic potential for waste remediation is often present in natural microbial communities, limiting factors such as an absence of electron acceptors or donors, poor nitrogen or phosphorous content of the waste being degraded, or a lack of induction of the metabolic pathways responsible for degradation can reduce the effectiveness of these communities in waste remediation. In these cases, the addition of exogenous nutrients can enhance the degradation of waste; this process is often termed 'biostimulation'. Biostimulation of *in situ* microbial communities has been successfully used to enhance the degradation of crude oil (Mills *et al.* 2002; Röling *et al.* 2004), tetrachloroethene (Major *et al.* 2002), diesel (Rivera- Espinoza & Dendooven 2004; Namkoong *et al.* 2002) and polyaromatic hydrocarbons (Yu *et al.* 2005).

Plastics constitute a large proportion of solid household and industrial waste in the developed world. The proportion of household waste made up of plastic in the average American home is greater than 30% (Kawai 1995a), and the total amount of plastic produced per year world-wide as of 2001 stood at over 140 million tonnes (Shimao 2001). Although the majority of plastics are physically and chemically recalcitrant, a few, such as the polyhydroxyalkanoates, polycaprolactone, polylactic acid, and polyvinyl alcohol (Shimao 2001) have been found to be susceptible to biodegradation in the environment.

This work focuses on polyester polyurethane (PU), which constitutes a major group of biodegradable plastics, and is used in the manufacture of foams, fibres and synthetic leather and rubber goods. Approximately 8 million tons are produced globally each year, a number that is increasing at the rate of 4-5% annually (Matsumura *et al.* 2005). The breakdown of PU by microorganisms is thought to primarily occur via the enzymatic hydrolysis of ester bonds present in abundance in the backbones of these compounds (Nakajima-Kambe *et al.* 1999). Hydrolysis products of PU degradation have

been found to be metabolised as carbon sources (Barratt *et al.*, 2003; Barratt 2003), potentially allowing for the efficient mineralization of PU waste.

Although the majority of PU waste is solid in form, no method exists to quickly or easily identify PU degrading organisms based upon their ability to degrade solid PU. Instead, colloidal liquid suspensions of PU are often employed in clearance assays to assign putative PU degrading ability. At the present time, little work has been done to determine if the degradation of colloidal dispersions of PU can be correlated with the degradation of solid PU.

In this work, the colloidal PU dispersion 'Impranil', yeast extract, or Impranil and yeast extract were periodically added to microcosms of soil under controlled conditions. The effects of these treatments on the native soil microbial communities were investigated using culture-based methods and denaturing gradient gel electrophoresis (DGGE). PU coupons were buried in treated soil, and the effect of each treatment on the microbial communities colonising the PU surface as well as the extent of degradation of buried PU was determined after three months.

Methods and Materials

Polyester Polyurethane

Two types of PU were employed in this work. The liquid PU dispersion Impranil DLN (Bayer GmbH, Dormagen, Germany) was added to soil and was also used in clearance assays to determine the numbers of putative PU degrading organisms. Solid PU (Elastogran GmbH Lemförde, Germany) was used to determine if soil treatment influenced the degradation of buried PU.

Establishment, Maintenance and Sampling of Soil microcosms.

Garden soil recovered from a site in Greater Manchester UK was used in this work. This soil had been used previously during *in situ* soil PU burial experiments (Cosgrove *et al.* 2006). Previous analysis revealed this soil to be a sandy loam, with a pH of 5.5, and contained 43.4g kg^{-1} organic carbon and 3g kg^{-1} nitrogen (analysis performed by Adas Laboratories, UK). Soil was divided into 330g samples and placed into plastic boxes measuring 16 x 12 x 5cm (termed 'microcosms'). Sufficient moisture was then added such that the water content of the soil in each microcosm was 40% of the maximum water holding capacity (whc) for the soil (whc is defined as the maximum amount of water the soil would hold at saturation). This moisture took the form of either sterile distilled water ('control' microcosms), a solution of 200 gl^{-1} yeast extract (YE) in water ('YE treated' microcosms), a 50% (v/v) aqueous solution of Impranil ('Impranil treated' microcosms), or an aqueous solution containing 200 gl^{-1} YE plus 50% (v/v) Impranil ('Impranil plus YE' treated microcosms). The weight of each microcosm was recorded at time zero. Each microcosm was weighed every 2-3 days, and water lost through evaporation was replenished with sterile distilled water, YE solution, Impranil solution or a YE/Impranil solution. Moisture addition was accompanied by thorough stirring and homogenization of each microcosm with a sterile spatula. At 3-4 week intervals 5g samples of soil from each microcosm were taken immediately following moisture replenishment. A portion of this soil was removed for plate counting and DGGE analysis.

Burial of solid PU in treated microcosms

After 12 weeks of soil treatment as described above, dumbbells (n=15) and rectangular coupons (n=3) of solid PU were buried in the treated soil microcosms for a further 12 weeks. Dumbells were of the following dimensions: width 2 mm, depth 1.5 mm, and length 20 mm. Rectangular coupons measured 7.5 x 4 x 0.15 cm. All PU pieces were surface sterilised by immersion in 70% (v/v) ethanol prior to burial. During the period of burial, the water content of the soil was maintained at 40% whc as described previously.

Recovery of biomass on buried PU

Biomass was recovered from buried PU after 12 weeks burial in order to analyse the microbial communities growing on the surface (Cosgrove *et al.* 2006). Briefly, loosely adhered soil particles were removed by agitating the PU coupons in sterile phosphate buffered saline (PBS) (Sambrook & Maniatis, 1989) for 5 min. Washed PU was then submerged in 20 ml sterile PBS, and the biomass was scraped from both sides of the PU into the PBS using a sterile scalpel blade. An aliquot of 1ml of this biomass suspension was reserved for viable counting. The remainder was centrifuged at 3000g for 30 min at 4°C, and the supernatant was discarded. The remaining biomass was then used for DNA extraction and DGGE analysis.

Microbial viable counts

Total viable counts of fungi and bacteria in the soil and on the surface of buried PU were determined by dilution plating in PBS onto soil extract agar (SEA) (Alef & Nanniperi, 1995) and colonies were counted after 5-7 days incubation at 25°C. Putative PU degraders were enumerated as colonies producing zones of clearance on Impranil agar plates (Crabbe et al. 1994) following 5-7 days incubation at 25°C. Media included either 50 $\mu g ml^{-1}$ chloramphenicol to select for fungi, or 50 $\mu g ml^{-1}$ nystatin to select for bacteria. The number of Impranil degrading organisms was then calculated as a percentage of the total number of colonies recovered.

DNA extraction

The FastDNA SpinKit for Soil (Q-Biogene, California, U.S.A) was used to extract total DNA from 0.4 g soil samples, or 0.5 g samples of biomass (wet weight) recovered from the surface of buried PU. To remove all traces of PCR inhibitory compounds, 20 µl of extracted DNA was run for ca. 15 min on a 1.0% (w/v) agarose/TAE gel. Bands of genomic DNA were then excised, and DNA was recovered using the Nucleospin Extract II gel extraction kit (Machery-Nagel, Düren Germany).

PCR amplification of fungal community DNA

PCR was employed to generate the necessary DNA fragments for fungal community DGGE analysis. The PCR DNA template consisted of approximately 50 ng per reaction of extracted DNA. Biomix Red PCR master mix (Bioline, London U.K) was employed in all reactions. Primers were present in each reaction at a concentration of 1 µM. Fungal DGGE fragments were generated using the GM2/JB206c primer set (GM2: 5'-CTGCGTTCTTCATCGAT-3', JB206c: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAAGTAAAAGTCGTAACAAGG-3'), which amplify the ITS1 region found in the fungal rDNA gene complex. The PCR regime employed was as follows: 94°C initial denaturation for 5 min; 20 'touchdown' cycles of 94°C for 30 s, annealing for 30 s at 59 to 49°C with annealing temperature being reduced by 0.5°C per cycle, extension at 72°C for 45 s; 30 cycles at 95°C for 30 s, annealing at 49°C for 30 s and extension at 72°C for 45 s; 1 final extension at 72°C for 5 min.

DGGE analysis of microcosm communities

The composition of the fungal communities in the soil and on the surface of buried PU were compared using DGGE (Muyzer *et al.* 1993). The D-Code universal mutation detection system (Biorad, Herts U.K.) was employed. Gels measured 16 cm x 16 cm x 1 mm and contained 10% (v/v) acrylamide in 1 x TAE (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA), with a parallel denaturant gradient of 25-55%. For all gels, approximately 500 µg of PCR product was used per lane; gels were run in 1 X TAE buffer at a constant temperature of 60°C for 16.5 h at 42 volts. Gels were stained with SybrGold (Molecular Probes, Netherlands) for 45 min and photographed under

ultraviolet light. Gels were analysed using the Phoretix 1D software package (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) to construct dendrograms showing the degree of similarity between the profiles.

Identification of Impranil enriched fungi via sequencing of bands excised from gels

Fungi that increased in numbers following YE and Impranil addition were identified by sequencing bands in the soil fungal DGGE community profiles that increased in intensity following treatment of soil with Impranil and YE (Cosgrove *et al.*2006). These bands were excised under blue-light illumination (UVP, California U.S.A.) using an ethanol-flamed scalpel blade. Excised bands were placed in 50 µl of sterile distilled water overnight at 4°C; 5 µl aliquots were then used as the template in DGGE PCR reactions as described previously. Products were run on DGGE to check for correct migration of products, and successful excisions were sequenced from the GM2 primer site using in-house sequencing facilities. Sequences were then used to interrogate the EMBL fungal database using the blastn algorithm.

Identification of Impranil enriched fungi via cloning

ITS-1 DGGE fragments generated from DNA extracted from fungal communities in Impranil treated soil were cloned into the pGEM-Teasy plasmid (Promega, UK) and transformed into *E.coli* strain JM109 as per manufacturer's instructions. Individual clones were screened using colony PCR to reamplify the ITS 1 fragments contained within them using the DGGE PCR regimen described above. These fragments were then run on a DGGE as described previously alongside whole soil community DGGE products. Clones producing bands that co-migrated to the same position as bands of interest within the soil community profiles were then selected for sequencing. These sequences were then used to interrogate the EMBL fungal database as described previously.

ITS sequencing of fungal isolates

Pure cultures of selected fungal isolates were grown in malt extract broth (MEB) (Oxoid, UK) at 25°C under aerobic conditions until sporulation or confluent growth had occured. Genomic DNA was then extracted using the method described by Anderson *et al.* (1996). The ITS1-5.8S-ITS2 region of the fungal rDNA gene complex was then PCR amplified using the ITS1/ITS4 primer set (ITS1: 5'-TCCGT AGGTGAACCTGCGG-3, ITS4: 5'-TCCTCCGCTTATTGATATGC-3') using the Expand High Fidelity PCR System (Roche, Mannheim, Germany). The PCR regime was as follows: 94°C for 3 min; 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min; a final extension at 72°C for 5 min). PCR products were then sequenced using in-house facilities.

Tensile strength determination of buried PU

The tensile strength of PU dumbbells recovered from the soil microcosms after 12 weeks burial was determined to assess the extent to which the PU was degraded. Dumbells were stretched at a rate of 200 mm min^{-1} and the tensile strength was determined using an Intstron 4301 (Instron Ltd, Swindon UK). The tensile strength of unburied control dumbbells was also determined.

Results.

The influence of Impranil and Yeast extract on fungal and bacterial viable counts in soil

The response of the cultivable portion of microbial soil communities to the addition of YE and/or Impranil to the soil was determined by plating soil samples onto SEA and Impranil agar. Table 1 shows the total viable fungi and bacteria and the numbers of putative PU degrading fungi in the soil after three months of soil treatment.

Soil treated with YE contained 5-fold more viable bacteria and 1.6-fold more viable fungi compared to untreated control soil. Treatment with YE and Impranil together increased the numbers of viable bacteria 29.4-fold and viable fungi 10.8-fold compared to untreated control soil. The addition of Impranil alone to soil increased the numbers of viable bacteria 224-fold; however the addition of Impranil alone did not significantly change the numbers of viable fungi compared to the control soil ($p>0.05$).

The numbers of putative PU degrading fungi and bacteria were determined by enumerating the number of colonies producing zones of clearance on Impranil agar. Very few putative PU degrading bacteria were observed, whereas putative PU degrading fungi were very common in the soil, with 39% of all of the viable fungi recovered from the control soil able to clear Impranil. Treatment with YE alone or Impranil alone did not significantly change the numbers of fungi able to degrade Impranil ($p>0.05$), whereas the addition of both Impranil and YE increased the numbers of putative PU degrading fungi 16-fold compared to the control. The majority of the fungi recovered from Impranil and YE co-treated soil was comprised of only two colony morphotypes. One of these morphotypes was found to clear Impranil; this morphotype accounted for 55% of the viable fungi recovered from soil treated with YE and Impranil.

In conclusion, treatment of soil microcosms with YE alone increased the number of viable fungi and bacteria in the soil, but a much greater increase in numbers of both fungi and bacteria was observed if both Impranil and YE were added simultaneously. Treatment with Impranil alone led to a large increase in the numbers of recoverable bacteria. In the absence of YE, Impranil treatment had no significant effect on the numbers of fungi recovered from the soil. No Impranil degrading bacteria were observed in any of the treatments. In contrast, very high numbers of Impranil degrading fungi were detected throughout, even in the control soil.

Table 1: Total numbers of bacteria and fungi, and numbers of Impranil degrading fungi after soil was treated with YE and Impranil. All values are given per gram of soil (n=3). Subscript letters indicate statistical significance: values with the same letter were not significantly different ($p>0.05$).

Treatment	Bacterial viable counts (cfu g^{-1})	Fungal viable counts (cfu g^{-1})	Impranil clearing fungal viable counts (cfu g^{-1})	Percentage of viable colonies clearing Impranil
dH_2O (control)	$8.5 \times 10^{6}{}_{a}$	$5.7 \times 10^{5}{}_{e}$	$2.2 \times 10^{5}{}_{g}$	$39\%_{i}$
Yeast extract	$4.1 \times 10^{7}{}_{b}$	$8.9 \times 10^{5}{}_{f}$	$2.0 \times 10^{5}{}_{g}$	$22\%_{j}$
Yeast extract plus Impranil	$2.5 \times 10^{8}{}_{c}$	$6.2 \times 10^{6}{}_{g}$	$3.4 \times 10^{6}{}_{h}$	$55\%_{k}$
Impranil alone	$1.9 \times 10^{9}{}_{d}$	$5.6 \times 10^{5}{}_{f}$	$2.4 \times 10^{5}{}_{g}$	$42\%_{i}$

The influence of Impranil and/or YE on the numbers of fungi and bacteria recovered from the surface of PU buried in treated soil.

The total numbers of fungi and bacteria recovered from the surface of the PU, and the numbers of putative PU degraders were determined as described previously for soil communities.

Although large increases in the numbers of bacteria were seen in the soil after all of the treatments (see Table 1) there was no significant difference in the numbers of bacteria on the surface of PU buried in the untreated control soil, soil treated with Impranil alone and soil co-treated with both YE and Impranil (Table 2). There was a 71% reduction in the number of viable bacteria on the surface of PU buried in soil treated with YE compared to untreated control soil. As in the soil, no putative PU degrading bacteria were observed on the surface of the PU.

Co-treatment of soil with YE and Impranil or YE alone increased the numbers of viable fungi on the surface of the PU by 24% and 18%, respectively, compared to the control. Furthermore, there was an approximate 45% increase in the number of putative PU degraders on the surface of PU buried in soil co-treated with Impranil and YE and

treated with YE alone compared to PU buried in untreated control soil. Treatment of soil with Impranil alone reduced the numbers of viable fungi on the surface of the PU by 25% compared to PU buried in untreated control soil, and there were 11% fewer putative PU degrading fungi present on the surface of PU buried in soil treated with Impranil alone. Hence, treatment with of soil with YE and YE plus Impranil increased the numbers of putative PU degrading fungi on the surface of buried PU, whilst treatment with Impranil alone led to a decrease in the numbers of PU degraders.

Table 2: Total numbers of fungi and bacteria, and the numbers of Impranil degrading fungi recovered from PU buried in treated soil. All values are given per square centimetre of PU surface (n=3). Subscript letters indicate statistical significance: values with the same letter were not significantly different ($p>0.05$).

Treatment	Bacterial viable counts (cfu cm^{-2})	Fungal viable counts (cfu cm^{-2})	Impranil clearing fungal viable counts (cfu cm^{-2})	Percentage of viable colonies clearing Impranil
dH_2O (control)	$5.6x\ 10^6{}_a$	$6.6 \times 10^4{}_c$	$2.1\ x10^4{}_f$	$31\%_j$
Yeast extract	$1.6\ x10^6{}_b$	$7.8 \times 10^4{}_d$	$3.0 \times 10^4{}_h$	$38\%_k$
Yeast extract plus Impranil	$4.51\ x10^6{}_a$	$8.2 \times 10^4{}_d$	$3.1\ x10^4{}_h$	$38\%_k$
Impranil alone	$4.1 \times 10^6{}_a$	$4.9 \times 10^4{}_e$	$1.87\ x10^4{}_i$	$37\%_k$

Identification of dominant cultivable fungi enriched in soil treated with both Impranil and YE

The high numbers of Impranil degrading fungi detected in the microcosm co-treated with both Impranil and YE were due to high counts of only two colony morphotypes, one of which could degrade Impranil. These two dominant morphotypes were present in approximately equal proportions, and were responsible for approximately 90% of the viable counts on SEA plates. After isolation, these two morphotypes were identified by sequencing as *Trichosporon multisporum* and an unidentified Zygomycete. The unidentified Zygomycete was able to degrade Impranil in solid medium, whereas *Trichosporon multisporum* did not produce any zones of clearing on Impranil agar.
The isolation of only two predominant Impranil degrading fungi indicated a strong selection for these two fungal species if both YE and Impranil were added to soil.

The effect of Impranil and YE amendment on the species composition of fungal communities in the soil and on the surface of buried PU

DGGE was used to detect changes in both the noncultivable and cultivable members of the soil fungal communities as a result of each soil treatment, and to determine both if specific members of the soil fungal populations were selected for during growth on buried PU, and if each soil treatment influenced which members of the soil consortia proliferated on the surface of the plastic. Since only fungi showed putative PU degrading ability, bacterial communities were not analyzed by DGGE. Fig 1 shows the DGGE profiles of the fungal communities in treated soil and on the surface of PU buried in treated soil. The dendrogram in Fig 2 shows the degree of similarity between each of the fungal communities.
The DGGE profiles for the untreated control soil and the soil treated with Impranil alone (Fig 2, lanes 1 and 2, respectively) were very similar, suggesting that treating soil with Impranil alone had little effect on the composition of the native soil fungal communities. Conversely, the DGGE profiles of fungal communities in soil treated with YE alone (Fig 2, lane 4) or with YE and Impranil together (Fig 2, lane 3) show little similarity to the DGGE profile of fungal communities in the control soil (Fig 2, lane 1).

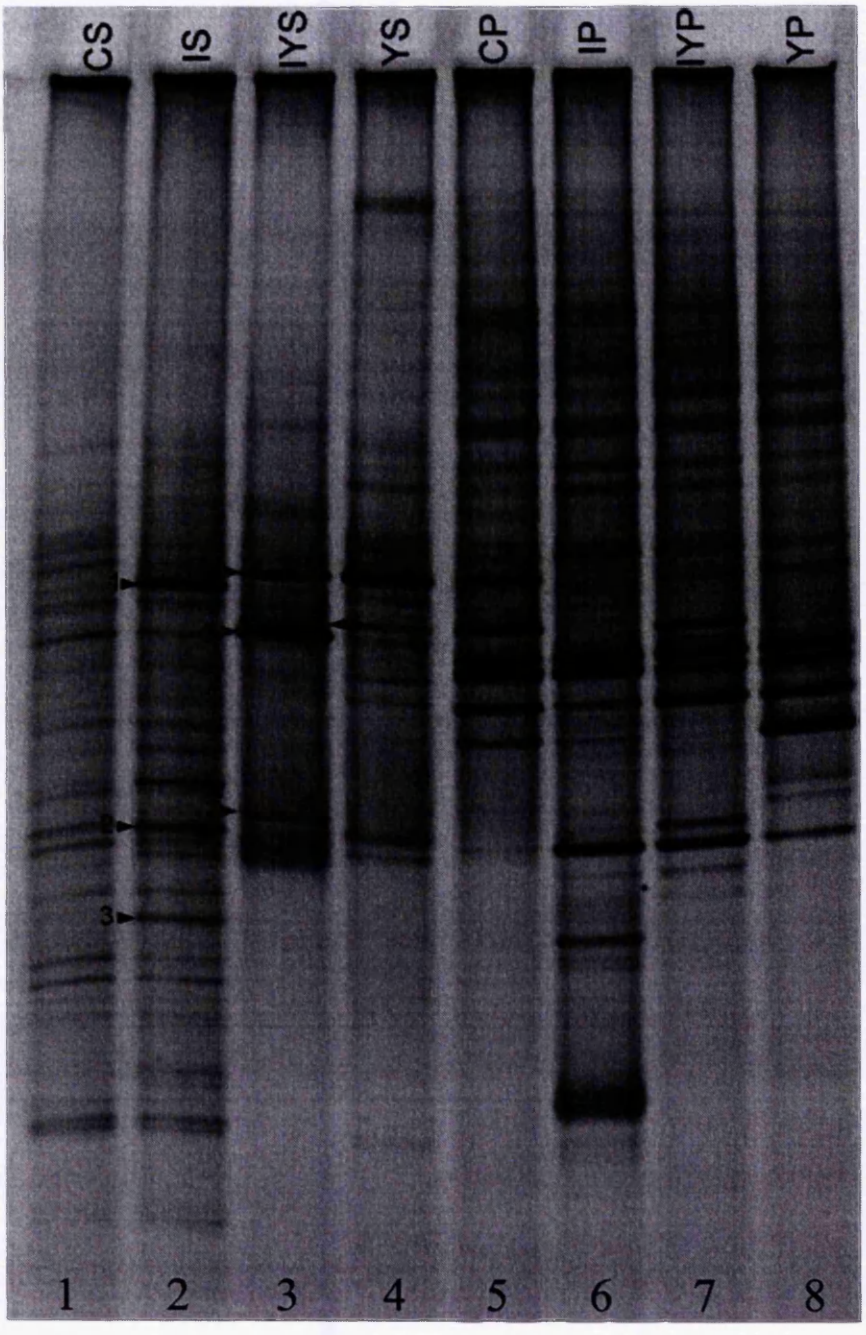

Figure 1: DGGE profiles of fungal communities in soil (S) and on PU (P) after treatment of soil microcosms with sterile distilled water (C), Yeast extract (Y), Impranil (I) or co-treatment with Impranil and YE (IY). Numbers indicate bands that were sequenced and identified. Lane numbers are referred to in the text.

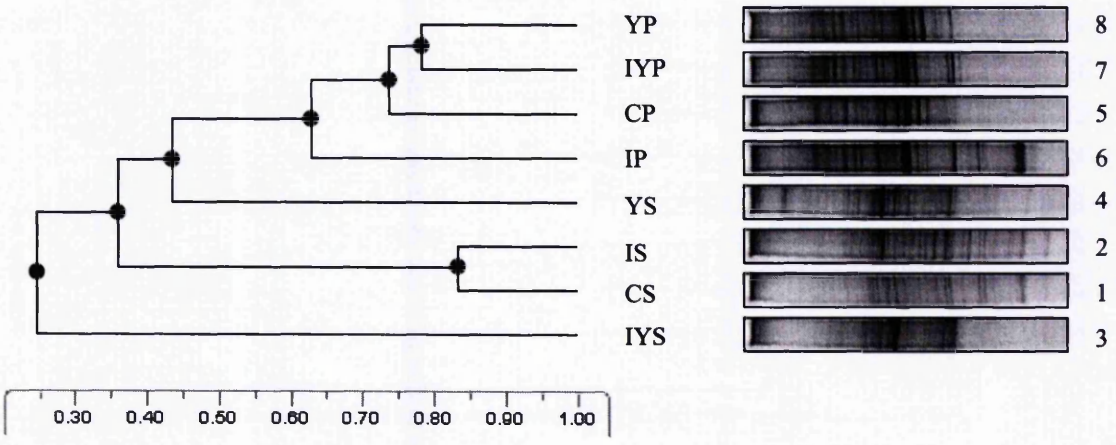

Figure 2: Dendrogram showing the degree of similarity between DGGE profiles of fungal communities in the soil (S) and on the surface of buried PU (P) following treatment of soil with dH_2O (C), Yeast extract (Y), Impranil (I) or yeast extract and Impranil (IY). Lane numbers are referred to in the text.

This indicated that both of these treatments led to large changes in the composition of the soil fungal communities, leading to the proliferation of specific members of the soil fungal consortia. Furthermore, treatment of soil with YE alone produced a different soil fungal DGGE profile than treatment with YE and Impranil together, suggesting that each treatment led to the proliferation of a different subset of the soil microflora. Treatment with Impranil and YE together appeared to cause the greatest change in the structure of the soil fungal community, as the DGGE profile for soil treated in this way clusters furthest away from all of the other soil profiles in Fig 2. The two dominant bands in this profile (Fig 1, bands 4 and 6) comigrate to the same position as bands produced by the two fungi comprising the majority of the cultivable fungi in soil treated with YE and Impranil together (data not shown).

In Fig 2, all of the DGGE profiles of the fungal communities colonising the surface PU buried in soil (Fig 2, lanes 5-8) clustered separately from the soil fungal community DGGE profiles (Fig 2, lanes 1-4), showing that the fungal communities on the surface of buried PU contained fungi that were not common members of the soil fungal communities. This suggests that growth on the surface of PU selected for specific members of the soil fungal communities. Although many bands were seen in the DGGE profiles of fungal communities on the surface of PU regardless of the treatment applied to the soil, there were some differences in the banding patterns between each of the PU community profiles, indicating that treatment type influenced which fungi colonised the surface of buried PU. However, with the exception of band 4 (Fig 2, lane 3), none of the bands seen to increase in intensity following treatment of soil with YE or YE and Impranil together were seen as major bands within the DGGE profiles of fungal communities on the surface of buried PU. This suggests that the majority of the fungi enriched for in the soil following YE or Impranil treatment could not go on to colonise the surface of buried PU. Treatment of soil with Impranil alone had the greatest effect on the composition of the fungal communities on the surface of buried PU (Fig 2, lane 6), as the Impranil only treated PU DGGE profile showed the least similarity to the profile for PU buried in untreated control soil (Fig 2, lane 5). Treatment of soil with YE (Fig 2, lane 8) or with both YE and Impranil (Fig 2, lane 7) also altered the composition of the fungal communities on the surface of buried PU compared to the control, although to a lesser degree than did Impranil alone.

In conclusion, treatment of soil with YE alone and with YE and Impranil together led to large changes in the composition of the native soil fungal communities. The addition of Impranil alone to soil, however, had very little effect on the soil fungal communities.

Specific members of the soil fungal communities were selected for during growth on buried PU, but few of the fungi colonising the surface of buried PU were those enriched for in the soil after treatment. Treatment type did influence the composition of fungal communities on buried PU, with Impranil alone having the greatest effect.

Identification of Impranil enriched soil fungi by DGGE band excision and cloning

Bands showing increased intensity in DGGE profiles of communities treated with Impranil (either alone or in conjunction with YE) were presumed to be representative of fungi increasing in number in the presence of Impranil. Hence the sequencing of excised DGGE bands and sequencing of cloned community DGGE fragments allowed the identification of organisms enriched in response to Impranil addition. The sequenced bands are numbered in Fig 1, and the identities of these organisms are given in Table 3.

The dominant bands in the DGGE profile of the fungal community in the Impranil and YE co-treated soil (Fig 1, Lane 3, bands 4 and 6) were found to co-migrate with bands produced by the two dominant cultivable morphotypes in this soil. Furthermore, upon sequencing these bands were found to belong to an unidentified Zygomycete (Fig 1, lane 3, band 4) and *Trichosporon multisporum* (Fig 1, lane 3, band 6), in agreement with the identities of the cultured isolates. However, unlike the culture based data, DGGE revealed an increase in the prevalence of at least two other fungi in soil treated with YE and Impranil, identified as *Mortierella cf. hyaline* (Fig 1, lane 3, band 7) and *Geomyces pannorum* (Fig 1, lane 3, band 5).

Because the DGGE profile of soil to which Impranil alone had been added contained many closely spaced bands of approximately equal intensity, band excision and sequencing was not possible for determining the identity of fungi enriched after the addition of Impranil alone to soil. Instead, cloning and sequencing of DGGE-PCR products was used to isolate and sequence bands that increased in intensity after the addition of Impranil alone. Using this method, three bands were recovered as clones (Fig 1, bands numbered 1-3 in lane 2) – sequencing identified these bands as *Hohenbuehelia sp* (band 1), *Trichosporon gracile* (band 2) and *Alternaria sp. 18-2* (band 3).

Hence, the addition of Impranil to soil enriched for specific members of the soil community. However, the addition of Impranil and YE together enriches for a different subpopulation than the addition of Impranil alone.

Table 3: Putative identities of fungal species selected for by the addition of Impranil to soil. Impranil was added alone, or with yeast extract.

Treatment	DGGE band*	Nearest match in database	Sequence homology (%)
Impranil alone	1	*Hohenbuehelia sp*	97.8
Impranil alone	2	*Trichosporon gracile*	99.1
Impranil alone	3	*Alternaria sp. 18-2*	99.4
Impranil plus YE	4	Unidentified Zygomycete	97.4
Impranil plus YE	5	*Geomyces pannorum*	100
Impranil plus YE	6	*Trichosporon multisporum*	98.3
Impranil plus YE	7	*Mortierella hyaline*	100

* Bands from DGGE profiles of fungal communities treated with YE and Impranil (Fig 1) were sequenced and identified.

The effect of soil treatment on the degradation of solid PU

Extensive degradation of buried PU, as indicated by a reduction in the tensile strength of PU dumbbells, was observed after burial in soil microcosms regardless of the treatment received. After three months burial in the control soil, PU lost 90% of its tensile strength compared to unburied PU (Figure 3). Pre-treatment of soil with YE alone or with both Impranil and YE led to a 96% reduction in the tensile strength of PU compared to unburied PU. PU buried in soil treated with Impranil alone was less degraded than PU buried in the control soil, with PU buried in soil treated with Impranil alone experiencing an 85% reduction in tensile strength compared to unburied PU.

Hence, treatment of soil with Impranil alone did not enhance PU degradation, but instead inhibited it. Furthermore, although some enhancement in degradation was observed when the soil was treated with YE or co-treated with YE and Impranil, this reduction in tensile strength was negligible compared to the severe degradation that occurred over the course of twelve weeks even in the untreated control soil.

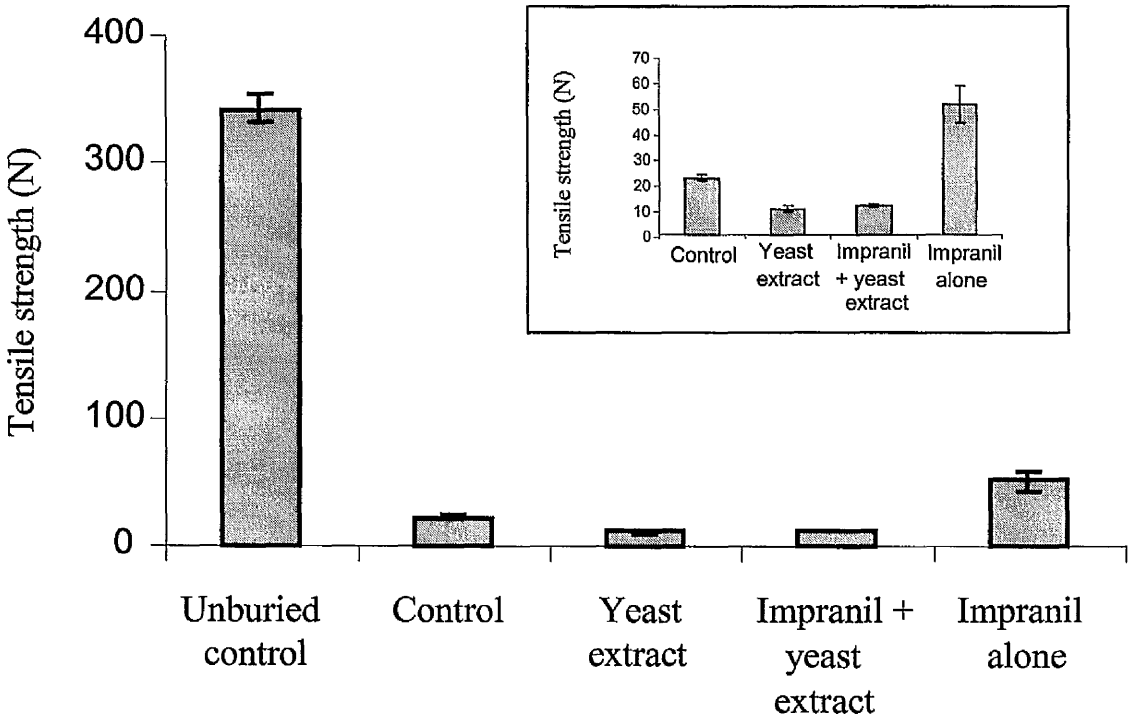

Figure 3: Tensile strength of PU after twelve weeks burial in soil treated with YE and/or Impranil. The tensile strength of unburied PU is also shown. Inset shows data minus that of the unburied control.

Discussion

The soil employed in this work possessed a large reservoir of putative PU degrading fungi, and PU was found to degrade readily even in untreated control soil. These observations are in agreement with previous work carried out using this soil (Cosgrove *et al.* 2006), and have also been seen for other soils (Cosgrove *et al.* 2006; Barratt *et al.* 2003; Barratt, 2003). The presence of such a large reservoir of fungi putatively possessing pathways for PU degradation suggests that this soil would make a good medium for biostimulation in PU degradation.

As well as being numerous in the soil employed in this work, Impranil degrading fungi are ubiquitous in a range of soil environments (Pathirana and Seal 1985; Crabbe *et al.* 1994; Bentham *et al.* 1987; Barratt *et al.* 2003). This ubiquity of putative PU degrading fungi in communities that are unlikely to have previously been exposed to PU suggests that the enzymatic degradation of Impranil, and PU in general, is likely to be a result of cometabolism. Cometabolism is defined as the fortuitous degradation of organic compounds of non-biological origin by enzymes that are usually produced by microbes to degrade biological compounds (Alexander 1999). A number of microbial esterases (Crabbe *et al.* 1994), proteases (Ruiz *et al.* 1999), lipases (Stern and Howard, 2000) and ureases (Pathirana and Seal 1984) have been found to have hydrolytic activity against Impranil *in vitro*. Since fungi are known to secrete high levels of a wide variety of lytic enzymes, it is likely that many fungi will possess at least one enzyme with Impranil degrading capability. In contrast, no significant numbers of PU degrading bacteria were seen in this work. Although bacterial PU degraders have been described in the literature, in the majority of cases these are recovered following enrichment and selection procedures (Nakajima-Kambe *et al.* 1995; Kay *et al.* 1991; Howard *et al.* 1999). Previous quantitative assessments of the prevalence of Impranil degrading bacteria in soil have shown that PU degrading bacteria are rare in soil environments (Barratt *et al.* 2003; Cosgrove *et al.* 2006).

Although putative PU degrading fungi were very common in the soil, only a small number of species were found to increase in number after Impranil was added to the soil. This suggests that the majority of fungi in the soil were not utilising the Impranil as a nutrient source. If Impranil degradation is occurring via cometabolism, it is possible that the breakdown products of Impranil degradation are not suitable as growth

substrates for the organisms responsible for degradation; this is frequently observed when compounds are degraded due to cometabolism (Alexander 1999). This may explain why only a specific subset of the fungal communities in the soil, revealed by DGGE to include a *Hohenbuehelia sp*, *Trichosporon gracile*, *Alternaria sp. 18-2*, an unidentified Zygomycete, *Trichosporon multisporum*, *Mortierella cf. hyaline* and *Geomyces pannorum*, proliferated in response to Impranil and YE amendment. Although the incidence of PU degrading fungi was high, the prevalence of organisms able to metabolise the breakdown products was likely to be much lower.

Although very few bacterial Impranil degraders were observed, some members of the bacterial soil consortium appeared to be able to use Impranil breakdown products as carbon sources, as bacteria increased markedly in numbers following the addition of Impranil to soil both with and without the concomitant addition of YE. Adipic acid and diethylene glycol are common breakdown products of PU hydrolysis (Akutsu *et al.* 1998), and diethylene glycol has been found to be a suitable carbon source for a number of soil bacteria, particularly *Pseudomonas* species (Kawai 1995b; Haines & Alexander 1975; Dwyer & Tiedje 1986). Hence, following hydrolysis of Impranil by soil fungi, bacteria may have been scavenging the breakdown products as nutrient sources.

A number of fungal species was seen to increase in numbers following the addition of Impranil and YE to soil (that were not also seen to increase in numbers when YE alone was added to soil), suggesting that they were capable of degrading Impranil and/or using the breakdown products as nutrient sources. However, with the exception of the unidentified Zygomycete, DGGE revealed that the majority of these fungi did not colonise the buried PU to a significant degree. Rather, colonisation was carried out by a mostly different set of soil fungi. The apparent inability of Impranil degrading/utilising fungi to colonise the surface of buried PU may be explained by the fact that the physicochemical characteristics of Impranil differ significantly than those of solid PU. Solid PU is highly hydrophobic, and Akutsu *et al.* (1998) have hypothesised that enzymes responsible for solid PU degradation may require hydrophobic binding domains to facilitate access of the enzyme to the plastic's surface. Such an interaction was found to be unnecessary for the hydrolysis of Impranil (Akutsu *et al.*, 1998). Hence, organisms possessing enzymes lacking these specialised binding domains may have been able to degrade Impranil upon its addition to soil, but may have been incapable of degrading solid PU, offering no selective advantage for proliferation on PU coupons.

Although both *G. pannorum* and *Alternaria sp18-2* were members of the fungal soil communities used in this work, comparison of soil DGGE profiles with PU profiles showed that neither of these fungi colonised the surface of the PU. Previous work involving burial of PU in this soil (Cosgrove *et al.* 2006, see chapter 2) found that these two fungi were major members of fungal communities on the surface of buried PU. However, the previous work was carried out *in situ* during primarily winter and spring months, whereas the microcosms in this work were incubated at 25°C in the laboratory; these differing conditions may have affected which species colonised the surface of buried PU. Furthermore, PU was buried for three months in this work and five months in the previous experiment. Colonisation sequences have been shown to occur during the development of fungal communities on the surface of plastic (Sabev *et al.* 2006; Webb *et al.* 2000), and *G.pannorum* or *Alternaria sp 18-2* may not colonise the surface of buried PU until sometime after three months burial.

PU buried in YE and YE plus Impranil treated microcosms showed a 96% reduction in tensile strength compared to unburied PU. However, the tensile strength of PU buried in untreated control soil was reduced by 90%, suggesting that, although YE and YE plus Impranil treatments increased the degradation of PU, this increase was relatively minor. Under these conditions therefore, the addition of nutrients to soil to enhance PU degradation appears to be unnecessary. However, caution must be employed when interpreting these data: only endpoint measurements were available for these samples. It has been shown previously that, whilst the amount of waste degraded at the end of an experiment may not be significantly different between the biostimulated communities and the untreated control, the actual rate of degradation may be significantly greater following biostimulation (Röling *et al.* 2004; Mills *et al.* 2004; Rivera- Espinoza & Dendooven 2004).

It is unclear how treating soil with YE or YE and Impranil enhanced, albeit slightly, the degradation of buried PU. The addition of nutrients often allows a given environment to support a generally larger microbial population, thus enhancing degradation due to the presence of larger numbers of waste degrading organisms (Sarkar *et al.* 2005; Rivera-Espinoza & Dendooven 2004). In support of this, a higher fungal titre was observed on plastic buried in soil treated with YE and YE plus Impranil. Alternatively, hydrocarbon contaminants such as PU are often poor in nitrogen and phosphorus, and

supplementation with these elements often enables contaminants to be degraded more easily (Roling *et al.* 2004; Xu and Obbard 2003).

It is interesting that treatment of soil with Impranil alone inhibited the degradation of buried PU. A similar effect was seen by Bento *et al.* (2005), in which adding fertilizer to soil contaminated with diesel reduced the rate at which diesel was degraded. They postulated that toxicity due to excess nitrates may have been responsible; however, Impranil has never been demonstrated to have significant biocidal properties. Nevertheless, fewer fungi were recovered from the surface of PU buried in Impranil only treated soil. Repeated Impranil addition may have influenced the soil's properties such that the physicochemical processes involved in initial attachment of fungi to the plastic were disrupted. Alternatively, competition with the extremely abundant bacteria in the soil following Impranil only treatment may have retarded the colonization of the PU by degrading fungi.

In conclusion, treatment of soil with YE or YE and Impranil caused a small but significant increase in the degradation of buried PU by altering the composition of the microbial communities in the soil and on the surface of buried PU. Further work using time point measurements will make it possible to determine if these treatments have a greater effect on the rate of degradation, as their influence on the final extent of degradation was relatively minor.

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Chapter 4:

Degradation of polyester polyurethane (PU) buried in soil bioaugmented with PU degrading fungi

Abstract

Biodegradation is an important method for waste remediation, and can be enhanced by the introduction of known waste degrading organisms. In this work, soil was inoculated with wheat individually colonised by the polyester polyurethane (PU) degrading fungal isolates *Geomyces pannorum*, *Penicillium inflatum*, *Nectria sp*, *Penicillium viridicatum*, *Penicillium ochrochloron*, an unidentified Zygomycete and two other isolates, 11n and 19n. PU was then buried in inoculated soil for four weeks. The influence of each inoculum on fungal community composition in the soil and on the surface of buried PU, and on the degradation of buried PU was investigated. Sterile wheat was added to soil as a control and increased the numbers of putative PU degrading fungi 38-fold in the soil and 35-fold on the surface of buried PU compared to untreated control soil. Of the isolates, only *P. ochrochloron* increased the numbers of putative PU degrading fungi compared to sterile wheat alone, increasing PU degraders 400-fold in the soil and 143-fold on PU. Denaturing gradient gel electrophoresis showed that inoculation with any of the isolates led to large changes in the fungal communities both in the soil and on the surface of buried PU; however, only *Nectria sp* and the unidentified Zygomycete could be detected in significant amounts in the soil after four weeks, and *P. viridicatum* and the Zygomycete were the only isolates that colonised buried PU. All treatments enhanced PU degradation after four weeks burial, compared to untreated control soil. Inoculation with *P. ochrochloron* lead to the greatest amount of degradation, with PU losing 85% of its tensile strength, compared to 24% in control soil, proving that bioaugmentation is an effective technique for enhancing PU degradation.

Introduction

The polyester polyurethanes (PU) are a diverse group of synthetic polymers employed in a number of industrial and commercial applications, including insulating foams, fibres, and synthetic leather and rubber goods (Matsumura *et al.* 2006). PU is a member of a relatively small group of synthetic polymers (including the polyhydroxyalkanoates, polycaprolactone, polylactic acid, and polyvinyl alcohol (Shimao 2001)) that are subject to degradation in the environment, a property which makes these plastics desirable from a waste management standpoint. The ability of a plastic to undergo degradation is of importance as the generation of plastic waste is a growing concern in the developed world. More than 30% of household waste in the USA is comprised of plastic refuse (Kawai 1995), and the global production of all plastics exceeded 140 million tonnes globally in 2001 (Shimao 2001). The majority of synthetic polymers are both chemically and physically recalcitrant, so these figures have serious environmental implications. There is therefore a need for the development of strategies to reduce persistent plastic waste. An increased use of biodegradable plastics would go some way to reducing the current ecological burden of plastic waste.

PU is produced via the condensation of poly- or di-isocyanates with polyols containing ester bonds (Nakajima-Kambe *et al.* 1999), leading to the formation of polymers containing an abundance of chemical linkages analogous to those found in molecules of biological origin, including ester and urethane bonds. PU is therefore often susceptible to microbial degradation, chiefly via hydrolytic enzymes produced by microorganisms during nutrient acquisition. Furthermore, products of PU hydrolysis often make suitable carbon sources (Pathirana and Seal 1985; Barratt *et al.* 2003), allowing PU waste to be partially or completely mineralised as biomass.

The biodegradation of pollutants such as PU within the environment is dependent on the presence of microbes at sufficiently high population densities and with the necessary metabolic pathways to ensure that degradation occurs within a reasonable timeframe. In some instances, native environmental microbial populations are sufficient to bring about biodegradation. When native microbial populations lack the capacity for efficient biodegradation, bioaugmentation may be employed. Bioaugmentation involves inoculating microorganisms possessing waste degrading phenotypes into polluted environments. Bioaugmentation has been successfully used to remediate a wide range of waste products, from hydrocarbons (Ueno *et al.* 2006; Bento *et al.* 2005) to organic pollutants (Fathepure *et al.* 2005; Garon *et al.* 2004; Da Silva *et al.* 2004), to heavy

metals (Jézéquel *et al.* 2005). To date, bioaugmentation as a strategy for plastic remediation has not been investigated.

Although factors such as pH, redox conditions and the presence of inhibitory compounds in the environment all influence the success of bioaugmentation (Alexander 1994), the survival and activity of the inoculum is often of vital importance; this is in turn heavily dependent on the initial strain selection (Thompson *et al.* 2005). Fortunately, a wide array of PU degraders has been isolated from a range of different environments (Ruiz *et al.*, 1999; Stern & Howard, 2000; Akutsu *et al.*, 1998; Pathirana & Seal, 1985; Crabbe *et al.*, 1994; Barratt *et al.*, 2003), providing a large reservoir of organisms which may be suitable for bioaugmentation.

In this work, bioaugmentation was assessed as a method for enhancing the degradation of PU buried in soil, employing putative PU degrading fungi isolated from a number of different soils. The primary objectives of this work were (i) to assess the PU degrading ability of each of the putative PU degrading isolates by cultivating them as monocultures on the surface of PU, (ii) to investigate the effect that inoculation of putative PU degraders into soil had on the fungal communities within the soil and on the surface of buried PU, and (iii) to determine if inoculating soil with putative PU degrading fungi increases the degradation of PU subsequently buried in that soil.

Materials and methods

Fungal strains

Putative PU degrading fungi were isolated as colonies producing zones of clearance on agar plates containing the colloidal PU dispersion Impranil DLN (Bayer GmbH, Dormagen, Germany). The majority of these isolates were recovered previously from the sandy loam soil used in this work ('acidic' soil, with a pH of 5.5 and 43.4g kg^{-1} organic carbon), or were recovered from the surface of PU buried in the same soil during previous experiments (Cosgrove *et al.* 2006a;b). One isolate was recovered from the surface of PU buried in a different sandy loam soil with a more neutral pH ('neutral' soil, with a pH of 6.8 and an organic carbon content of 78g kg^{-1}) (Cosgrove *et al.* 2006a), and one was recovered from the surface of PU buried in John Innes N°2 compost (Barratt *et al.* 2003). The identities of each isolate and their origins are detailed in Table 1.

Table 1: Origins and identities of the putative PU degrading fungal isolates used in this work.

Putative PU degrading fungal isolate	Origin	% homology with database sequence	Accession number
Penicillium ochrochloron	PU : John Innes Compost (Barratt *et al.* 2003)	98.6	AJ509865
Geomyces panorum	PU : acidic soil (Cosgrove *et al.* 2006a)	99.2	DQ779788
Penicillium viridicatum	PU : acidic soil (Cosgrove *et al.* 2006a)	99.1	DQ779779
Isolate 19n	PU : acidic soil (This work)	N/A	N/A
Penicillium inflatum	PU : organic soil (Cosgrove *et al.* 2006a)	99.7	DQ779783
Nectria sp. BC11	Soil: acidic soil (Cosgrove *et al.* 2006a)	100	DQ779785
Isolate 11n	Soil: acidic soil (This work)	N/A	N/A
Unidentified Zygomycete*	Soil : acidic soil* (Cosgrove *et al.* 2006b)	97.9	

* This isolate was enriched in acidic soil following the addition of Impranil and YE to the soil.
'PU' before the colon in the origins column indicates isolate was recovered from the surface of PU buried in soil; 'soil' indicates that the isolate was recovered from the soil in which the PU was buried. The soil in question is indicated after the colon.

Preparation of inoculum

Isolates were grown on malt extract agar (MEA, Oxoid, UK) at 25°C in preparation for spore or mycelium harvesting. Once sporulation or confluent growth had occurred, 25ml of sterile phosphate buffered saline (PBS) (Sambrook & Maniatis, 1989) containing 0.1% (v/v) Tween 80 was poured onto the agar surface and biomass was dislodged into the PBS using a Pasteur pipette. The dislodged biomass was then washed three times in fresh PBS (without Tween) via centrifugation at 3000g for 30min followed by re-suspension of the pellet in fresh PBS. Samples of washed biomass were dilution plated onto MEA to determine viable c.f.u. per ml.

Inoculation of sterile PU with putative PU degrading fungal isolates

PU (Elastogran GmbH Lemförde, Germany) coupons measuring 4x7x0.15cm (giving a total surface area of 59.3cm^2) were surface sterilised in 70% ethanol. Spore or mycelium suspensions of putative PU degrading fungal isolates were inoculated as monocultures onto the plastic at a density of 1x10^3 cfus cm^{-2}. This was done by adding 100µl of a 1x10^4 cfu ml^{-1} suspension of isolate biomass in PBS to each side of the PU coupon as 20 evenly spaced 5 µl drops, allowing each side of the coupon to dry before applying the isolate to the second side. Additional coupons were inoculated at the same density with an equal mixture of all 8 isolates, with equal volumes of all 8 isolates combined and mixed thoroughly prior to application to the plastic surface. Un-inoculated negative controls were prepared by adding 100 µl of sterile PBS to coupons. Six replicates of each inoculated coupon were prepared.

Preparation, maintenance and sampling of artificial soil microcosms containing PU inoculated with monocultures of putative PU degrading fungi.

Since the soil used in this work was not amenable to complete sterilisation, and in order to maintain monocultures on the surface of inoculated PU coupons, coupons inoculated with putative PU degrading fungi were buried in sterile synthetic soil consisting of a 50:50 mixture of perlite and vermiculite sterilised by autoclaving at 121°C for 1 h. The sterilised perlite and vermiculite mixture was dried at 85°C for 72 h. A small sample of this mixture was removed to determine its water holding capacity (whc) by adding water to a given amount until saturation occurred. Sufficient soil extract (Alef &

Nanniperi 1995) was added to dried perlite/vermiculite to reach 40% of the whc. Petri plates were then filled with this mixture, and a single inoculated PU coupon was buried in each. These 'microcosms' were sealed with parafilm and incubated at 25°C for four weeks. There were six replicate microcosms for each inoculated PU coupon.
After four weeks burial, PU coupons recovered from three replicates were scraped to recover biomass on the surface of the coupon; the remaining three coupons were immersed in 70% (v/v) ethanol to halt further biodegradation and their tensile strength was determined.

Cultivation of putative PU degrading fungal isolates on sterile wheat grains

In order to conveniently generate large quantities of biomass for inoculation into non-sterile soil, putative PU degrading fungal isolates were cultivated on the surface of sterile wheat grains. Wheat grains (100 g) were placed into 250 ml Erlenmeyer flasks and were sterilised by autoclaving for 20 min. Sterilised grain was then moistened using mineral salts medium (containing, per litre of H_2O: 7 g K_2HPO_4; 3 g KH_2PO_{4l}; 0.1 g $MgSO_4.7H_2O$; 1 g $(NH_4)_2SO_4$) supplemented with 2 gl^{-1} of D-glucose. Wheat was then inoculated with spores or mycelia of a given putative PU degrading isolate, and was incubated at 25°C for 4 weeks, at which point abundant growth of biomass had occurred. Uninoculated sterile wheat was also incubated as a negative control.

Preparation, maintenance and sampling of soil microcosms inoculated with putative PU degrading fungi grown on wheat.

An acidic garden soil with a sandy loam consistency and with no previous exposure to PU was employed in this work (Cosgrove *et al*, 2006a). This soil was heavily inoculated with putative PU degrading fungal isolates by mixing with wheat that had been colonised by these isolates. Soil was divided into 200 g samples, and each was mixed with 50 g of wheat on which a given isolate was cultivated. An additional 200 g of soil were combined with 50 g of a mixture of all of the variously inoculated wheat grains combined in equal proportions. Petri plates were filled with these soil/wheat mixtures, and a single ethanol sterilised PU coupon (4 x 7 x 0.15cm) was buried in each. In addition, controls were prepared wherein PU coupons were buried in petri plates containing soil mixed with sterile wheat and soil to which no wheat had been added.

Plates were sealed with parafilm and incubated at 25°C for four weeks. Each mixture of inoculated wheat and soil was prepared in replicates of six. After four weeks, PU coupons recovered from three of these replicates were scraped to recover biomass on the surface of the coupon; the remaining three coupons were immersed in 70% (v/v) ethanol to halt further biodegradation and their tensile strength was determined.

Tensile strength determination of buried PU

The tensile strength of PU coupons after burial in test microcosms was determined to assess the extent of degradation. Coupons were cut into dumbbell shapes with the following dimensions: width 2 mm, depth 1.5mm, length 20 mm. Dumbbells were stretched at a rate of 200 mm min^{-1} and the maximum load at breaking (the tensile strength), in kilonewtons, was determined using an Intstron 4301 (Instron Ltd, Swindon UK). Breaking strength was considered to be inversely proportional to the extent of degradation. The tensile strength of unburied control dumbbells was also determined.

Recovery of biomass from the surface of buried PU

At the end of each experiment, biomass was recovered from PU coupons buried in microcosms in order to analyse the fungal communities growing on the surface. For coupons buried in soil, loosely adhered soil particles were removed by agitating the PU coupons in sterile phosphate buffered saline (PBS) (Sambrook & Maniatis, 1989) for 5 min. These coupons were submerged in 20ml sterile PBS, and the biomass was scraped from both sides of the PU into the PBS using a sterile scalpel blade. An aliquot of 1ml of this biomass suspension was reserved for viable counting. The remainder was centrifuged at 3000g for 30 min at 4°C, and the supernatant was discarded. The biomass remaining was then used for DNA extraction and DGGE analysis.

Fungal viable counts

Viable counts of fungi in the soil and on the surface of buried PU were determined on solid culture media. Samples of soil in which the PU was buried, and samples of biomass recovered from the surface of buried PU, were dilution plated in PBS onto soil extract agar (SEA, Alef & Nanniperi, 1995) and Impranil agar (Crabbe *et al.* 1994). Colonies were counted after 5-7 days incubation at 25°C. Total viable fungi were

enumerated on SEA, whilst putative PU degrading fungi were enumerated as colonies producing zones of clearance on Impranil agar. Media included 50 μgml^{-1} of chloramphenicol to inhibit bacterial growth. The number of Impranil degrading fungi was then calculated as a percentage of the total number of colonies recovered.

DNA extraction

The FastDNA SpinKit for Soil (Q-Biogene, California U.S.A) was used to extract total DNA from 0.4 g soil samples, or 0.5 g samples of biomass (wet weight) recovered from the surface of buried PU. To remove all traces of PCR inhibitory compounds, 20 µl of extracted DNA was run for ca. 15 min on a 1.0% agarose/TAE gel. Bands of genomic DNA were then excised, and DNA was recovered using the Nucleospin Extract II gel extraction kit (Machery-Nagel, Düren, Germany).

PCR amplification of fungal community DNA

PCR was employed to generate the necessary DNA fragments for fungal community denaturing gradient gel electrophoresis (DGGE) analysis. PCR DNA template consisted of approximately 50 ng per reaction of extracted DNA. Biomix Red PCR master mix (Bioline, London U.K) was employed in all reactions. Primers were present in each reaction at a concentration of 1 µM. Fungal DGGE fragments were generated using the GM2/JB206c primer set (GM2: 5'-CTGCGTTCTTCATCGAT-3', JB206c: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAA GTAAAAGT CGTAACAAGG-3'), which amplify the ITS1 region found in the fungal rDNA gene complex. The PCR regime employed was as follows: 94°C initial denaturation for 5 min; 20 'touchdown' cycles of 94°C for 30 sec, annealing for 30s at 59 to 49°C with annealing temperature being reduced by 0.5°C per cycle, extension at 72°C for 45 s; 30 cycles at 95°C for 30 s, annealing at 49°C for 30 s and extension at 72°C for 45 s; 1 final extension at 72°C for 5 min.

DGGE analysis of microcosm fungal communities

The compositions of the fungal communities in the soil and on the surface of buried PU were compared using DGGE (Muyzer *et al.* 1993). The D-Code universal mutation detection system (Biorad, Herts U.K.) was employed. Gels measured 16cm x 16cm x 1mm and contained 10% (v/v) acrylamide in 1 x TAE (40 mM Tris base, 20 mM glacial acetic acid, 1mM EDTA). A denaturant gradient of 25-55% was used. For all gels, approximately 500 μg of PCR product was used per lane; gels were run in 1 X TAE buffer at a constant temperature of 60°C for 16.5 h at 42 volts. After electrophoresis was complete, gels were stained with SybrGold (Molecular Probes, Netherlands) for 45 min and photographed under ultraviolet light.

Results

Growth of Impranil degrading isolates following inoculation onto PU buried in sterile artificial soil

Putative PU degrading fungal isolates were inoculated onto sterile PU as monocultures and as an equal mixture of all 8 isolates. The growth of each isolate after four weeks incubation in sterile perlite and vermiculite was determined by plating biomass from the PU surface onto MEA (Fig 1).

Each PU coupon was inoculated at a density of $1x10^3$ cfus cm^{-2} at the onset of the experiment; the lowest values for cfus recovered after four weeks were for isolate 11n at $3.9x10^4$ cfu cm^{-2} and *Nectria sp BC11* at $5.9x10^4$ cfu cm^{-2} (these two values were not significantly different, $p>0.05$), indicating that all of the isolates tested could proliferate on the surface of PU as monocultures. *P. inflatum*, *G. pannorum* and *P. ochrochloron* displayed intermediate growth, with an average of 7.2 $x10^5$ cfus per sq cm ($p>0.05$ for these three isolates). The unidentified Zygomycete showed the greatest amount of growth of all of the monocultures at $1.3x10^6$ cfus cm^{-2}. The most growth was observed when a mixture of all 8 isolates was applied to the PU, with $2.3x10^6$ cfus cm^{-2} recovered after four weeks burial.

In conclusion, all of the isolates employed could proliferate on the surface of PU in the absence of any competition; however, some isolates could colonise the surface of PU in greater numbers than others.

Degradation of PU coupons by defined cultures of putative PU degrading fungi.

Putative PU degrading ability was assigned to the isolates used in this work according to their ability to degrade the colloidal PU dispersion Impranil in solid medium. In order to determine if these isolates could degrade solid PU, each was grown as a monoculture on the surface of PU buried in sterile perlite and vermiculite for four weeks. If isolates were capable of degrading solid PU, a decrease in tensile strength of the PU was expected.

The tensile strength of PU after burial for four weeks in perlite and vermiculite was measured (Fig 2). Non-degraded, unburied control PU coupons had a tensile strength of 330N. There was no significant difference in the tensile strength of un-inoculated

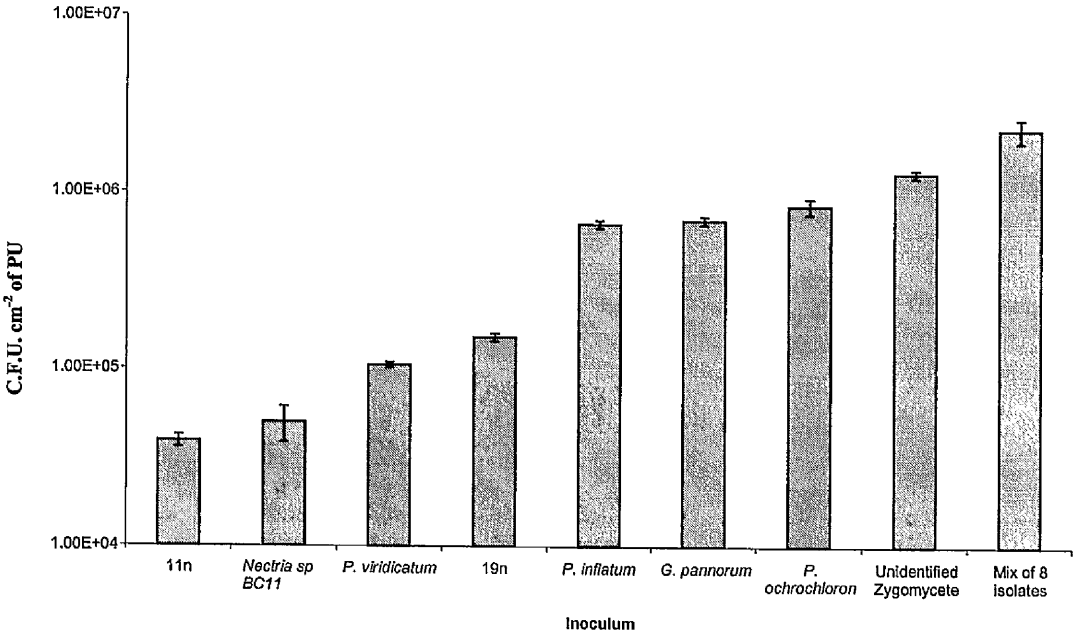

Figure 1: Colonisation of solid PU by pure cultures of putative PU degrading fungi after four weeks burial in sterile perlite:vermiculite microcosms. Error bars indicate standard error of the mean (S.E.) (n=3).

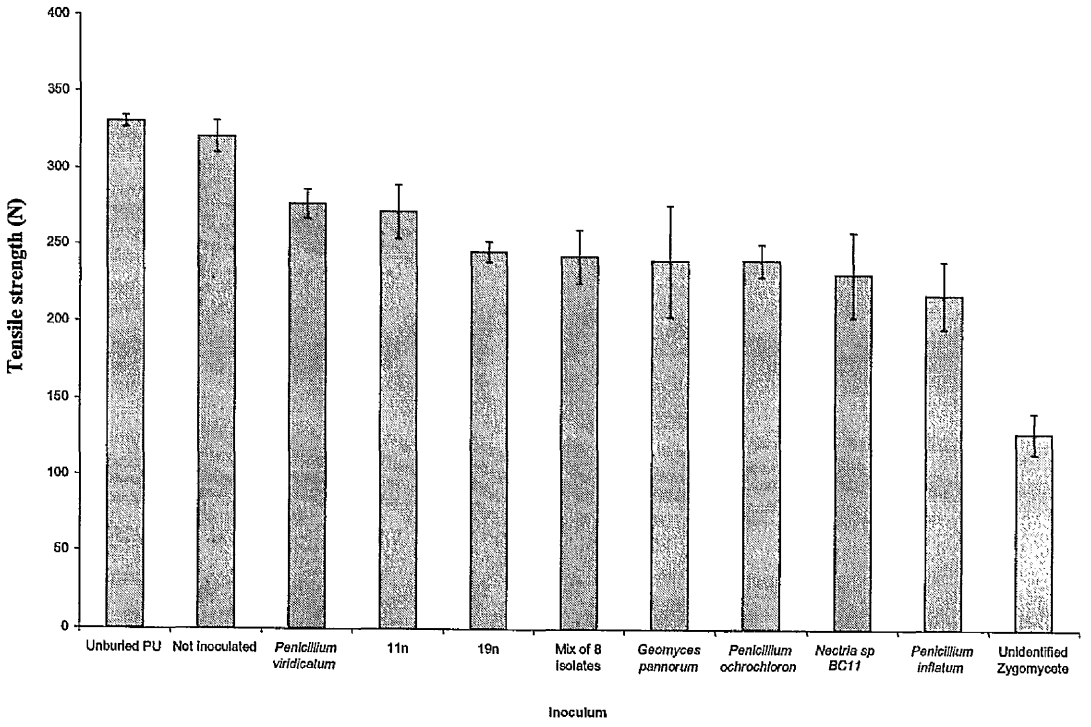

Figure 2: Loss of tensile strength of PU coupons after colonisation by monocultures of putative PU degrading fungi. PU coupons inoculated with putative PU degrading fungal isolates were buried in sterile perlite:vermiculite for four weeks. Unburied PU and PU buried in sterile perlite:vermiculite but without any fungal inocula were included as controls. Error bars indicate +/- S.E. (n=3).

PU buried in sterile microcosms and unburied PU ($p>0.05$). All of the inoculated PU coupons had a reduced tensile strength compared to the uninoculated control, indicating that all of the isolates were capable of degrading solid PU. The least degradation occurred when the PU was inoculated with *P. viridicatum* or isolate 11n, where tensile strength was seen to decrease by approximately 17%. PU showed the greatest degradation when inoculated with the unidentified Zygomycete, showing a 61% reduction in tensile strength compared to the unburied control. PU inoculated with the remaining isolates and with an equal mixture of all isolates showed an intermediate degree of degradation, with reductions in tensile strength of 26-34% compared to the control.

In conclusion, all of the isolates employed in this study were shown to be able to degrade solid PU. Although many of the isolates showed a similar level of activity against PU, examples of both relatively poor and especially active PU degrading fungi were found.

Numbers of fungi and numbers of Impranil clearing fungi recovered from soil four weeks after inoculation with putative PU degrading isolates

The total numbers of fungi and the numbers of putative PU degrading fungi in the soil four weeks after inoculation were determined using soil extract agar and Impranil agar, respectively. All colony morphotypes were counted after recovery from the surface of PU on solid media, and not just those initially inoculated into soil on wheat.

Control soil, to which no wheat had been added, contained $7.5x10^5$ fungal cfus g^{-1} (Fig 3), of which $2.4x10^5$ (32%) produced clear zones on Impranil agar. This soil therefore contained a large population of indigenous putative PU degrading fungi. The addition of sterile wheat increased the numbers of viable fungi in the soil by a factor of 26 to $2x10^7$ cfu per gram. In the majority of cases, inoculation of soil with wheat colonised with putative PU degrading fungi did not lead to a significantly different number of fungal cfus in the soil compared to sterile wheat alone ($p>0.05$). In three instances, however, a greater number of fungi was recovered if wheat was colonised with PU degrading fungi. If the unidentified Zygomycete, *P. ochrochloron* or an equal mixture of all 8 isolates were added to the soil, fungal cfus were increased by a factor of 2.7, 4.5 and 3.4, respectively, compared to soil treated with sterile wheat alone.

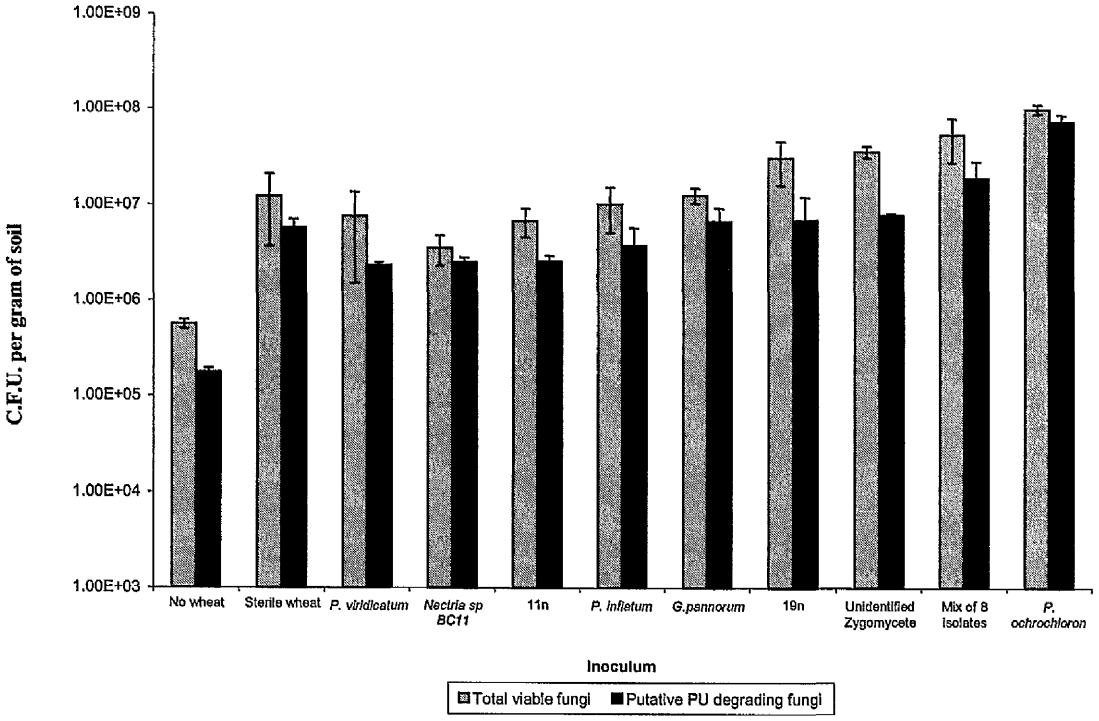

Figure 3: Numbers of viable fungi and numbers of Impranil degrading fungi in soil microcosms four weeks after inoculation with wheat colonised by putative PU degrading fungi. Total numbers of CFUs of fungi per gram of soil recovered on SEA (dark bars) and numbers of colonies producing clearing zones on Impranil agar (light bars) are shown. Error bars indicate +/- S.E. (n=3)

The number of putative PU degrading fungi in the soil was increased compared to untreated control soil, regardless of the inoculum. The addition of sterile wheat to soil increased the numbers of putative PU degrading fungi approximately 38-fold compared to untreated control soil. Of the isolates, *P. ochrochloron* was found to produce the largest increase in the numbers of putative PU degraders in the soil, with 400-fold more Impranil-clearing fungal cfus recovered compared to untreated control soil. An investigation of colony morphology, however, revealed that the majority of these Impranil clearing colonies were not *P. ochrochloron*, but were instead a mixture of morphotypes. Inoculation with *P. viridicatum, Nectria sp BC11* and isolate 11n all led to approximately 2.5-fold fewer putative PU degrading fungi in soil compared to soil treated with sterile wheat. *P. inflatum*, *G. pannorum*, isolate 19n and the unidentified Zygomycete did not significantly increase the numbers of putative PU degrading fungi compared to the addition of sterile wheat alone. Adding all 8 isolates to soil together increased the numbers of putative PU degraders 3-fold compared to soil treated with sterile wheat alone. In all cases a diverse range of colony morphotypes were seen on Impranil agar plates, and the majority of the fungi recovered after 4 weeks were not the isolates inoculated into the soil at the onset of the experiment.

In conclusion, putative PU degrading fungi are naturally present in high numbers in the soil employed in this work and these fungi can be increased in number by the introduction of sterile wheat. If wheat was colonised with *P. ochrochloron* or a mixture of all 8 isolates, the numbers of putative PU degrading fungi in the soil could be increased still further. However, colonising the wheat with the remaining PU degrading isolates did not significantly increase the numbers of PU degrading fungi in the soil compared to adding sterile wheat alone.

Survival of PU degrading fungi after inoculation into soil.

DGGE was used to determine if isolates introduced on wheat could survive in the soil for the four weeks of the experiment. DGGE profiles of fungal soil communities (Fig 4) were also compared to determine how each inoculum influenced the composition of fungal communities in the soil.

Although some bands were found in both the control soil DGGE (Fig 4, lane 1) and the DGGE of soil to which sterile wheat had been added (Fig 4, lane 2), there were numerous differences in the banding pattern between these two profiles, indicating

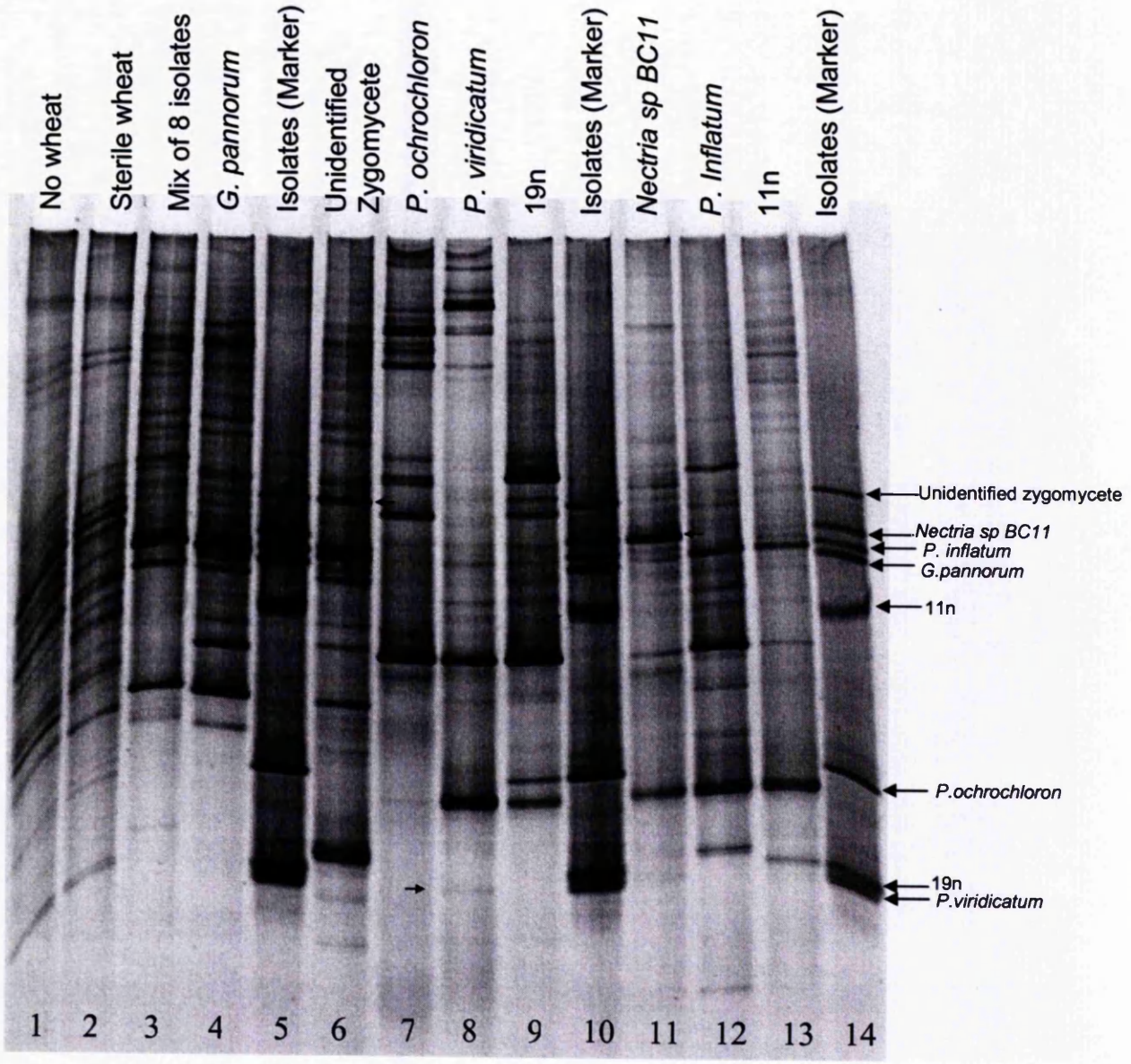

Figure 4: DGGE comparison of fungal soil communities 4 weeks after the addition of wheat colonised with PU degrading fungal isolates. Marker lanes (5, 10 and 14) contain DGGE products from all 8 isolates. Arrows in lanes 6, 9 and 11 indicate bands that comigrate with bands produced by isolates.

that the addition of sterile wheat to soil led to changes in the fungal communities in the soil. The DGGE profiles of soil communities that had been inoculated with putative PU degrading isolates (Fig 4 lanes 3 and 4, 6-9, and 11-13) showed clear differences in banding patterns compared to the sterile-wheat treated soil. Different inocula produced different banding patterns, proving that the nature of the inoculum influenced the composition of the soil fungal communities. Lanes 5, 10, and 14 (Fig 4) contain a mixture of DGGE products generated by each of the isolates, with each isolate producing a distinct band (which band each isolate produced is indicated by arrows in Fig 4, lane 14). The lanes for soil inoculated with *G. pannorum*, *P. ochrochloron*, isolate 19n, *P. inflatum* and isolate 11n did not contain any bands that migrated to the same position as the bands produced by those same isolates. Hence, although each isolate had a different effect on the composition of the soil community, in most cases there was no evidence of significant survival of isolates four weeks after their inoculation into soil. Only the Zygomycete and *Nectria sp BC11* showed clear evidence of survival – intense bands representing these isolates can be seen in the appropriate lanes in Figure 4 and are indicated by arrows. A faint band that migrates to the same position as that produced by *P. viridicatum* was seen in the DGGE profile of fungal communities in soil inoculated with this isolate (Fig 4, lane 8, arrow). The faintness of the band suggests that *P. viridicatum* was able to survive in the soil, albeit at a low level. In conclusion, the addition of putative PU degrading isolates to soil leads to clear changes in the fungal soil communities. However, DGGE indicated that only two of the isolates survived to a significant degree in the soil four weeks after inoculation.

Numbers of fungi recovered from PU buried in soil inoculated with PU degrading fungal isolates.

The total numbers of fungi and the numbers of putative PU degrading fungi on the surface of PU after four weeks burial in soil inoculated with putative PU degrading fungi were determined using SEA and Impranil agar (Fig 5). All colony morphotypes were counted after recovery from the surface of PU on solid media, and not just those initially inoculated into soil on wheat.

PU coupons buried in untreated soil were colonised by approximately $4.5x10^3$ fungal cfus cm^{-2} (Fig 5, 'no wheat'). There were $2x10^3$ Impranil clearing colonies cm^{-2} of PU

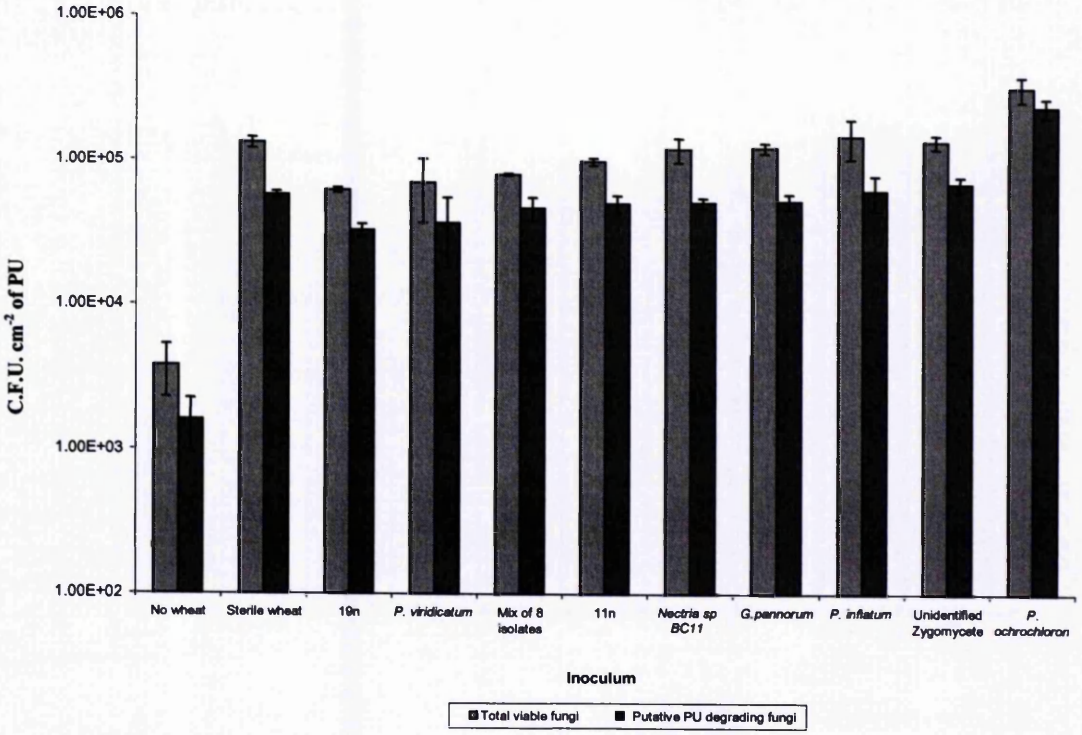

Figure 5: Total numbers of viable fungi (light bars) and numbers of Impranil degrading fungi (dark bars) on PU after four weeks burial in soil inoculated with wheat colonised by PU degrading fungi. Error bars indicate +/- S.E. (n=3).

buried in untreated control soil. As with the soil populations, the addition of sterile wheat increased the numbers of fungi present. Both total viable fungi and the numbers of putative PU degrading fungi colonising the surface of buried PU increased 35-fold compared to the control. Although inoculation of soil with *P. viridicatum*, isolate 11n, *Nectria sp BC11*, *G. pannorum*, *P. inflatum*, the unidentified Zygomycete and a mixture of all eight isolates increased the numbers of PU degrading fungi on the surface of buried PU compared to the control soil, none produced an increase that was significantly different from that seen when sterile wheat alone was added ($p>0.05$). Significantly fewer (1.7-fold) putative PU degrading fungi were recovered from PU buried in soil inoculated with isolate 19n compared to soil to which sterile wheat alone was added. Only inoculation with *P. ochrochloron* significantly ($p<0.05$) increased the numbers of PU degrading fungi on the surface of buried PU compared to sterile wheat alone - in this case 4-fold more putative PU degraders were recovered compared to PU buried in soil treated with sterile wheat alone.

Effect of inoculating soil with PU degraders on the colonisation of buried PU

DGGE profiles of fungal communities recovered from the surface of PU buried in soil inoculated with putative PU degrading isolates (Fig 6) were analysed to determine if the inoculated isolates colonised the surface of buried PU. The effect of each inoculum on the structure of the fungal communities colonising the surface of PU was also determined.

A band that migrated to the same position as the band produced by the isolate *P. viridicatum* (shown in marker lanes 7 and 13, Fig 6) was seen in the DGGE profile of the fungal PU community colonising the surface of PU buried in soil inoculated with *P. viridicatum* (Fig 6, lane 8, arrow). This suggests that *P. viridicatum* was capable of colonising the surface of buried PU. There was also some evidence that the unidentified Zygomycete was capable of colonising buried PU, as a faint band migrating to the same position as this isolate was visible in the DGGE profile of communities colonising PU buried in soil inoculated with the unidentified Zygomycete (Fig 6, lane 5, arrow). However, there was no evidence of colonisation of buried PU by the remaining isolates that had been inoculated into the soil.

The addition of sterile wheat to soil affected the species composition of the fungal communities that colonised the surface of buried PU, as many of the bands seen in the

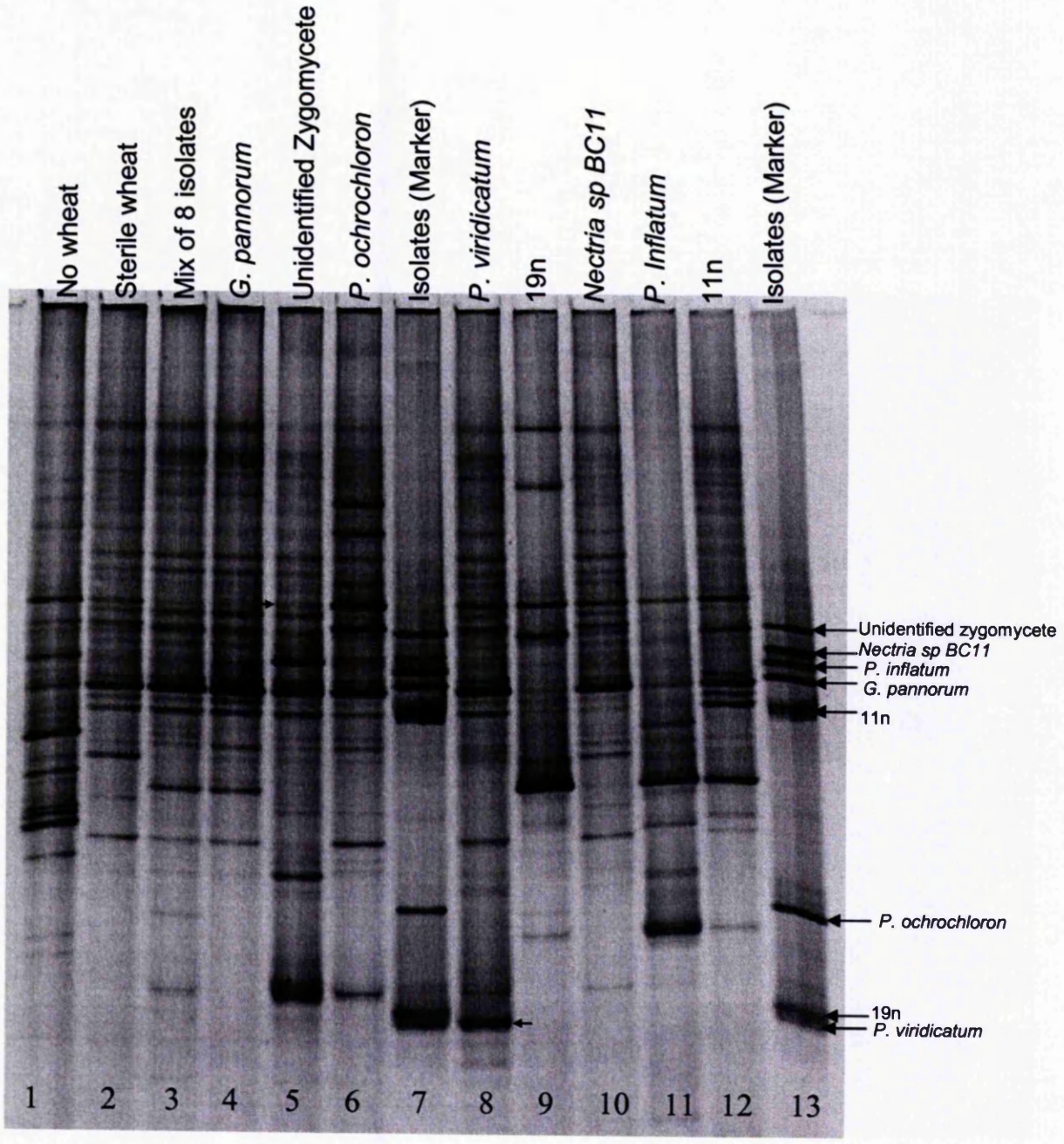

Figure 6: DGGE comparison of fungal communities on PU after 4 weeks burial in soil inoculated with wheat colonised with PU degrading fungal isolates. Marker lanes (7 and 13) contain DGGE products from all 8 isolates. Arrows in lanes 5 and 8 indicate bands that comigrated with bands produced by the unidentified Zygomycete and *P. viridicatum*, respectively.

DGGE of PU communities in untreated control soil (Fig 6, lane 1) are absent in the DGGE profile of PU communities in soil to which sterile wheat had been added (Fig 6, lane 2), and vice versa. The addition of PU degrading isolates to soil also altered the composition of the fungal communities on the surface of the PU (Fig 6, lanes 3-6 and 8-12) compared to the PU communities in untreated control soil. DGGE profiles of PU communities in isolate inoculated soil were also different to those in soil inoculated with sterile wheat, indicating that the effect of introducing PU degrading isolates growing on wheat on PU communities was not solely due to the concomitant addition of wheat grains. Each isolate produced a different PU community DGGE profile, suggesting that each isolate had a different effect on how fungal communities developed on the surface of buried PU.

In conclusion, the inoculation of soil with putative PU degrading fungal isolates influenced the composition of fungal communities on the surface of PU subsequently buried in the soil. However, with the exception of *P. viridicatum* and the Zygomycete, these isolates were unable to colonise and proliferate on the surface of buried PU to any significant degree after four weeks.

Degradation of PU coupons in soil inoculated with large quantities of putative PU degrading fungi cultivated on wheat

The extent of PU degradation after four weeks burial in soil inoculated with wheat colonised with putative PU degrading fungi was assessed by determining the tensile strength of buried PU coupons (Fig 7). Tensile strength was inversely proportional to extent of degradation.

PU buried in untreated soil with no wheat added had 24% less tensile strength than unburied PU, indicating that the indigenous soil organisms could degrade PU. PU buried in soil to which sterile wheat had been added had 45% the tensile strength of unburied PU, suggesting that the addition of wheat to soil enhances PU degradation compared to untreated control soil. PU buried in soil inoculated with *G. pannorum*, *P. inflatum*, and isolate 19n cultivated on wheat grains was not significantly more degraded than PU buried in soil to which wheat alone was added ($P>0.05$). Inoculation with isolate 11n or an equal mixture of all isolates led to an approximate 62% reduction in tensile strength compared to untreated control soil, and *Nectria sp BC11*, *P. viridicatum*, and the unidentified Zygomycete produced a 77% reduction in tensile strength.

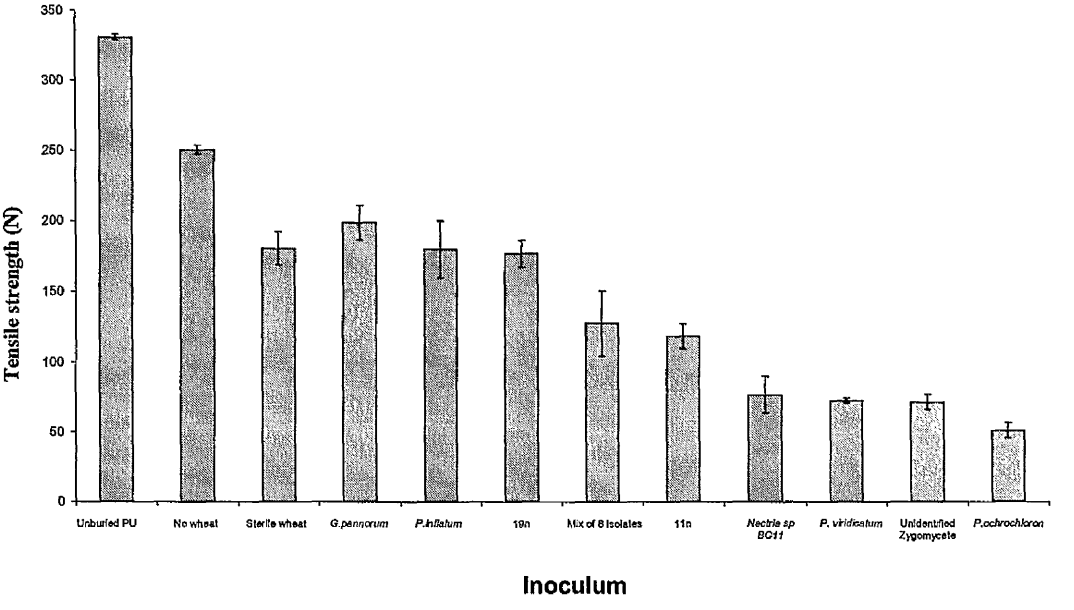

Figure 7: Loss of tensile strength of PU after 4 weeks burial in soil inoculated with wheat colonised by putative PU degrading fungi. Unburied PU, PU buried in soil containing sterile wheat and PU buried in soil with no wheat were included as controls. Error bars indicate +/- S.E. (n=3).

The greatest loss of tensile strength occurred when the soil was inoculated with *P. ochrochloron*, where buried PU lost 85% of its tensile strength over the course of four weeks.

It is unclear as to whether the presence of any particular fungal species on the surface of PU lead to more extensive degradation. Although the DGGE profiles of the fungal communities on PU showing the greatest amount of degradation (Fig 6, lanes for unidentified Zygomycete (lane 5), *P. ochrochloron* (lane 6), *P. viridicatum* (lane 8), and *Nectria sp BC11* (lane 10)), possessed some bands not present in communities on less degraded plastic, these bands were generally not shared amongst all of the communities on well degraded plastic. Also, strong bands found to be shared between communities associated with greater degradation are also present in communities where less PU degradation occurred.

In conclusion, the native soil microbial populations could degrade PU, although there did not appear to be any particular fungal species strongly associated with extensive degradation. Degradation could be enhanced by the addition of sterile wheat grains. Although no benefit was gained by colonising the wheat with *G. pannorum*, *P. inflatum*,

and isolate 19n before adding it to soil, the remaining isolates proved useful for increasing the rate of PU degradation, with *P. ochrochloron* showing the greatest effect.

Summary of the behaviour of each isolate in sterile perlite-vermiculite and soil microcosms

The survival of each of the isolates in pure culture and in soil, and the degradation of PU in the presence of these isolates is summarised in Table 2.

Geomyces panorum, Isolate 19n, and *Penicillium inflatum* were the least suitable of all of the isolates tested as potential strains for bioaugmentation. Monocultures of each produced only moderate degradation of PU in sterile microcosms. Neither of these strains survived in soil four weeks after burial, nor could they colonise the surface of PU buried in soil inoculated with these isolates. Futhermore, the inoculation of these strains on wheat into soil did not significantly enhance the degradation of PU compared to when sterile wheat alone was added to soil.

Although isolate 11n was found to slightly increase the degradation of PU buried in soil inoculated with this strain, this isolate showed poor degradation of PU as a monoculture and was also incapable of surviving in soil after inoculation or of colonising the surface of buried PU.

P. viridicatum both survived in soil four weeks after inoculation and colonised the surface of buried PU. PU buried in soil inoculated with this isolate was also severely degraded; however, this isolate was only capable of causing slight degradation of PU as a monoculture in sterile microcosms.

Although *Nectria sp BC11* was not seen in the soil four weeks after inoculation, it was capable of colonising the surface of buried PU. PU was also severely degraded when buried in soil that had been inoculated with this fungus.

Although *P.ochrochloron* was found to produce the greatest amount of PU degradation when inoculated into soil (Figure 7), a monoculture of this isolate in sterile microcosms degraded PU only to a moderate degree. Furthermore, this isolate did not survive in soil four weeks after inoculation and was not found to colonise the surface of PU buried in soil inoculated with this fungus. It is unclear, therefore, how inoculation of soil with this fungus led to such severe degradation of PU.

Table 2: Colonisation of PU by PU degrading fungal isolates in sterile perlite and vermiculite microcosms and non-sterile soil microcosms. Survival of isolates in non-sterile soil four weeks after inoculation (as indicated by DGGE) is shown. The degradation of PU buried in artificial and true soil microcosms inoculated with isolates is also indicated.

Isolate	Colonisation of PU buried in artificial soil*	Colonisation of PU buried in non-sterile soil*	Survival in soil after four weeks*	Degradation of PU buried in artificial soil microcosms ‡	Degradation of PU buried in non-sterile soil ‡
Unidentified Zygomycete	+	+	+	+++	+++
Penicillium ochrochloron	+	-	-	++	+++
Nectria sp. BC11	+	-	+	++	+++
Penicillium viridicatum	+	+	+	+	+++
Isolate 11n	+	-	-	+	++
Geomyces panorum	+	-	-	++	-
Isolate 19n	+	-	-	++	-
Penicillium inflatum	+	-	-	++	-

*** + = colonisation/surivival, - failure to survive/colonise.**

‡ - = tensile strength of PU not significantly reduced, + = tensile strength of PU reduced by less than 25%, ++ = tensile strength of PU reduced by 25 - 50%, +++ = tensile strength of PU reduced by greater than 50%. PU degradation is presented relative to the controls, comprising uninoculated PU for sterile artificial soil microcosms and PU buried in soil to which sterile wheat had been added for the true soil microcosms.

Of all of the isolates tested, the unidentified Zygomycete showed the greatest promise for bioaugmentation. This strain was found to survive in soil following inoculation and could colonise the surface of PU buried in soil inoculated with this isolate. The Zygomycete was also found to degrade PU in pure culture more than any other isolate, and PU buried in soil inoculated with this strain was severely degraded.

Discussion

Bioaugmentation, in which microorganisms with pollutant degrading phenotypes are introduced into contaminated sites, is receiving increasing attention as a potential method for waste remediation. In this work, 8 known PU degrading fungi were added to soil in large quantities in an attempt to accelerate the degradation of PU buried in the soil.

The isolates used in this work were initially assigned putative PU degrading phenotypes based on their ability to clear the colloidal PU dispersion 'Impranil'. To determine if each isolate could colonise and degrade solid PU, each was inoculated onto the surface of PU coupons. All of the isolates were able to proliferate as monocultures on PU buried in artificial soil microcosms, with the unidentified Zygomycete showing the greatest amount of growth. It is unclear whether the fungal isolates were using the PU as a sole carbon source, since soil extract was added to the artificial soil microcosms to more closely mimic real soil. Soil extract contains a mixture of organic constituents including humic compounds, which have been found to be suitable as carbon sources for a wide variety of soil fungi (Gramss & Ziegenhagen, 1999; Steffen *et al.* 2002). PU was significantly degraded following colonization by monocultures of all of the isolates compared to uninoculated control PU, proving that all of the isolates could degrade solid PU. The unidentified Zygomycete was particularly effective, degrading PU twice as effectively as any of the other fungi tested. This fungus was also found previously to proliferate massively after the addition of Impranil to soil (Cosgrove *et al.* 2006b); these results therefore support the use of Impranil clearance assays as a screen for putative PU degrading fungi.

PU buried in soil was degraded more rapidly than PU that had been colonized by monocultures of known PU degraders and buried in artificial soil microcosms; this was the case even though there were fewer putative PU degrading fungi per square centimeter on PU buried in soil. DGGE showed that the fungi colonizing the surface of soil buried PU were not those introduced into the soil; these unknown fungi may have possessed more efficient PU degrading abilities than the isolates. Alternatively, the mixed communities on the surface of the soil buried PU may have been acting synergistically, leading to more efficient PU degradation. Although such synergism was not observed when mixed communities were artificially constructed (since PU

colonized with a mix of all isolates in artificial soil microcosms was not more degraded than PU inoculated with monocultures), synergy may have been more likely to occur in communities allowed to develop naturally. Walter *et al.* (2004) found that successful bioaugmentation of a PCP contaminated soil required interactions between introduced fungi and the native soil population. It is unlikely that abiotic processes in the soil were responsible for the increased degradation of PU, as PU has been found to be generally resistant to abiotic degradation (Bentham *et al.* 1987; Dale & Squirrell, 1990; Woods 1990).

Both DGGE and observation of the colony morphotypes recovered on agar plates showed that the majority of the isolates did not survive in the soil in significant numbers four weeks after inoculation. Both biotic factors such as competition with native soil communities and abiotic factors such as soil pH, soil pore size and other physicochemical soil properties have been found to influence survival in the soil (van Veen *et al.* 1997, van Elsas *et al.* 1986). It seems likely that the isolates recovered from soils different to that used in this work (*P. ochrochloron* and *P. inflatum*) would be the most susceptible to these factors, and neither of these fungi survived in the soil. Similarly, although isolates *G. pannorum* and *P. viridicatum* were both native to the soil used in this work, they were only very minor members of the soil consortia and were only recovered after being enriched on the surface of buried PU (Cosgrove *et al.* 2006a). Therefore *G. pannorum* and *P. viridicatum* may also represent fungi poorly adapted to growth in the soil; these isolates also showed little evidence of survival in soil, although there was evidence of limited survival for *P. viridicatum*. Only the unidentified Zygomycete and *Nectria sp BC11* showed clear evidence of survival in soil, and both of these were initially isolated from soil populations in the soil used in this work.

The failure of the majority of the isolates to survive in the soil was unexpected, as many studies show inocula persisting for some time, or actually increasing in numbers after inoculation (Mishra *et al.* 2004; Yu *et al.*, 2005; Das *et al.*, 2006; Jézéquel *et al.*, 2005). This was the case even if the inoculum was not native to the environment to which the inoculum was introduced. In all of these studies, however, the native communities lacked significant numbers of organisms with waste degrading phenotypes; the introduced strains, which can usually exploit waste as a nutrient source, therefore had a selective advantage. Impranil clearance assays revealed that putative PU degraders were common amongst the native fungi in the soil used in this work. Under these

conditions, inoculated isolates may have had no selective advantage other than initially superior numbers; this, combined with the factors previously described may explain the poor survival of the isolates in the soil.

The addition of fungal isolates to the soil led to clear changes in both the native soil fungal communities and the communities that developed on the surface of buried PU, as revealed by both agar plate counts and DGGE. This is not surprising - the influx of such a large quantity of biological matter would be highly likely to perturb the native microbial communities. Many fungi also produce antimicrobials and other secondary metabolites, hence stimulating the growth of some soil species whilst inhibiting the growth of others. Furthermore, these changes are likely to persist long after the inoculum disappeared. Wenderoth *et al.* (2003) found that inoculation of chlorobenzene degraders into groundwater led to perturbed microbial communities, even after all traces of the inoculum had disappeared from DGGE profiles of the microbial communities.

Although there was no evidence that the majority of the inoculated isolates (with the exception of *P. viridicatum* and the Zygomycete) colonised the surface of the PU to a significant degree, there was nevertheless an increase in the degree of PU degradation when soil was inoculated with wheat grains colonized by these isolates. It is likely that the concomitant addition of wheat was at least partially responsible for the enhanced degradation. The addition of organic matter has been found to enhance bioremediation in previous studies (Cosgrove *et al.* 2006b; Rivera- Espinoza & Dendooven 2004; Sarkar *et al.*, 2005) and is usually attributed to the stimulation of the of the microbial communities present due to the influx of nutrients. Such stimulation of the numerous putative PU degrading fungi found to be native to the soil used in this work may explain some of the enhancement of degradation observed. Indeed, the addition of wheat alone was found to increase the numbers of putative PU degrading fungi in the soil 21-fold and on the surface of the PU 35-fold, and increased PU degradation 35% compared to PU buried in control soil. However, stimulation by wheat alone cannot explain the even more pronounced PU degradation that occurred when isolates 11n, *Nectria sp*, *P.viridicatum*, the unidentified Zygomycete and *P. ochrochloron* were added to the soil. Although apparently incapable of causing PU degradation by colonising its surface, these isolates may have degraded PU via less direct means. Isolates growing on wheat grains may have secreted PU lytic enzymes into the soil prior to their disappearance from the soil; high levels of enzyme secretion by fungi growing on the surface of wheat

products has been described previously (Ullah *et al.*, 2000). The action of these enzymes may have enhanced the degradation of PU directly via hydrolysis of the PU backbone, and may have also conditioned the surface of the PU allowing native PU degrading soil fungi to colonize the PU earlier than they otherwise would have, allowing a greater amount of time for degradation. Inoculation with fungal isolates also influenced which of the native soil fungi colonized the surface of the PU, which would have influenced its degradation. Finally, the introduction of such large quantities of actively growing fungi and their metabolites may have altered the physicochemical environment of the soil, making conditions more amenable to PU degradation. It is likely that a combination of these factors, as well as other unknown factors may explain the enhanced PU degradation.

In conclusion, the addition of PU degrading fungal isolates 11n, *Nectria sp BC11*, *Penicillium viridicatum*, an unidentified Zygomycete, and especially *Penicillium ochrocholoron* to soil can be used to enhance the degradation of buried PU. However, the mechanism by which this occurs remains unclear, as introduced fungi, with few exceptions, did not persist in the soil for a significant period of time, and did not colonise the surface of buried PU. Although the microbial communities native to the soil used in this work were able to bring about the degradation of PU readily without the need for bioaugmentation, this work has shown that bioaugmentation can be used to enhance the degradation of plastic waste, and may prove useful in environments that are less favorable to plastic degradation.

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Chapter 5:
General conclusions
and future work

General conclusions and future work.

This study investigated the microbial colonisation and degradation of PU following the burial of PU in soil. PU was found to be highly degraded in soil, and fungi were found to be the most important microorganisms involved in the colonisation and degradation of PU under natural, *in situ* conditions (Chapter 2). Attempts to increase the degradation of PU during burial in soil were made, with nutrients added to soil in an effort to stimulate the activities of the native PU degrading populations (Chapter 3); however, this was found to have little effect on the degradation of buried PU. Greater success was observed with the addition of large quantities of known PU degrading fungi into the soil (Chapter 4), with PU undergoing much more rapid degradation in soil heavily inoculated with certain PU degrading fungi. The primary conclusions from each of these chapters, as well as potential avenues of future work are given below.

Chapter 2. Fungal communities associated with the degradation of PU in soil

PU coupons were buried *in situ* in garden soil in order to determine which microorganisms were associated with the degradation of PU within the environment. Two different soils were employed in this work, differentiated primarily by pH – one soil had an acidic pH (5.5), whilst the other was almost neutral (pH 6.7). As well as differing in pH, DGGE showed that each soil type contained a different native soil fungal population.

PU was highly susceptible to degradation in the soil, losing more than 90% of its tensile strength after five months burial. That PU was so readily degraded in these soils was unsurprising, as clearance assays using the colloidal PU dispersion Impranil revealed that putative PU degrading fungi made up approximately 40% of the recoverable fungi in these soils, suggesting that fungi were the principle agents of PU degradation. In contrast, very few putative PU degrading bacteria were seen throughout, and bacteria probably play only a minor role in PU degradation.

Although putative PU degrading fungi were numerous in the soil, DGGE showed that only a limited subset of the soil communities could colonise and proliferate on the surface of buried PU. Possessing PU hydrolytic enzymes, as indicated by the clearing of Impranil, was therefore insufficient to allow the colonisation of buried PU. Further

characterisation of fungi that successfully colonised the surface of buried PU, such as the interactions that occur between the fungi and the PU surface may offer insights on the characteristics possessed by important PU degraders.

The ITS 1 region of the fungal rRNA gene complex was used for DGGE analysis of the fungal communities in the soil and on the surface of buried PU. This made it possible to identify the fungi most likely to be involved in PU degradation in these soils as *Nectria sp. BC11, Geomyces pannorum, Neonectria ramulariae* and *Alternaria sp. 18-2.* However, the role of these fungi as PU degraders was inferred from their ability to colonise and proliferate on the surface of buried PU. Although DGGE using the rRNA genes makes it possible to identify the microorganisms in a community, it is not usually possible to infer phenotype from this data. By using metabolic gene fragments in DGGE analysis, however, it is possible to generate DGGE profiles based on function. The use of polyurethanase genes as targets for DGGE may make it possible to unambiguously investigate PU degrading organisms in the environment. However, currently no conserved motifs have been described for PU degrading enzymes, making this sort of DGGE analysis impossible at the present time.

This work employed garden soil for reasons of convenience and accessibility during the *in situ* soil burial experiments. However, garden soil is not generally the principle site at which PU is degraded. Rather, PU waste is often deposited in landfill sites, where conditions may be quite different. The use of landfill soil in future work may give a more accurate representation of the microbes involved in the degradation of PU waste in the environment.

Chapter 3. Biostimulation of soil microbial communities and the degradation of buried PU

The results from Chapter 2 established that a substantial community of putative PU degrading fungi existed in the garden soils used in this work. Attempting to enhance PU degradation by stimulating these communities with nutrients in the form of yeast extract (YE) or Impranil was met with limited success. PU buried in the acidic soil (Chapter 2) that had been treated for one month previously with YE or a combination of YE and Impranil lost 96% of its tensile strength after one month's burial. However, PU buried in untreated control soil experienced a 90% reduction in tensile strength, meaning that

treatment of soil with YE and a combination of YE and Impranil only slightly enhanced the already severe degradation that occurred in the absence of treatment. Interestingly, treatment of soil with Impranil alone inhibited the degradation of buried PU, with the tensile strength of PU buried in soil so treated reduced by 85%.

Treatment of soil with YE or co-treatment with YE and Impranil caused particular members of the soil community to increase in number. This was especially marked in soil treated with both Impranil and YE, where two fungal species, *Trichosporon multisporum* and a Zygomycete increased in numbers to such a degree that 90% of the cultivable fungi in the soil belonged to one of these species. However, with the exception of the Zygomycete, none of the fungi seen to increase in numbers following soil treatment were seen to colonise the surface of buried PU in significant numbers. However, the numbers of putative PU degrading fungi on the surface of buried PU was increased following these treatments, which may explain the slightly enhanced degradation.

Fungal communities in soil treated with Impranil alone were almost unchanged compared to fungal communities in the untreated control soil. Yet, this treatment had an effect both on the fungal communities that colonised the surface of buried PU and the extent to which PU was degraded. A possible explanation for this may lie in the response of the bacterial soil communities to the treatment of soil with Impranil alone, with bacterial numbers in the soil massively increased. Such high numbers of bacteria may have retarded the colonisation of PU by the soil fungi due to competition between bacteria and fungi in colonising the surface of buried PU. However, there was no evidence of elevated numbers of bacteria on the surface of the PU buried in Impranil only treated soil after one months burial compared to the control, suggesting that this competition, if present, may have been limited to the time period shortly after the PU was buried. Unfortunately, no data describing the early colonisation events were gathered, and future work monitoring the colonisation of PU over time may be helpful in elucidating any colonisation sequences that may occur on the surface of buried PU.

Although biostimulation was of limited use in enhancing PU degradation in this work, the conditions employed were highly conducive to PU degradation even in untreated control soil. For example, a high population of PU degrading fungi was present in soil, with tests taking place under laboratory conditions at 25°C. Biostimulation may be of

greater use under less favourable conditions, such as in less controlled *in situ* environments, or in environments with a lower population of PU degraders. Furthermore, this work is the first offering proof of principle that biostimulation may be used to enhance the degradation of plastic waste; further work using different plastic formulations should be carried out to determine whether biostimulation is a viable method for enhancing the degradation of plastic waste.

Chapter 4. Degradation of PU buried in soil bioaugmented with PU degrading fungi.

Work on the previous chapters yielded a library of fungal isolates with putative PU degrading abilities, namely *Geomyces pannorum*, *Penicillium inflatum*, *Nectria sp*, *Penicillium viridicatum*, *Penicillium ochrochloron*, a Zygomycete and isolates 11n and 19n. However, the putative PU degrading ability of these isolates was determined using Impranil clearance assays. In order to unambiguously determine whether the fungi could degrade solid PU, each isolate was cultivated as a monoculture on the surface of solid PU, and the degradation of the PU after one month was determined. All of the fungal isolates were found to be able to degrade the PU to varying degrees. The Zygomycete produced the greatest amount of degradation, with PU coupons showing a 61% reduction in tensile strength. The remaining isolates produced more modest amounts of degradation, with PU losing 25-34% of its tensile strength.

Each isolate was inoculated into soil (so-called 'bioaugmentation') to determine if the degradation of subsequently buried PU could be enhanced. Wheat colonised by these isolates was used as the inoculum for the sake of convenience. PU buried in soil inoculated with wheat colonised by isolates 11n, *Nectria sp*, *P. viridicatum*, the Zygomycete and *P. ochrochloron* was significantly more degraded than PU buried in soil to which sterile wheat alone had been added, proving that bioaugmentation could be used to enhance the degradation of PU.

Interestingly, the addition of sterile wheat alone led to PU being 45% more degraded compared to PU buried in untreated control soil. This was probably due to a similar biostimulating effect as seen in Chapter 2, with the sterile wheat acting as a nutrient source. This biostimulating effect made it difficult to separate the effects of the addition

of sterile wheat and the addition of PU degrading fungi on PU degradation. It may be more viable in the future to simply add fungal spores or mycelia directly to the soil.

Although bioaugmentation was seen to enhance the degradation of PU, there was little evidence that the majority of the isolates survived in soil in significant amounts one month after their inoculation, or that they could colonise the surface of the buried PU. In fact, only the Zygomycete displayed a straightforward mechanism for the effectiveness of bioaugmentation, i.e. it showed good degradation of PU as a monoculture, persisted in soil following its inoculation, and colonised the surface of buried PU. None of the remaining isolates had such an obvious mode of action. This was especially true of *P. ochrochloron*, which showed no evidence of survival either in the soil or on the surface of buried PU, but nevertheless led to the greatest amount of PU degradation following its inoculation into soil.

Alternative explanations for the efficacy of these isolates in enhancing PU degradation were necessary. In all cases, Impranil agar clearance assays revealed that inoculation increased the numbers of putative PU degraders by at least 38-fold in the soil and 35-fold on the surface of buried PU. Although DGGE showed that in the majority of cases these putative PU degraders were native to the soil and were not the isolates inoculated into the soil, this increase may nevertheless have led to greater PU degradation. Alternatively, although they were incapable of colonising the surface of the PU, some of the isolates may have been secreting PU degrading enzymes into the soil following their inoculation. Impranil clearance assays using filter sterilised supernatant from a slurry of inoculated soil could determine if isolates were secreting PU degrading enzymes into the soil.

Appendix

Appendix 1: Characterisation of Impranil clearing enzymes secreted by *Geomyces pannorum* during growth

Introduction

The fungal isolate *Geomyces pannorum* has been associated with the degradation of PU in both of the soils used in this work (Cosgrove *et al.*2006) as well as in an unrelated soil (John Innes N°2 compost) in a previous work (Barratt *et al.*, 2003), suggesting that this fungus may be especially well suited for the remediation of PU waste under a variety of conditions. An understanding of the PU degrading enzymes secreted by this fungus may help to determine what factors are responsible for the activity of this fungus against PU. *G. pannorum* was found to be the most active of all of the isolates recovered in this work at clearing the colloidal PU dispersion Impranil. An especially active strain, *G. pannorum* ANPCGP1, was chosen for further analysis. Clear zones around colonies of *G. pannorum* ANPCGP1 growing on Impranil agar extended up to 10mm away from the colony's periphery (data not shown), suggesting that this fungus was secreting at least one highly active Impranil degrading enzyme. In order to characterise these putative Impranil degrading enzymes, products secreted by *G. pannorum* ANPCGP1 during growth were recovered by cultivating the fungus on Impranil-based media using poloxamer as the gelling agent. Poloxamers are a group of surfactants that display thermo-reversible gelation at high concentrations in aqueous solutions (Rickard *et al.* 2004). In other words, they are liquid at low temperatures and solid at higher temperatures. The poloxamer used in this work transitions between solid at liquid at approximately 15°C in a 40% (w/v) aqueous solution. Products secreted by *G. pannorum* ANPCGP1 during growth on poloxamer in its solid phase were separated from *G. pannorum* ANPCGP1 biomass by aspirating the medium after it was liquefied by exposure to low temperature. Secreted products recovered in this way were then used in Impranil clearance assays in the presence of various enzyme inhibitors. In this way, the probable mode of action of Impranil clearing enzymes secreted by *G. pannorum* was determined.

Methods and Materials

Recovery of products secreted by *G. pannorum* during growth on solid media.

G. pannorum ANPCGP1 was grown on the surface of a poloxamer-based medium in Petri plates. The medium contained the same components as Impranil agar (Crabbe *et al.*, 1994), but with the agar substituted with 40% (w/v) Synperonic PEF 127 poloxamer (ICI Chemicals and Polymers Ltd, France). After one month's growth at 25°C, clearance of the medium was observed, and the poloxamer was liquidised by placing the plates at 4°C. Liquidised medium was then drawn off with a syringe, leaving biomass behind. The contents of the syringe were centrifuged at 3000g for 30 min at 4°C to pellet any remaining biomass. The supernatant was then recovered to give a 'slurry' of secretory products generated by *G. pannorum* during growth. Finally, the slurry was passed through a 0.22μm filter (Millipore Corporation, U.S.A.) to completely remove any remaining biomass.

Impranil clearance by *G. pannorum* slurry in the presence of enzyme inhibitors.

The slurry secreted by *G. pannorum* was then used in Impranil clearance assays in order to characterise the components of the slurry that were responsible for Impranil degradation. Assays were set up at 4°C in order to maintain the poloxamer in a liquid state. Poloxamer medium containing $4gl^{-1}$ of Impranil was aliquoted five times in 1ml volumes into centrifuge tubes. To three of the tubes one of the following enzyme inhibitors was added: the esterase inhibitor phenylmethylsulfonyl fluoride (PMSF, 1mM); the lipase inhibitor Tween 80 (5% v/v); and the urease inhibitor acetohydroxamic acid (AHA, 1mM). 75μl of the *G. pannorum* slurry was then added to all of the tubes, with the contents of all tubes being thoroughly mixed. One of the two tubes without enzyme inhibitors was then boiled at 100°C for 20 min to determine the thermostability of the active ingredients in the slurry. The remaining tube (i.e. that had not been boiled, or had no inhibitors added) was the positive control, and was used to determine the basic Impranil clearing rate of the slurry. Microtitre plate wells were filled with 150μl of these mixtures. Microtitre plates were then placed at 37°C for ten minutes to solidify the poloxamer. Optical density (OD) at 450nm was then monitored in a microtitre plate reader (Biotek Instruments Inc. U.S.A.) at 7 minute intervals for 13h. The assay was carried out at 25°C.

Results and conclusions.

A linear relationship existed between the concentration of Impranil in the poloxamer medium and OD (Fig 1), making it possible to monitor the degradation of Impranil over time by measuring changes in OD. In the absence of inhibitors (Fig 2 a-c, blue line) the *G. pannorum* slurry was found to be highly active against Impranil, causing a 30% reduction in OD over the course of 800 min. This was extremely rapid compared to the action of similar slurries produced by any of the other fungal species isolated in this work, where clearance rates an order of magnitude less were typical (data not shown). Boiling the slurry reduced the rate of Impranil degradation (Fig 2a), suggesting that the active compound or compounds in the slurry were proteinaceous in nature. The addition of the urease inhibitor AHA did not reduce the rate of Impranil clearance (Fig2b), indicating that the active component in the slurry was not a urease. Similarly, adding Tween had no effect (Fig 2c), indicating that lipases were not responsible for the clearance of Impranil. Adding the esterase inhibitor PMSF caused a significant reduction in the rate of Impranil clearance (Fig 2d), suggesting that at least one enzyme with esterase activity was causing Impranil clearing in the *G.pannorum* slurry.

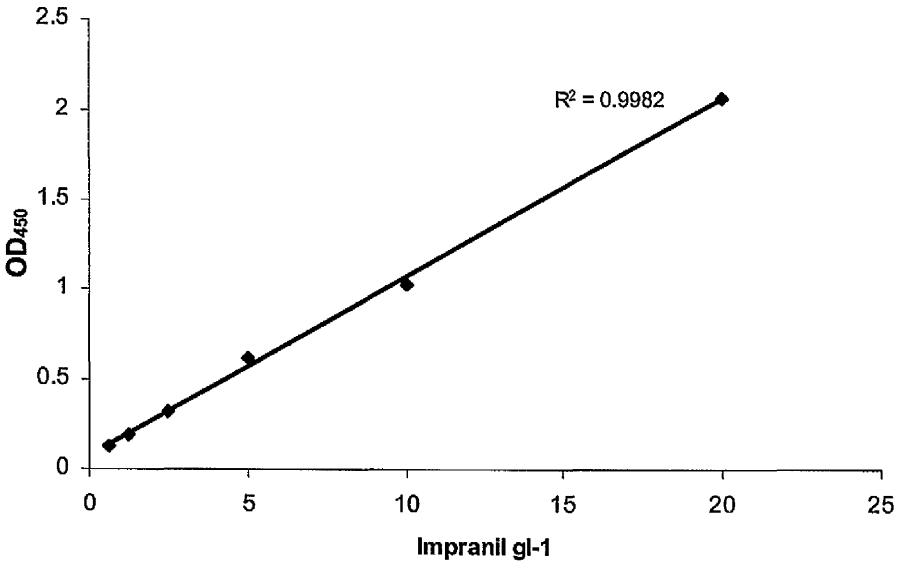

Figure 1: Calibration curve showing the linear relationship between Impranil concentration in poloxamer and OD at 450nm.

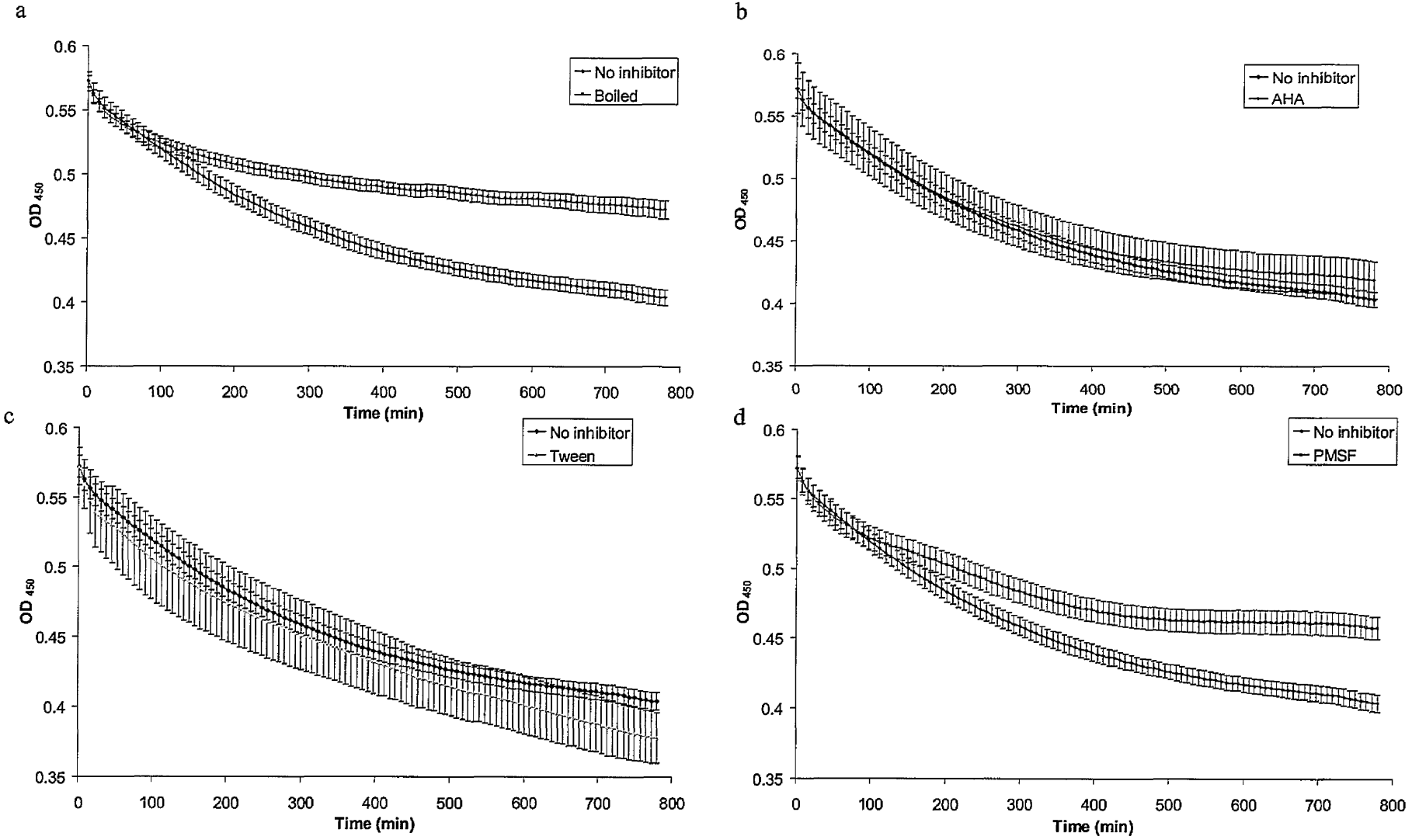

Figure 2: Impranil clearing activity of products secreted by *Geomyces pannorum* ANPCGP1 during growth on solid media. Secreted products were tested after boiling (a), or the in the presence of the urease inhibitor acetohydroxamic (AHA) (b), the lipase inhibitor Tween (c) or the esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) (d). Impranil clearing is indicated by a reduction in OD_{450}.

It therefore appears that *Geomyces pannorum* is secreting at least one PU degrading esterase during growth on solid substrates. To date, only one other fungal PU degrading enzyme has been described (Crabbe *et al.*, 1994). This enzyme was isolated from the fungus *Curvularia senegalensis*, and was found to primarily possess lipase activities, and interestingly was also found to form multimers. This is of relevance to this work as native Impranil-containing PAGE gels showed zones of clearance between the stacking gel and the resolving gel when a sample of the slurry was run (data not shown), indicating that the agent degrading Impranil either has a high molecular weight or forms multimers.

This study is the first to demonstrate the versatility of poloxamer for investigating crude enzyme slurries excreted by fungi during growth. The reversible phase change that poloxamer can be made to undergo offered numerous benefits. The solid phase made it possible to cultivate the fungus on a solid medium, and also prevented the Impranil from settling during the assay's run, which could have given a false impression of degradation. The liquid phase made it possible to recover from a culture on solid media what is essentially analogous to culture supernatant in liquid cultures. This may be especially useful in studies where a secreted protein of interest is only produced during growth on a solid substrate.

Although it has been shown that *G. pannorum* is secreting esterases capable of degrading the colloidal PU dispersion Impranil, as has been previously noted these results are not necessarily applicable to solid PU. Further work investigating whether the slurry can degrade solid PU, perhaps by monitoring changes in the tensile strength of PU in the presence of the slurry, should be carried out to ensure that these results are relevant to solid PU waste degradation.

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