FUNGAL BIOREMEDIATION OF CREOSOTE-CONTAMINATED SOIL: A LABORATORY SCALE BIOREMEDIATION STUDY USING INDIGENOUS SOIL FUNGI

HARRISON IFEANYCHUKWU ATAGANA[1,*], R. J. HAYNES[2] and F. M. WALLIS[2]
[1]*School of Earth Sciences, Mangosuthu Technikon, P.O.Box 12363, Jacobs, Durban 4026, South Africa;* [2]*School of Applied Environmental Sciences, University of KwaZulu-Natal, Pietermaritzburg 3209, South Africa*
*(*author for correspondence, e-mail: atagana@yahoo.com)*

(Received 3 February 2005; accepted 19 December 2005)

Abstract. The aim of the study is to determine the efficacy of indigenous soil fungi in removing (PAHs) from creosote-contaminated soil with a view to developing a bioremediation strategy for creosote-contaminated soil. Five fungal isolates, *Cladosporium, Fusarium, Penicillium, Aspergillus* and *Pleurotus*, were separately inoculated onto sterile barley grains and incubated in the dark. The colonized barley was inoculated onto creosote-contaminated (250 000 mg kg^{-1}) soil in 18 duplicate treatments and incubated at 25 °C for seventy days. The soil was amended with nutrient supplements to give a C:N:P ratio of 25:5:1 and tilled weekly. Creosote removal was higher (between 78 and 94%) in nutrient supplemented treatments than in the un-supplemented ones (between 65 and 88%). A mixed population of fungi was more effective (94.1% in the nutrient amended treatment) in creosote removal than single populations wit a maximum of 88%. Barley supported better fungal growth and PAH removal. *Pleurotus sp.* removed the creosote more than the other isolates. Two and three-ring PAHs were more susceptible to removal than the 4- and 5-ring PAHs, which continued to remain in small amounts to the end of the treatment. Reduction of creosote in the present study was higher than was observed in an earlier experiment using a consortium of microorganisms, mainly bacteria, on the same contaminated soil (Atagana, 2003).

Keywords: bioremediation, creosote, fungi, nutrients, PAHs, soil

1. Introduction

Fungi are known for their diversity and remarkable ability to degrade complex and persistent natural materials such as lignin, chitin, and microcrystalline cellulose. In contrast to bacteria, fungi are able to extend the location of their biomass through hyphal growth. They are able to grow under environmentally stressed conditions such as low nutrient availability, low water activity and at low pH values where bacterial growth might be limited (Davis and Westlake, 1978).

The ability of mycelial fungi to penetrate insoluble substances such as oil tarballs initiates a succession of other microorganisms and enhances degradation of the oil. Such a relationship was observed by Kirk (1969) who found that bacteria became attached to the mucilaginous hyphae of *Corollospora maritima* during the degradation of oil. The low specificity of the enzymes produced by the white rot

Water, Air, and Soil Pollution (2006) 172: 201–219
DOI: 10.1007/s11270-005-9074-x

fungi such as *Phanerochaete chrysosporium* is believed to enable them degrade a range of recalcitrant, anthropogenic compounds (Field *et al.*, 1992; Bogan and Lamar, 1995).

Bioremediation technologies have commonly relied on bacterial activity to decontaminate the environment. While knowledge of bacterial bioremediation has continued to improve, relatively less attention has been given to fungal bioremediation of contaminated environments. Although many reports claim the success of fungi in metabolising different hydrocarbons, relatively few reports are available where direct application of fungi in bioremediation has been carried out. The potential for these organisms to degrade high molecular mass polycyclic aromatic hydrocarbons (PAHs) (MacGillivray and Shiaris, 1993; Cerniglia, 1997; Andersson and Henrysson, 1996) and other recalcitrant organic compounds in the laboratory, through the use of their extracellular and other enzyme systems, presents an attractive area for investigation. Thus the application of fungi in remediating soil contaminated with creosote containing high molecular mass PAHs, which are resistant to bacterial degradation constitutes the focus of this study. Cerniglia (1997) presents a list of PAHs that have been reported to successfully support the growth of a large number of fungi and yeasts and in the last five years this list has grown (Clemente *et al.*, 1999; Harmsen *et al.*, 1999). This report gives a further indication that these organisms, if managed under appropriate conditions, will help address the issue of the recalcitrance of some of the PAHs in soil.

The aim of this study is thus to determine the capability of the fungal strains isolated from creosote-contaminated soil in degrading PAHs in soil with a view to developing a cost effective treatment technology for bioremediation of creosote-contaminated soils.

2. Materials and Methods

2.1. Enrichment and isolation of soil fungi

2.1.1. *Soil Samples*

Creosote-contaminated Mispah form (FAO:lithosol) soil (>250 000 mg kg^{-1}) was collected from a creosote wood treatment site in KwaZulu-Natal, South Africa. Uncontaminated soil was collected from the same site but with no visible trace of creosote contamination. The soil pH was 5.5, total nitrogen 0.08%, extractable phosphorus 4.7 mg kg^{-1}, clay 18.8%, silt 18.8% sand 62.5%. The samples were separately homogenized and stored in heat-sealed polyethylene bags at 4 °C until required.

2.1.2. *Batch Culture Enrichment*

Ten gram samples of the contaminated soil and uncontaminated soil were placed in separate sterile 250 ml Erlenmeyer flasks containing 100 ml of sterile mineral

salts medium (Coutts *et al*., 1987). Penicillin (0.01 g); chloramphenicol (0.5 g); and streptomycin (0.025 g) were dissolved in 2.5 ml of distilled water, filter sterilized through a 0.4 μm millipore filter (Raymond *et al.*, 1976) and incorporated into both cultures to inhibit bacterial growth. The medium was aerated by connecting an aquarium pump through a system of tubes to air stones, which were submerged in the medium in the different flasks. All flasks were then incubated for 72 h at 32 °C. The garden soil extract broth was prepared by mixing fresh garden soil (1 kg) with 1 l of tap water and autoclaving at 121 °C (15l b psi) for 30 min. To buffer the system, calcium carbonate ($CaCO_3$) (3 g) was added to the mixture and stirred. The soil suspension was allowed to settle before the supernatant was filtered through a double thickness of Whatman's No. 1 filter paper. The filtration process was repeated until the filtrate became clear. The garden soil extract was autoclaved again and the pH was adjusted to about 4.6 with HCl. Antibiotics, as described in Section 2.1.2 above were incorporated into the soil extract after cooling to about 40 °C to inhibit bacterial growth. Four flasks, each containing 200 ml of the soil extract diluted to 350 ml with deionised water, were each separately inoculated with 10g of one of the two soil types and incubated under the same conditions, as described above for the mineral salts medium cultures. All the antibiotics used were obtained from Hoechst™.

2.1.3. *Hydrocarbons*

The PAHs used were naphthalene, anthracene, phenanthrene, pyrrole, fluorene, pyrene, chrysene, fluoranthene and benzo (a) pyrene and all were of analytical grade. All hydrocarbons used were obtained from Sigma-Aldrich™. The creosote was of commercial grade.

2.1.4. *Solid Media*

Five grams of each PAH and of creosote, were separately added to 15 ml aliquots of diethylether and then 5 g fine colloidal silica was added to the mixture. Each mixture was slurried in a mortar and placed in a fume cabinet until the solvent had evaporated to leave the PAHs and creosote adsorbed to the silica. The PAH- and creosote-adsorbed silica were then added to the nutrient medium, which was modified from Czapeks medium containing per litre deionised water: 1 g K_2HPO_4, 3 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 20 g agar, to facilitate even distribution of the hydrocarbons in the nutrient medium (Lees, 1996). The nutrient medium was then autoclaved for 15 minutes at 121 °C (15lb psi) and cooled to about 40 °C before antibiotics as described above was incorporated into it (Raymond *et al*., 1976; Lees, 1996). The pH was adjusted to 4.6 with conc. HCl. The individual nutrient media containing the different hydrocarbons were then poured into separate plates.

2.1.5. *Strain Selection*

Aliquots (0.1 ml) of the two enrichment culture types described in Section 2.1.2 were then inoculated onto the different hydrocarbon spiked plates described in

Section 2.1.4 and incubated at 25 °C for five days. Active mycelia of about 1 cm^2were aseptically cut from five-day-old cultures and inoculated onto similar hydrocarbon supplemented agar plates described above and incubated at 25 °C. Growth was monitored daily and the rate of growth was measured by measuring the diameters of the expanding colonies over a period of 10 days. Cultures were maintained on Potato Dextrose Agar (PDA) plates and identification was done by cultural and morphological characteristics of isolates using microscopes (Olympus™) with reference to Barnett and Hunter (1998) and Raper and Thom (1968). The chemicals used were obtained from Merck™ and the PDA was from Oxoid™.

2.2. Soil reactors

Thirty-six 3-litre polyvinyl chloride (PVC) vessels, with a diameter of about 33 cm and depth of 13 cm, were each filled with 2.5 kg (fresh weight) of PAH-contaminated soil from the experimental site. Perforated polystyrene bases were fitted into the vessels and supported from below to prevent sagging, to allow passage of leachate, Black polyethylene sheeting was used to cover the vessels to prevent photo-oxidation of the creosote components. Distilled water was used to maintain the soil at 70% water holding capacity determined by using the method described by Forster (1995). The pH was measured at the start of the experiment using a Crison Micro pH 2000.

2.3. Fungal bulking

About 300 g of barley grain, soaked in water for 48 hours and drained, were placed in each of 30 polyethylene bags. The bags were plugged with cotton wool stoppers before being sterilized at 121 °C for 15 min. (15 Ib psi) in an autoclave. Each bag was aseptically inoculated with one of the selected fungal isolates (Table I) by slicing the PDA colonized by the organisms into small 5 mm pieces. Subsequent bags were inoculated with 50 g of the colonized grains. The bags were incubated in the dark for three weeks at 25 °C to allow complete colonization of the grains. The colonized grains were then inoculated into the contaminated soil, after a number of trials, at the rate of 10 g of barley to 100 g of soil (fresh weight).

2.4. Treatments

Eighteen duplicate treatments were established in the soil reactors described above (Table II). The nutrient amendment applied to the soil was a C:N:P ratio of 25:5:1, as recommended for aerobic metabolism (Baker and Herson, 1995; Alexander, 1999), by adding NH_4NO_3 (7.5 g) and K_2HPO_4(1.5 g) (dissolved in 150 ml of distilled water) and mixing it into the soil after each sampling event. Moisture in all treatments was maintained at 70% of field capacity for the duration of the experiment.

TABLE I

Growth of fungal isolates on solid media spiked with creosote and selected PAHs

Organism	Naphthalene	Anthracene	Phenanthrene	Pyrrole	Fluorene	Pyrene	Chrysene	Fluoranthene	Benzo(a)pyrene	Creosote
Aspergillus	++	+	++	++	++	++	+	+	+	++
Cladosporium	+++	++	++	++	+++	++	++	++	++	+++
Fusarium	++	−	−	+	+	+	−	+	−	+
Candida	+	+	++	−	−	−	+	−	−	+
Monicillium	+	+	+	+	−	++	−	+	−	+
Trichoderma	++	+	−	−	++	+	+	−	++	+
Penicillium	+	+	+	+	++	++	+	+	+	++
Pleurotus	++	+	+	+	++	++	+	+	+	++
Phanerochaete	+	−	−	−	−	+	−	−	++	+++

*Profuse growth^{+++}, Moderate growth^{++}, Sparse growth+, No growth^{-}.

TABLE II
Experimental design showing the different treatments in each set of treatment reactor

Treatments in duplicates	Barley addition	Fungal inocula from isolates from contaminated soil	Nutrient addition (NH_4NO_3+K_2HPO_4)
1	yes	*Cladosporium sp.*	no
2	yes	*Fusarium sp.*	no
3	yes	*Penicillium sp.*	no
4	yes	*Aspergillus sp.*	no
5	yes	*Pleurotus sp.*	no
6	no	no	no (control 1)
7	yes	*Cladosporium sp.*, *Fusarium sp.*, *Penicillium sp.*, *Aspergillus sp.*, *Pleurotus sp.*	no
8	no	no	yes (control 2)
9	sterile barley	no	no (control 3)
10	yes	*Cladosporium sp.*	yes
11	yes	*Fusarium sp.*	yes
12	yes	*Penicillium sp.*	yes
13	yes	*Aspergillus sp.*	yes
14	yes	*Pleurotus sp.*	yes
15	sterile barley	no	yes (control 4)
16	yes	*Pleurotus sp. (from Cedara)*	yes
17	yes	*Pleurotus sp. (from Cedara)*	no
18	yes	*Cladosporium sp. Fusarium sp. Penicillium sp. Aspergillus sp. Pleurotus sp.*	yes

Soil in the reactor was aerated by tilling with a small garden trowel once a week. All treatments were incubated at ambient temperature for 70 days.

2.5. SAMPLING

Once every two weeks, five random samples were collected as cores (100 mm × 15 mm) from each reactor, using a 300 mm × 15 mm glass tube. Each set of samples was mixed together thoroughly, placed in a plastic bag and heat sealed before storing at approximately −17 °C. Soil samples were also taken before and after nutrient addition, for pH measurements.

2.6. ANALYSES OF THE SAMPLES

Soil extraction. Soxhlet extraction was performed, as outlined in EPA Method 3540. A duplicate soil sample (5 g), described above, was placed in an extraction thimble

(Whatman cellulose). A glass wool plug was placed above the sample to prevent dispersion of the soil. Dichloromethane (75 ml) was placed in a round-bottom flask containing two boiling chips. The flask was attached to a Soxhlet extractor fitted with a condenser and the sample was extracted for 8 h. After cooling, the extract was passed through magnesium sulphate ($MgSO_4$) to remove residual water. The extract was filtered and the total volume reduced to about 5 ml in a rotary evaporator (Heidolph B.NR.51111). Residual dichloromethane was evaporated under a low flow of nitrogen by passing gaseous nitrogen through the extract. The method was modified, in that $MgSO_4$ was not added to the sample before extraction. All experiments were duplicated.

Infrared spectrophotometry. Total creosote concentration in the soil was determined by using the USEPA 418.1 (1986) method. The extract was finally filtered through a Whatman GF/C glass fibre filter. The filtrate was made up to 10 ml in a volumetric flask and the absorbance determined with a Nicolet Avater 320 Infra-red Spectrophotometer at wave numbers between 400 and 4000 cm^{-1}. Calibration of the reference creosote was done by diluting commercial grade creosote with carbon tetrachloride to a series of five working standards (10; 100; 250; 500; 1000 mg L^{-1}) and a calibration curve was derived by determining the absorbance of each standard. A calibration plot of the absorbance versus mg creosote (100 ml L^{-1}) solution was generated with Omnic™ software. The concentration of creosote in each extract was determined by comparing the response with the calibration plot. To calculate the results, a linear equation ($y = 1.192x + 1.363$) was generated by Omnic™ software, where x = creosote concentration of sample (kg $^{-1}$ soil) and y = peak area. The actual creosote concentration in mg kg^{-1} soil was then calculated by multiplying x by a factor of 50, which compensates for the cell path-length, sample size and dilution factor. This formula was then programmed into a spreadsheet, which automatically calculated the creosote concentration in mg kg^{-1} from the infrared absorbance values as they were entered.

Gas chromatography. The extracts were analysed by GC/FID. The GC was a Varian-3800 with argon as the carrier gas and fitted with a 30 m capillary column with 0.25 mm internal diameter and 0.25μm film thickness, and a flame ionisation detector (FID). Two temperature programmes were run in order to obtain a good separation and quantification of the more volatile compounds. The first temperature programme was: 60 °C, 4 min., 10 °C/min. up to 235 °C, kept for 40 min.; injector temperature: 220 °C. The second temperature programme was used for the analysis of the more volatile compounds at 20 °C: 40 °C, 1min., 10 °C/min., up to 200 °C, kept for 20 min., injector temperature 220 °C (Eriksson *et al.,* 2000).

3. Results

3.1. Enrichment cultures

Cultures from the mineral salts medium and the soil extract broth containing the uncontaminated soil grew slowly and had fewer strains on the hydrocarbon spiked solid media. This is probably due to the absence of hydrocarbons in the culture in which they grew. Cultures containing the contaminated soil grew faster and showed more diversity on the hydrocarbon spiked plates. Generally, the soil extract was more restrictive than the mineral salts medium in supporting fungal growth. Growth from both cultures occurred on the plates containing naphthalene, anthracene, phenanthrene, pyrole, fluorene and whole creosote between day 14 and 15, becoming prolific in anthracene and naphthalene by day 15 and in creosote by day 18. Fungal growth appeared in the pyrene and benzo(a)pyrene cultures on day 18. Growth occurred in fluoranthene and chrysene spiked plates on day 21 but was poor.

3.2. Isolation, identification and strain selection

Table I shows the growth of the fungi on the different hydrocarbons. From both the mineral salts medium and the soil extract broth, seventeen strains of fungi were isolated on hydrocarbon spiked solid media. The isolates were identified as belonging in the following genera: *Penicillium, Aspergillus, Fusarium, Trichoderma, Pleurotus, Cladosporium, Phanerochaete*, *Candida* and *Monicillium.*

Cladosporium and *Aspergillus* grew most successfully on the 2- and 3-ring PAHs, with *Cladosporium* showing the best growth on naphthalene and fluorene. The growth of the other organisms on these compounds ranged from moderate to no growth. *Phanerochaete* grew only on naphthalene, pyrene, benzo(a)pyrene and whole creosote. Growth of these fungi on the 4- and 5- ring PAHs was less prolific than on the lower hydrocarbons studied. *Penicllium* and *Candida* grew on only pyrene and chrysene. Of all the fungi investigated, *Cladosporium* showed the greatest metabolic diversity, growing well to moderately on all the compounds tested. *Pleurotus* showed sparse to moderate growth on all of the compounds tested (Table I). Naphthalene, which is known to be mineralised by representatives of several genera of fungi (Cerniglia, 1997; Kotterman *et al.*, 1999) was utilized by all the fungi tested in this experiment. *Cladosporium, Phanerochaete and Pleurotus*, grew well on whole creosote.

Based on the growth of the organisms on the creosote and PAHs amended solid media, *Aspergillus, Cladosporium, Fusarium, Penicillium* and *Pleurotus* were selected for trial in reactors containing creosote-contaminated soil.

3.3. GROWTH OF FUNGI IN SOIL REACTORS

Growth was first observed after 14 days in all treatments inoculated with non-basidiomycete fungi. After 20 days of incubation, mycelium had spread profusely but leaving patches of uncolonised soil in the treatments, which were not nutrient supplemented. There was no observable growth within 20 days in the treatments inoculated with basidiomycetes. In Treatment 7, *Cladosporium* sp., *Fusarium* sp., *Penicillium* sp. and *Aspergillus* sp. became established first, while *Pleurotus* sp. only became noticeable after 21 days.

All treatments except the controls showed prolific fungal growth by day 38, with *Pleurotus* sp. becoming dominant in Treatments 7 and 18. During tilling it was observed that fungal mycelium did not penetrate the entire soil mass at the same rate. There was more mycelial proliferation in the upper soil layer (3–5 cm depth) than in the middle and bottom layers. However, with tilling, hyphae were distributed to the lower levels of the soil system.

Treatments 9 and 15 (controls 3 and 4), to which barley was added, exhibited large fungal growth in spite of not being inoculated with any fungus while Treatments 6 and 8 (controls 1 and 2), without barley showed very sparse growth. Samples from both the latter treatments revealed the presence of some bacteria, mycelial fungi and yeast species. Growth of *Pleurotus* sp. in Treatments 16 and 17 was similar and profuse by day 25. Fungal growth in these two treatments continued until the end of the experimental period.

3.4. DEGRADATION OF CREOSOTE IN SOIL REACTORS

The treatments resulted in between 65% and 94% removal of creosote from the soil in the reactors (Figure 1). Removal from the controls ranged from 26% to 58% (Figure 1). Reduction in creosote concentration became more rapid after 14 days as the fungal population became better established (Figure 2 and 3).

The highest reduction in total creosote (94.1%) was observed in the mixed population amended with nutrient (Treatment 18) (Figure 3). The least reduction among the treated reactors was observed in the reactor inoculated with *Aspergillus* sp. but not supplemented with nutrient (Treatment 4) (Figure 2). Reduction in creosote concentrations in the treatments not supplemented with nutrient (Treatments 1–7 and 17), was found to be generally lower (between 65% and 88%) than in those supplemented (Treatments 8–16 and 18), which showed between 78% and 94% reduction (Figure 1).

ANOVA carried out on the changes in total creosote and PAHs concentrations with time showed that Treatment 18 was the most effective in removing the creosote from the contaminated soil. Overall, at p 0.05, all treatments that received nutrient amendment and bulking were more effective than those that were not amended. Treatment 7 and 14, were ranked the next most effective in the removal of creosote from the soil. There was no significant difference in the performance of Treatments

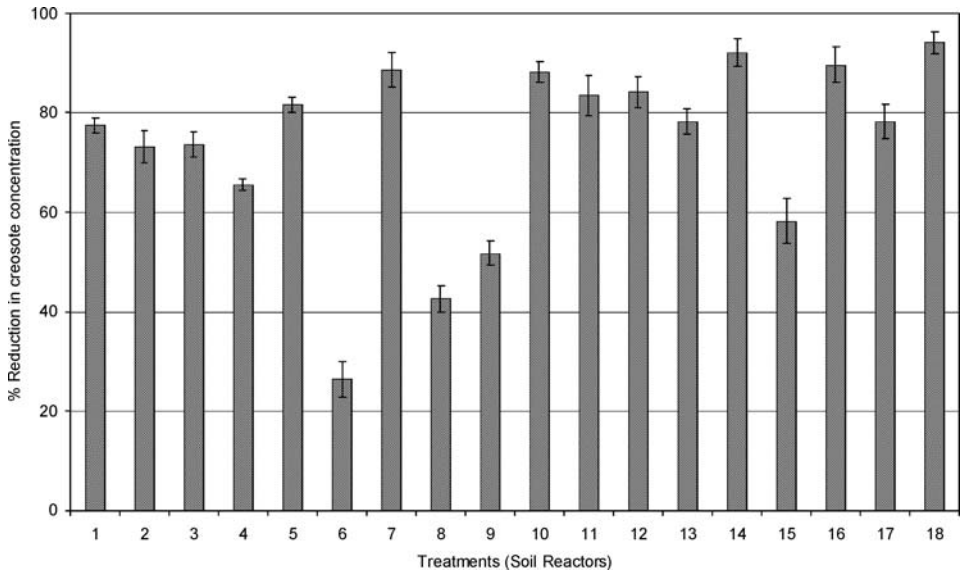

Figure 1. Percentage reduction in creosote concentration in contaminated soil in treatment reactors after seventy days of treatment. Values are means of two ± 1 Standard Error.

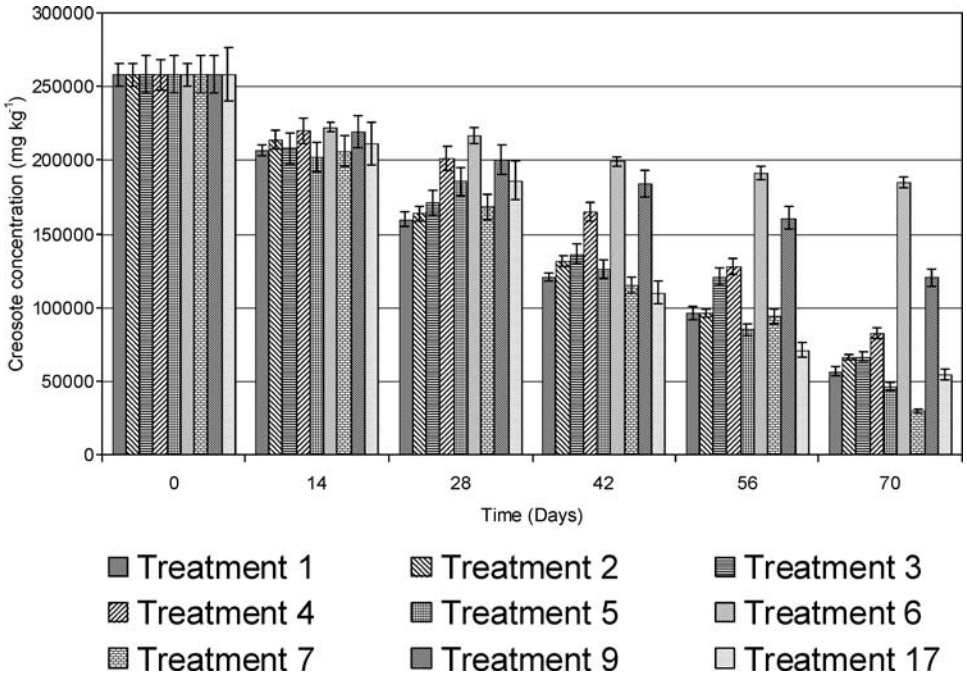

Figure 2. Changes in the concentration of creosote in the soil during the treatment period in Treatments not supplemented with nutrients. Values are means two ± 1 Standard Error.

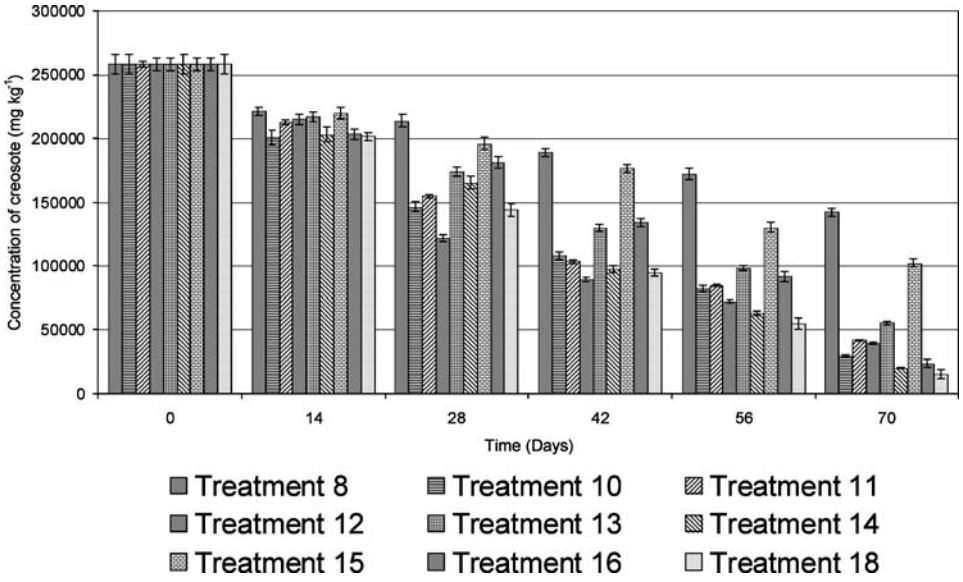

Figure 3. Changes in the concentration of creosote in the soil during the treatment period in Treatments supplemented with nutrients. Values are means two ± 1 Standard Error.

10, 16 and 17,which were ranked third in performance. All other treatments were significantly lower than the treatments described above. The control reactors (6, 8, 9, and 15) were least efficient in the removal of creosote from the soil.

3.5. Degradation of individual PAHs in the soil reactors

The 2- ring PAH (naphthalene) was completely removed from both the nutrient supplemented and non-supplemented treatments (Table III). The 3-ring PAHs (anthracene, phenanthrene, pyrrole and fluorene) were actively removed in both sets of treatments (Table III). Anthracene was removed below detection limit in all nutrient supplemented treatments and in the mixed population without nutrient supplementation. These results contrast with those obtained in an earlier study using a consortium of microorganisms, mainly bacteria, in which anthracene was much less actively removed over a period of 16 weeks (Atagana, 2003). Phenanthrene removal reached 98% in Treatment 18. Pyrrole and fluorene, both with similar heterocyclic structures, were degraded to below detection limits in some of the treatments supplemented with nutrients. The 4- and 5- ring PAHs continued to remain in reasonable amounts at the end of the experimental period with pyrene, fluoranthene and benzo(a)pyrene being more actively degraded (from 155 mgkg^{-1}, 168 mg kg^{-1} and 53 mg kg^{-1} to 9.6 mg kg^{-1}, 10.2 mg kg^{-1} and 2.0 mg kg^{-1}, respectively) by the organisms than chrysene (from 85mg kg^{-1} to 7.5 mg kg^{-1}) (Table III). Overall, the treatments that received nutrient supplements showed more

TABLE III
Residual concentrations of selected PAHs in contaminated soil after seventy days of treatment

	Naph-thalene	Anth-racene	Phena-nthrene	Pyrrole	Fluorene	Pyrene	Chrysene	Fluora-nthene	Benzo(a) -pyrene
Initial Conc. (mg kg^{-1})	125	65	215	67	45	155	85	168	53
Treatments									
1	0	3.2	12.8	1.6	0.5	18.2	14.2	15.3	6.5
2	0	5.3	21.3	2	1.3	21	16.1	17.2	8.6
3	0	5.5	26	3.6	1.2	23	16	15	8.2
4	0	7.4	23.5	6	1.5	45.5	19.5	21	10.8
5	0	3	9.8	2.5	0.5	15.2	12.6	13.8	3.2
6	98.6	58.5	192.7	48.1	39.5	138.6	67	138	41.7
7	0	0	6.4	1.2	0	10.5	9.7	12.4	3.2
8	63.5	51.2	169.3	45	33.8	133.1	63.7	126.7	38.8
9	61.5	34.7	138.2	41.5	28.7	126.7	58.5	110.5	35.6
10	0	0	9.8	0.3	0	15.6	12.5	12.1	4.5
11	0	0	15.8	0.5	8	15.8	12.8	12.9	6.5
12	0	0	21	0.3	1.2	18	13.5	12.8	6.8
13	0	0	21	0.7	0.8	18.7	13	13	7.8
14	0	0	8.5	0.2	0	9.7	9.5	10.1	3
15	28.9	31.6	135.5	27.8	25.5	98.7	53.3	101.6	33.9
16	0	0	7.2	0.8	0	11.5	12.5	13.9	3.5
17	0	0	9.3	0.6	0	13.2	12.8	13.8	3.2
18	0	0	0	0	0	9.6	7.5	10.2	2

Values are means of two $\pm$ 1 Standard Deviation.

reduction in PAH concentrations than the ones that did not receive any supplementation. This pattern of reduction is similar to the one observed in the reduction in total creosote concentration. However, the largest reduction (93.8%, 91.2%, 93.9% and 96%) in pyrene, chrysene, fluoranthene and benzo(a) pyrene respectively, observed in the nutrient supplemented treatment containing the mixed population of fungi (Treatment 18), was much higher than the results obtained in the earlier experiment (Atagana, 2003).

3.6. Changes in pH in the soil reactors during treatment

The initial soil pH in all the reactors ranged between 3.8 and 4.9. Apart from occasional slight fluctuations, the pH generally tended to rise (Table IV). Changes in pH in Treatment 1 were erratic, measuring 5.3 on day 14, falling to 4.9 by day 28, rising again to 5.9 by day 56 and falling again to 5.7 on day 70 (Table IV).

TABLE IV
Changes in pH of contaminated soil during the period of treatment

	Days					
Treatments	0	14	28	42	56	70
1	4.5	5.3	4.9	5.7	5.9	5.7
2	4.3	5.5	5.7	6.1	6.3	6
3	4.6	4.8	5.2	5.7	5.5	5.5
4	4.5	5.1	5.5	5.7	5.8	5.9
5	4.4	4.6	5.2	5.8	6.2	6.1
6	4.5	4.3	4.3	4.5	4.6	4.7
7	4.7	4.6	4.8	5.5	5.8	5.7
8	4.5	4.6	5	5	5.2	5
9	4.1	4.4	4.6	5.2	5.6	5.4
10	4.5	5.3	5.6	5.8	6	6.2
11	4.8	5.5	5.4	5.9	6.3	6.6
12	3.8	5.3	5.4	5.4	5	5.9
13	4.5	4.5	4.5	4.9	5.7	5.9
14	5	4.7	5.3	6.1	6.2	5.3
15	4.5	4.3	4.2	4.3	4.6	4.8
16	4.1	4.6	5.2	5.6	6.1	5.7
17	4.9	4.5	5.3	5.5	5.7	5.5
18	4.3	4.6	4.9	5.1	5.3	5.5

Values are means of two ± 1 Standard Deviation.

Treatments 6 and 15 showed a slight decrease in pH, followed by a gradual increase. These two treatments were not inoculated with any fungi, but fungal growth was detected in Treatment 15 during the later stages of the experiment. Treatments 4, 6, 10, 11, 12, 13, 15 and 18 showed increases in pH until the end of the incubation period. Treatments 1, 2, 5, 7, 8, 9, 14 and 16 decreased slightly between day 55 and 70. The highest pH value reached was less than pH 6.6 and was observed in Treatment 11, which was treated with nutrients and inoculated with *Fusarium.*

4. Discussion

Fungal growth was found in both sets of cultures. The poor growth in soil extract was attributed to its poor nutrient status. This type of restrictive medium helps in eliminating opportunistic organisms during studies requiring the isolation of only adapted organisms (Baker and Herson, 1995; Alexander, 1999). Growth of

opportunistic organisms has been reported in nutrient broth spiked with aliphatic hydrocarbons in spite of the antibiotics present. (Lees, 1996). It was evident that the higher molecular mass PAHs supported much less growth than the lower molecular mass compounds. This is attributed to the differences in the physicochemical properties of the two groups of compounds.

The growth of the tested fungi in the 2- and 3- ring compounds tested supports earlier reports in the literature (Bazalel *et al.* 1996; Casilas *et al.* 1996; Cerniglia 1997). The lignin-degrading enzymes of the white rot fungi have been particularly employed in the degradation of high molecular mass PAHs. Field *et al.* (1992) suggested that since the enzyme system of the white rot fungi is non-specific in action, it should be able to act non-specifically on a variety of hydrocarbon substrates. Pyrene, chrysene, benzo(a)pyrene and fluoranthene have been reported to support the growth of different fungi (Launen *et al.*, 1994; Sack and Fritsche, 1997).

A few of the isolates grew profusely and sporulated over the ten-day period, giving rise to many daughter colonies. It can be argued that the organisms were able to partially utilize the substrate and hence they were able to initiate such growth. However, the production of spores and subsequent appearance of daughter colonies could possibly be a response to the unavailability of the creosote for utilization, as many fungal species are known to produce spores under conditions of nutrient deprivation (Alexopoulos *et al.*, 1996). That the colonies continued to thrive for up to ten days is an obvious indication that they were progressively adapting to the creosote components.

Most of the organisms, on the other hand, produced very dense, compact, circular colonies indicating more ready adaptation to the xenobiotic carbon substrate. Based on these observations, it was difficult to eliminate any of the isolates from the biodegradation trial on creosote-contaminated soil. However, it was decided that only those organisms that produced discrete dense colonies would be used in the subsequent trial investigating the degradation of creosote in soil.

The initial delay in the growth of fungi in the treatment reactors was presumed to be due to the time taken for the fungi to become established in the soil possible because they were reintroduced into the contaminated soil after their isolation and purification on specially constituted media.

The fungi in the reactors that contained sterile barley (control 3 and 4) may have arisen from spores previously present in the contaminated soil, as the treatments were not inoculated with cultivated fungi. The abundant growth in the barley-containing treatments confirms the findings of other researchers, who reported improved growth of fungi following addition of substances such as straw, pine bark, wheat, alfalfa and barley to sterile soil (Boyle, 1995; Novotný *et al.*, 1999). The use of fungi grown in bulking material such as barley in remediating contaminated soil has previously been practiced and has produced both satisfactory and disappointing results in the bioremediation of different substances in soil (Loske *et al.*, 1989; Šašek *et al.*, 1993; Sack *et al.*, 1997). The absence of barley in this experiment affected the development of a fungal population in treatments not containing barley and

not inoculated with any fungal isolates (control 1 and 2). Creosote is naturally inhibitory to microbial growth. Thus it was no surprise to find that such unaided natural conditions such as lack of barley in the control experiments, failed to support fungal growth in such a short period.

The similarities in the results obtained from treatments 7 and 18, both inoculated with all five fungal isolates (Table III and Figure 1), are attributed to the fungal composition of both treatments (Table II). However, supplementary nutrient proved to be advantageous, as more reduction in creosote concentration was achieved in the nutrient supplemented reactor, confirming an earlier report by Lamar *et al.* (1987). Pollutant hydrocarbons results in an excessively high C:N:P ratios, which can be unfavourable for microbial growth in the environment (Atlas 1981). Hence it is necessary to increase concentrations of N_2 and P to offset the imbalance in order to promote microbial growth and biodegradation. Although fungi are known to grow in low nutrient media (Davis and Westlake, 1978; Rodriguez *et al.*, 1999), many workers have shown that nutrient-rich media support better growth (McGugan, 1997; Sack *et al.*, 1997; Eggen *et al.*, 1999; Podznyakova *et al.*, 2001).

In the present study, a combination of inorganic nutrients and bulking agent (viz. barley) supported prolific fungal growth and resulted in a large reduction in creosote concentrations in the soil. Most fungi are not known to utilize PAHs and other hydrocarbons as their sole source of carbon and energy and, as a result, the medium must be supplemented with an additional carbon source to allow fungi to metabolize them (Cerniglia, 1997). Since fungi are known to metabolise their substrate by non-specific extracellular enzymes (Field *et al.*, 1992; Fritsche, 1992; Martens and Zadrazil, 1992), the enzymes produced during growth on one substrate, in this case barley, can readily facilitate the metabolism of the creosote. Thus the initial reduction in the concentration of creosote by the fungal isolates was probably mainly by co-metabolization. This may explain the relatively high rate of reduction in creosote concentration during the first four weeks (Figures 2 and 3).

Although fungal biomass continued to increase in subsequent weeks, the reduction in creosote concentrations was not as pronounced as during the first four weeks. This may be attributable to a decrease in quantity of carbohydrate base (barley) present, which forced the organisms to adapt to the hydrocarbon substrate effectively, during which period enzyme activity decreased. The increase in creosote removal during the last two weeks may be an indication that the fungi were becoming better adapted to the creosote as a carbon source.

Although it can be argued that *Pleurotus* sp. was responsible for the improved performance of the treatments containing the mixed population of fungi, a comparison with the results from the reactor supplemented with nutrient and inoculated with only *Pleurotus sp.* suggests that the improved activity of the mixed population could have been the result of all the organisms contributing, albeit perhaps in a minor way, in a synergistic action with the dominant *Pleurotus* sp. There was no significant difference at p 0.05 in the performance of the *Pleurotus* sp. isolated from the creosote-contaminated soil (Treatment 14) and that from the non-contaminated

soil (Treatment 16) (Figure 1). However, the slow adaptation of the fungus to the creosote and the delayed removal observed, in the present study, could have been a result of the presence of an alternative carbon source (barley). These results suggests that factors such as the strain of the organism, and hence the particular type of enzyme produced, may greatly affect the rate of pollutant oxidation by a given species.

The high level of removal of 2- and 3- ring PAHs from the soil is attributed to their relatively high solubility in aqueous media compared to the other PAHs studied making them more available for microbial attack as better desorption from soil particles is achieved. Earlier reports have shown that ligninolytic and non-ligninolytic fungi could degrade naphthalene through a number of intermediates to form carbon dioxide and water (Ferris *et al.*, 1973; Cerniglia and Gibson, 1977). In the present study the degradation of naphthalene by the fungi was comparable with that achieved by a consortium of microorganisms in an earlier study (Atagana 2003).

The complete removal of anthracene in this study contrast with results obtained from the previous study, where anthracene was much less actively degraded. However, high anthracene degradation by fungi including *Pleurotus ostreatus* has been reported in the literature (Andersson and Henrysson, 1996; Novotný *et al.*, 1999).

The more than 90% removal observed in the concentrations of the 4- and 5-ring PAHs in 70 days, about half the time taken by a consortium of microorganisms, predominantly bacteria, to achieve the same or less level of removal in a previous study (Atagana, 2003), shows the capability of the fungi in utilizing the tested high molecular mass PAHs. Fungi can oxidize some of these compounds that are usually resistant to bacterial attack because of their non-specific enzymes.

The incomplete removal of these compounds from the experimental soil could be due to the age of the contamination, which presumably resulted in strong sorption to the soil particles (Field *et al.*, 1995; Eggen *et al.*, 1999) as was discussed earlier. Typically, the 4- and 5- ring PAHs, due to their high molecular mass and relatively low water solubility, a property which contributes to their poor desorption from soil matrices, renders them unavailable for microbial attack. However, the effective removal of these compounds in this experiment (Table IV) can be attributed to the ramification of the fungal mycelia, which was enhanced by the bulking and regular tilling of the soil and probably the secretion of non-specific extracellular enzymes. The difference in the level of oxidation between the 2- and 3- ring, and the 4- and 5- ring PAHs, is a function of their initial concentration as well as their physicochemical properties, which include their water solubility, as earlier mentioned, ionisation potential and adsorption and desorption properties (Alexander, 1999; Eriksson *et al.*, 2000).

The changes in pH may be due to the production of acidic intermediaries and their subsequent oxidation. It could also be due to changes in the concentration of enzyme produced during growth of the fungi (Clemente *et al.*, 1999). The general increase in pH is attributed to complete oxidation of some of the creosote com-

ponents present in the soil leaving the soil, with a relatively less acidic medium than it was before treatment. The changes in pH during the study did not affect the fungal growth and the removal of creosote or its components from the soil. The effect of pH on the degradation of pollutant hydrocarbons has not been extensively studied. However, it is possible that soil type, the chemical(s) under survey and a number of other physico-chemical factors will influence the pH of the system over time.

This study established that the white rot fungus, *Pleurotus*, was a better creosote-degrading organism than the other fungi studied. Although all the pure cultures actively degraded creosote, mixed fungal cultures were more effective. The *Aspergillus* isolate had the least degradative potential of the fungi studied.

The more prominent influencing factors in the oxidation of the PAHs were the presence of additional nutrients and an additional source of carbon (barley). In addition, the ability of fungal mycelia to ramify through the soil to reach the substrate, even when it is strongly adsorbed to soil surfaces, is possibly another reason why the degradation of these compounds was successful. This study has shown, however, that complete removal of these compounds is possible with both pure cultures of ligninolytic and non-ligninolytic fungi and mixed cultures of fungi, provided growth conditions are favourable. Although there are no government-set standards for such compounds in soil in South Africa, many of the compounds have been removed to below 1.0 mg kg^{-1}.

References

Alexander, M.: 1999, *Biodegradation and Bioremediation*, Academic Press, San Diego, pp. 10–75.

Alexopoulos, C. J., Mims, C. W. and Blackwell, M.: 1996, *Introduction to Mycology*, John Wiley & Sons, New York, pp. 45–145.

Andersson, B. E. and Henrysson, T.: 1996, 'Accumulation and degradation of dead-end metabolites during treatment of soil contaminated with polycyclic aromatic hydrocarbons with five strains of white rot fungi', *Appl. Microbiol. Biotechnol.* **46**, 647–652.

Atagana, H. I.: 2003, 'Bioremediation of creosote-contaminated soil: A pilot-scale landfarming evaluation', *World J. Microbiol. Biotechnol.* **19**, 571–581.

Atlas R. M.: 1981, 'Microbial degradation of petroleum hydrocarbons: an environmental perspective', *Microbiol. Rev.* **45**, 180–209.

Baker, K. H. and Herson, D. S.: 1995, *Bioremediation.* McGraw-Hill, Toronto, pp. 56–152.

Barnett, H. L. and Hunter, B. B.: 1998, *Illustrated genera of imperfect fungi.* APS Press, St. Paul, pp. 15–77.

Bazelel, L., Hadar, Y. and Cerniglia C. E.: 1996, 'Mineralization of polycyclic aromatic hydrocarbons by the white rot fungus *Pleurotus ostreatus*', *Appl. Environ. Microbiol.* **62**, 292–295.

Bogan, B. W. and Lamar, R. T.: 1995, 'One-electron oxidation in the degradation of creosote polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium*', *Appl. Environ. Microbiol.* **61**, 2631–2635.

Boonchan, S., Britz, M. L. and Stanley, G. A.: 2000, 'Degradation and mineralization of high-molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures', *Appl. Environ. Microbiol.* **60**, 1007–1019.

Boyle, C. D.: 1995, 'Development of a practical method for inducing white rot fungi to grow into and degrade organopollutants in soil', *Can. J. Microbiol.* **41**, 345–353.

Casillas, R. P., Crow Jr, S. A., Heinze, T. M., Deck, J. and Cerniglia, C. E.: 1996, 'Initial oxidative and subsequent conjugative metabolites produced during the metabolism of phenanthrene by fungi', *J. Ind. Microbiol.* **16**, 205–215.

Cerniglia, C. E. and Gibson, D. T.: 1977, 'Metabolism of Naphthalene by *Cunninghamella Elegans*', *Appl. Environ. Microbiol.* **34**, 363 –370.

Cerniglia, C. E.: 1997, 'Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation', *J. Ind. Microbiol. Biotechnol.* **19**, 324–333.

Clemente, A. R., Falconi, F. A., Anasawa, T. A. and Durrant, L. R.: 1999, 'Degradation of aromatic pollutants by a non-basidiomycete ligninolytic fungus', in A. Leeson and B.C. Alleman (eds.), *Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds*, Battelle Press, Columbus.

Coutts, D. A. P., Senior, E. and Balba, M. T. M.: 1987, 'Multistage hemostat investigation of interspecies interactions in a hexanoate-catabolizing microbial association isolated from anoxic landfill. *J. Appl. Bact.* **62**, 251–260.

Davis, J. S. and Westlake, D. W. S.: 1978, 'Crude oil utilization by fungi', *Can. J. Microbiol.* **25**, 146–156.

Eggen, T., Araneda, E., Vethe, Ø. and Sveum, P.: 1999, 'Degradation of aged creosote-contaminated soil by *Pleurotus ostreatus'*, in A. Leeson and B.C. Alleman (eds.) *Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds*, Battelle Press, Columbus.

Eriksson, M., Dalhammar, G. and Borg-Karlson, A. -K.: 2000, 'Biological degradation of selected hydrocarbons in an old PAH/creosote contaminated soil from a gas work site', *Appl. Microbiol. Biotechnol.* **53**, 619–626.

Ferris, J. P., Fasco, M. J., Stylianopoulou, F. L., Jerina, D. M., Daly, J. W. and Jeffrey, A. M.: 1973, Monooxygenase activity in *Cunninghamella bainieri:* evidence for a fungal system similar to liver microsomes. *Arch. Biochem. Bioph.* **156**, 97–103.

Field J. A., De Jong, E, Costa, G. F. and de Bont, J. A. J.: 1992.: 'Biodegradation of polycyclic aromatic hydrocarbons by new isolates of white rot fungi', *Appl. Environ. Microbiol.* **58**, 2219–2226.

Forster, J. C.: 1995, 'Determination of the water-holding capacity of soils', in Alef, K. and Nannipieri, P. (eds.) *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press, London.

Fritsche, W.: 1992, 'Degradation of xenobiotics by fungi', in Preprints of International Symposium on Soil Decontamination Using Biological Processes, Karlsruhe, Germany. 6–9 December 1992. pp 31–36. Frankfurt am Main: Dechema.

Harmsen, J., van den Toorn, A., Heersche, J., Riedstra, D. and van der Kooij, A.: 1999, 'Use of residual substrate from mushroom farms to stimulate biodegradation of poorly available PAH', in Leeson, A. and Alleman, B.C (eds.) *Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds*, Battelle Press Columbus.

Hofrichter, M., Günther, T. and Fritsche, W.: 1993, 'Metabolism of phenol, chloro- and nitrophenols by the *Penicillium* strain *Bi 7/2* isolated from a contaminated soil', *Biodegradation* **3**, 415–421.

Kirk, P.W.: 1969, 'Isolation and culture of lignicolous marine fungi', *Mycologia* **16**, 174–177.

Kotterman, K., van Lieshout, J., Grotenhuis, T. and Field, J.: 1999, 'Development of white rot fungal technology for PAH degradation' in Leeson, A. and Alleman, B.C (eds.), *Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds*, Battelle Press, Columbus.

Lamar, R. T., Larsen, M. J., Kirk, T. K. and Glaser, J. A.: 1987, 'Growth of the white rot fungus *Phanerochaete Chrysosporium* in soil', in *Land Disposal, Remedial Action, Incineration and Treatment of Hazardous Waste*: Proceedings of the 13th Annual Research Symposium. US EPA.

Launen, L., Pinto, L., Wiebe, C., Kiehlmann, E. and Moore, M.: 1995, 'The oxidation of pyrene and benzo(a)pyrene by nonbasidiomycete soil fungi', *Can. J. Microbiol.* **41**, 477–488.

Lees, Z. M.: 1996, 'Bioremediation of oil-contaminated soil: A South African case study', PhD thesis University of Natal, Pietermaritzburg, South Africa.

Loske, D., Hütterman, A., Majcherczyk, A., Zadrazil, F., Lorsen, H. and Waldinger, P.: 1989, 'Use of white rot fungi for the clean-up of contaminated sites', in Coughlan, M.P. and Amaral Collaço, M.T. (eds.) *Advances in Biological Treatments of Lignocellulosic materials*, Elsevier, London.

MacGillivray, A. R. and Shiaris, M. P.: 1993, 'Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments', *Appl. Environ. Microbiol.* **59**, 1613–1618.

Martens, R. and Zadrazil, F.: 1992, 'Screening of white rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil, in *Preprints of International Symposium on Soil Decontamination Using Biological Processes*, Karlsruhe, Germany. 6–9 December 1992. Dechema, Frankfurt am Main.

McGugan, B. R.: 1997, 'Exploitation of indigenous fungi in low-cost ex-situ attenuation of oil-contaminated soil', M.Sc. Thesis, University of Natal, Pitermaritzburg, South Africa.

Novotný, C., Erbanová, P., Šašek, V., Kubátová, A., Cajthaml, T., Lang, E., Krahl, J. and Zadrazil, F.: 1999, 'Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi', *Biodegredation* **10**, 159–168.

Pothuluri, J. V., Freeman, J. P., Evans, F. E. and Cerniglia, C. E.: 1990, 'Fungal transformation of fluoranthene', *Appl. Environ. Microbiol.* **56**, 2974–2983.

Pothuluri, J. V., Freeman, J. P., Evans, F. E. and Cerniglia, C. E.: 1992, 'Fungal metabolism of acenaphthene by *Cunninghamella Elegan*', *Appl. Environ. Microbiol.* **58**, 3654–3659.

Pozdnyakova, N., Turkovskaya, O., Ignatov, V.: 2001, 'Degradation of oil hydrocarbons by White-Rot fungi' in *Proceedings of the First International Congress on Petroleum Contaminated soils, Sediments and Water*, London. 14–17 August, 2001.

Raper, K. B. and Thom, C.: 1968, *A manual of the Penicillia*, Hafner Publishing Company, New York.

Raymond, R. L., Hudson, J. O. and Jamieson, V. W.: 1976, 'Oil degradation in soil', *Appl. Environ. Microbiol.* **31**, 522–535.

Rodriguez, R., Montalvo, C. P., Dendooven, L., Esparza, F. G. and Fernandez, L. L.: 1999, 'Degradation of benzo(a)pyrene by white rot fungi' in Leeson, A. and Alleman, B.C. (eds.), *Bioremediation Technologies for Polycyclic Aromatic Hydrocarbon Compounds* Battelle Press, Columbus.

Sack, U. and Fritsche, W.: 1997, 'Enhancement of pyrene mineralization in soil by wood-decaying fungi', *FEMS Microb. Ecol.* **22**, 77–83.

Sack, U., Heinze, T. M., Deck, J., Cerniglia, C. E., Martens, R., Zadrazil, F. and Fritsche, W.: 1997, 'Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi', *Appl. Environ. Microbiol.* **63**, 3919–3925.

Šašek, V., Volfová, O., Erbanová, P., Vyas, B. R. M. and Matucha, M.: 1993, 'Degradation of PCBs by white rot fungi, methylotrophic and hydrocarbon utilizing yeasts and bacteria', *Biotechnol. Lett.* **15**, 521–526.

USEPA: 1986, *Test Methods for Evaluating Soil Waste* SW-846, Vol. I and II Nov. 1986