

## CHAPTER 2

# BIODEGRADATION OF PAHS IN SOIL BY TWO DEUTEROMYCETE FUNGI

A.R. Clemente; L.R. Durrant

*Universidade Estadual de Campinas - UNICAMP*

**Abstract:** The fungal strains used in this work, namely, 984 and 1040, were isolated from soil samples collected at the Jureia-Itatins Ecological Reserve, São Paulo-Brazil. Following microscopical examinations these strains were classified as *Aspergillus* sp. (984) and *Verticillium* sp. (1040). The degradation of PAHs in soil contaminated with 5 mg naphthalene/g soil; 1.0 mg anthracene/g soil or 0.5 mg pyrene and/or benzo[a]pyrene/g soil, was verified. These strains were grown in wheatbran:water for 3 days, inoculated in sterilized and non-sterilized soil, and cultivated for 2, 4, 6, and 8 weeks. The PAHs were then extracted and degradation was determined by HPLC. The best degradation, in sterilized soil was obtained after 8 weeks for the two strains: naphthalene (64.50 - 65.43%), anthracene (77.35 - 85.83%) and pyrene (73.01 - 78.78%). When benzo[a]pyrene was used the best degradation shown by strain 984 was 89.62% in six weeks and 78.06% by strain 1040 in eight weeks. In non-sterilized soil the strains exhibited lower growth and degradation than in sterilized soil, with the exception for benzo[a]pyrene (82.3 - 82.6%). Our results indicate that these two fungal strains have potential for application in the bioremediation of soils contaminated with PAHs.

**Keywords:** PAHs, biodegradations, fungi, soil.

### 1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent a class of nonionic, poorly water-soluble, and toxic organic compounds which occur in environmental matrices due to natural causes such as forest fires or anthropogenic processes such as urban and industrial activities (Conte et al., 2001). Commonly used remediation techniques including land removal and incineration or landfilling are now less environmentally acceptable or cost effective than they used to be. Hence, interest in bioremediation as an alternative approach for the cleaning up of contaminated environments has

increased (Canet et al., 2001). Research on biodegradation has demonstrated the potential of fungi to degrade PAHs (Canet et al., 1999).

Several factors affect the persistence of PAHs in the environment. Wilcock et al., (1996) reported that low molecular weight PAHs is rapidly lost from sediments, whereas the high molecular weight ones have larger persistence. Ghoshal & Luthy (1996) showed that a mechanism of mass transfer of aromatic hydrocarbons (in particular naphthalene) is responsible for the rate of biomineralization of such contaminants in system containing single coal tar globules. Gustafson & Dickhut (1995) showed that the concentration of hydrophobic PAHs in water depended mainly on their hydrophobicity. Physical-chemical properties of soils have also been considered responsible of the retention of PAHs in soil matrices. The amount of organic carbon (Weissenfelds et al., 1992), and the hydrophobicity of soil organic matter (Murphy et al., 1990), were estimated to be the most significant parameters in decreasing the environmental availability of PAHs.

Several studies have shown that diverse fungi are capable of PAH mineralization, and that rates of mineralization correlate with the production of ligninolytic enzymes (Field et al., 1992; Sack et al., 1997). These microorganisms naturally decompose lignin to obtain the cellulose inside the wood fiber using a non-specific enzymatic complex, which also enables them to degrade a wide range of contaminants if they are introduced to soils. In soil, the fungi release these enzymes to the extracellular medium, allowing the fungi to degrade large molecules that they would otherwise be unable to incorporate across cell walls. Such metabolism has further advantages in so far as the fungi avoid the uptake of potentially toxic substances and the non-specific action of the enzymes involved makes precondition to individual pollutants unnecessary. Furthermore, induction of the extracellular enzyme system is independent of the presence of the contaminants, therefore the fungi can degrade contaminants at extremely low concentrations (Canet et al., 2001).

To date, most investigations of PAHs degradation by fungi have been carried out *in vitro* by inoculating artificially contaminated liquid media, which do not provide a reliable indication as to their likely success under field conditions (Canet et al., 1999). Here, the degradation of PAHs in soil by two ligninolytic fungi is demonstrated.

## **2. MATERIAL AND METHODS**

### **2.1 Fungal strains and inoculum preparation.**

The fungal strains isolated from soil samples collected at the Jureia/Itatins ecological reserve (São Paulo - Brazil) and classified as deuteromycetes, were a gift from the Tropical Culture Collection - Fundação Tropical André Tosello, Campinas-SP. The strains were maintained on PDA (potato dextrose agar-DIFCO) slants. Petri dishes containing PDA were inoculated with a mycelium portion of each strain and incubated at 30°C for 8-10 days and used as the inocula for the experiments described below. These strains have previously been reported as ligninolytic (Clemente et al., 2001 and Clemente & Durrant, 2003). Following microscopically examinations were classified as *Aspergillus* sp. (984) and *Verticillium* sp. (1040), (Clemente & Durrant, 2003).

### **2.2 Soil characteristics and soil contamination (Andersson & Henrysson, 1996).**

A sandy soil having an organic matter content of 3.1% and pH of 6.5, was used throughout this work. This soil type was chosen because of its properties, which made it easier to handle and the extraction of the PAHs more reliable. The soil was divided into two fractions. One fraction was stored in sterile flasks at +4°C, while the other was autoclaved at 121°C for 15 minutes, stored at room temperature for a week, sterilized again and then stored at +4°C. Individual soil fractions were contaminated with naphthalene (5.0%), anthracene (0.75%), pyrene (1.0%) or benzo[a]pyrene (0.5%), which had been previously dissolved in a 250 mL mixture of hexane and acetone 3:1 (v/v), except for anthracene which was dissolved only in acetone. The solutions were added to the soil to give the final concentration of 5.0 mg naphthalene/g soil; 1.0 mg anthracene/g soil or 0.5 mg pyrene or benzo[a]pyrene/g soil, and mixed for a total time of 2 hours. When all the solvents had evaporated the fungi grown in wheatbran:water for 3 days were inoculated into the flasks containing the contaminated soils and were incubated for 2,4, 6 and 8 weeks at 30° C.

### **2.3 HPLC analyses of the extracted PAHS for the determination of degradation (Andersson & Henrysson, 1996)**

At two-week interval samples, consisting of the whole flasks were collected and toluene (100 mL), was added to each flask, which were thoroughly mixed and placed in a water bath at 100°C for 3 hours. When the flasks had reached room temperature, 2 mL of the toluene mixture was removed, filtered with a 0.22 µm membrane using a glass syringe and used for the HPLC analyses.

### **2.4 High performance liquid chromatography (HPLC).**

All HPLC analyses, for the determination of degradation of PAHs, were performed with a Zorbax ODS (0.46 x 15 cm) C<sub>18</sub> reverse-phase column (SUPELCO Chromatography Products). Separation was achieved by isocratic elution in acetonitrile:water (70:30), with a flow rate of 1.0 mL/min and UV absorbance detector set at 254 nm.

## **3. RESULTS AND DISCUSSION**

In non-sterilized soil, the strains exhibited lower visual growth and degradation than in sterilized soil, with the exception of benzo[a]pyrene. As shown in figure 1, both strains were able to degrade naphthalene when present in sterile soil. Best degradation rates (~65% for both strains), were obtained from the 6<sup>th</sup> to 8<sup>th</sup> week of incubation. Very low degradation was observed following the HPLC analyses of samples from the non-sterile soil. It is possible that the indigenous microorganisms present in the non-sterile soil compete for nutrients or have some kind of antagonism with the fungal strains used here. However, when anthracene was used as the contaminant (figure 2), degradation occurred under both conditions, sterile (~80%) after 8 weeks and non-sterile (~70%) from the 6<sup>th</sup> to the 8<sup>th</sup> week. When pyrene was used (figure 3), degradation took place under both conditions, but was superior under sterilized condition for both strains (~70%) than under non-sterile (~50%). Benzo[a]pyrene was the only PAH best degraded by both strains (~82%), under both conditions. Growth of both strains in the sterilized soil may have been stimulated by the effective death of the indigenous microflora but also due to changes in the physicochemical properties of the soil after sterilization, a process that can bring about an increase in the concentration of soluble nutrients and organic matter (Lynch,

1998). Anderson et al., (2000), have observed an increase in the visual growth of *Pleurotus ostreatus* and *Phanerochaete chrysosporium* which showed a significant correlation with the degradation of the PAHs present in the growth medium. The low visual growth and degradation of PAHs in non-sterile soils could be explained by the low ability of fungi to compete with the existent microorganisms, or it may be that their extracellular enzymes are non-active in the soils used or may not be produced (Matens & Zadrazil, 1992, and In der Wiesche et al., 1996). It is also possible that other factors such as soil temperature and moisture could have a negative effect in the growth of the fungi (Canet et al., 2001). In the bioremediation of contaminated soils, it is important that any PAH present be removed by the soil microflora or by any inoculated microorganism or that a consortium can be established among them. When the ligninolytic white-rot fungus *Bjerkandera* sp was inoculated in soils containing high molecular weight PAHs, such as benzo[a]pyrene, it was able to completely degrade this compound following 15 days of incubation (Kotterman et al., 1998). Similarly, the results presented here show that *Aspergillus* sp and *Verticillium* sp were also able to degrade benzo[a]pyrene after 4 weeks. Our results indicate that these two fungal strains have potential for application in the bioremediation of soils contaminated with PAHs.

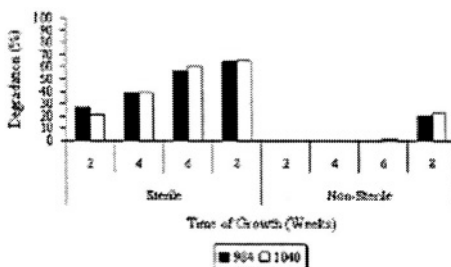


Figure 1. Degradation of Naphthalene in Sterile and Non -sterile Soils following Growth of *Aspergillus* sp and *Verticillium* sp

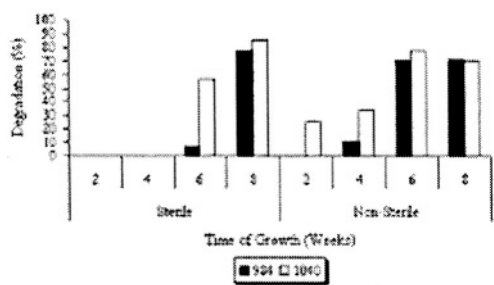


Figure 2. Degradation of Anthracene in Sterile and Non -sterile Soils following Growth of *Aspergillum sp* and *Verticillium sp*

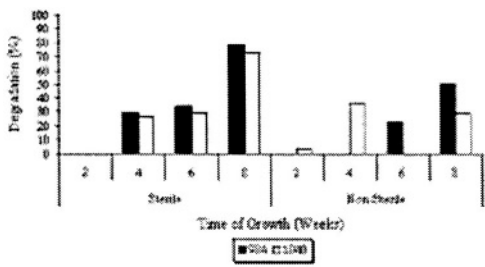


Figure 3. Degradation of Pyrene in Sterile and Non -sterile Soils following Growth of *Aspergillum sp* and *Verticillium sp*

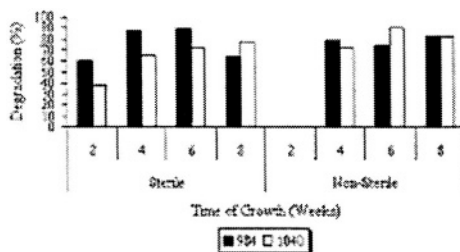


Figure 4. Degradation of Benzo[a]pyrene in Sterile and Non-sterile Soils following Growth of *Aspergillum sp* and *Verticillium sp*

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