EFFECTS OF CYCLODEXTRIN ON EXTRACTION AND FUNGAL REMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBON-CONTAMINATED MAHONING RIVER SEDIMENT

by

Sowmya Pabba

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Effects of Cyclodextrin on Extraction and Fungal Remediation of Polycyclic Aromatic Hydrocarbon-contaminated Mahoning River sediment

Sowmya Pabba

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Sowmya Pabba, Student Date

Approvals:

_____

Carl G. Johnston, Thesis Advisor Date

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Roland Riesen, Committee Member Date

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Daryl W. Mincey, Committee Member Date

_____

Peter J. Kasvinsky, Dean of Graduate Studies Date

Abstract:

The effects of *β*-cyclodextrin on the extraction and fungal remediation of PAHs in historically contaminated river sediment were examined in this study. Sediment collected from Lowellville, Ohio were incubated for 42 days and amended with paper, sawdust nitrogen supplement to stimulate fungal growth. The surfactant, *β*-cyclodextrin was added to increase the availability of non-polar PAHs from the sediment. The samples were extracted via a sonication method based on the USEPA method 3550, purified and analyzed by GC-MS. The total low molecular weight PAHs showed degradation of 64% and the total high molecular weight PAHs, a degradation of 57% for the sediment treated with the fungi *Pleurotus ostreatus,* sawdust and nitrogen. The low molecular weight compound fluorene degraded by 76% for sawdust-treated samples amended with fungi and *β* -cyclodextrin whereas the high molecular weight PAH chrysene showed only 8% degradation for the same treatment. Overall the effect of β-cyclodextrin on the PAH degradation was inconsistent, it did not improve the most effective treatment (sawdust, fungi and nitrogen), but did improve the degradation in less effective treatments.

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Chapter 1: Introduction

1.1. Mahoning River History:

Contamination of soils, sediments, ground water and air with toxic materials has become a major concern today. The Mahoning River has been a dumping site for industrial sewage from the early 70's and its lower region is regarded to be the most polluted site. The lower branch of the Mahoning River starts at Winona, flows through Leavittsburg, and continues south east through Girard, Youngstown, and Lowellville into PA. It finally joins the Ohio River by first joining with the Shenango River to form the Beaver River, which empties into the Ohio River.

In the early 70's, the Mahoning River has been significantly altered by the construction of numerous large reservoirs and low-head dams. The dams were constructed to provide a reservoir of cooling waters for the hot steel and machinery in the steel industries that used the Mahoning River as an 'industrial sewer'. (Ohio EPA, 1996). The water thus heated and filled with chemicals was directly poured into the river. This dumping resulted in elevated river water temperatures thus depriving it of any life. The list constitutes of heavy metals, oils, petroleum, polycyclic aromatic hydrocarbons (PAHs), (PCBs) polychlorinated biphenyl compounds and many other carcinogenic and mutagenic compounds, thus polluting the river.

Between 1900 and the 1970's the Mahoning received up to and over 70,000 lbs of oil and grease each day (USACE, 1999).

The late 70's brought an improved look to the Mahoning River as some of the major steel industries were brought down, thus cutting down on the sewage deposition into the river. Early in 2004, there was a drastic decrease in pollutant discharge into the river and the water quality improved appreciably. The sediment however, remained

highly contaminated with PAHs and PCBs because of their tendency to bind to the sediment particles, and their low solubility rates (MRC, 2005). When introduced into aquatic environments, the sediments serve as a repository for the majority of these toxins (OEPA, 1996).

The fish returned, but still a portion of them suffered from DELT (deformities, fin erosions, lesions and tumours). For these reasons, the Ohio Department of Health has issued a contact advisory and Fish consumption advisory against the river.

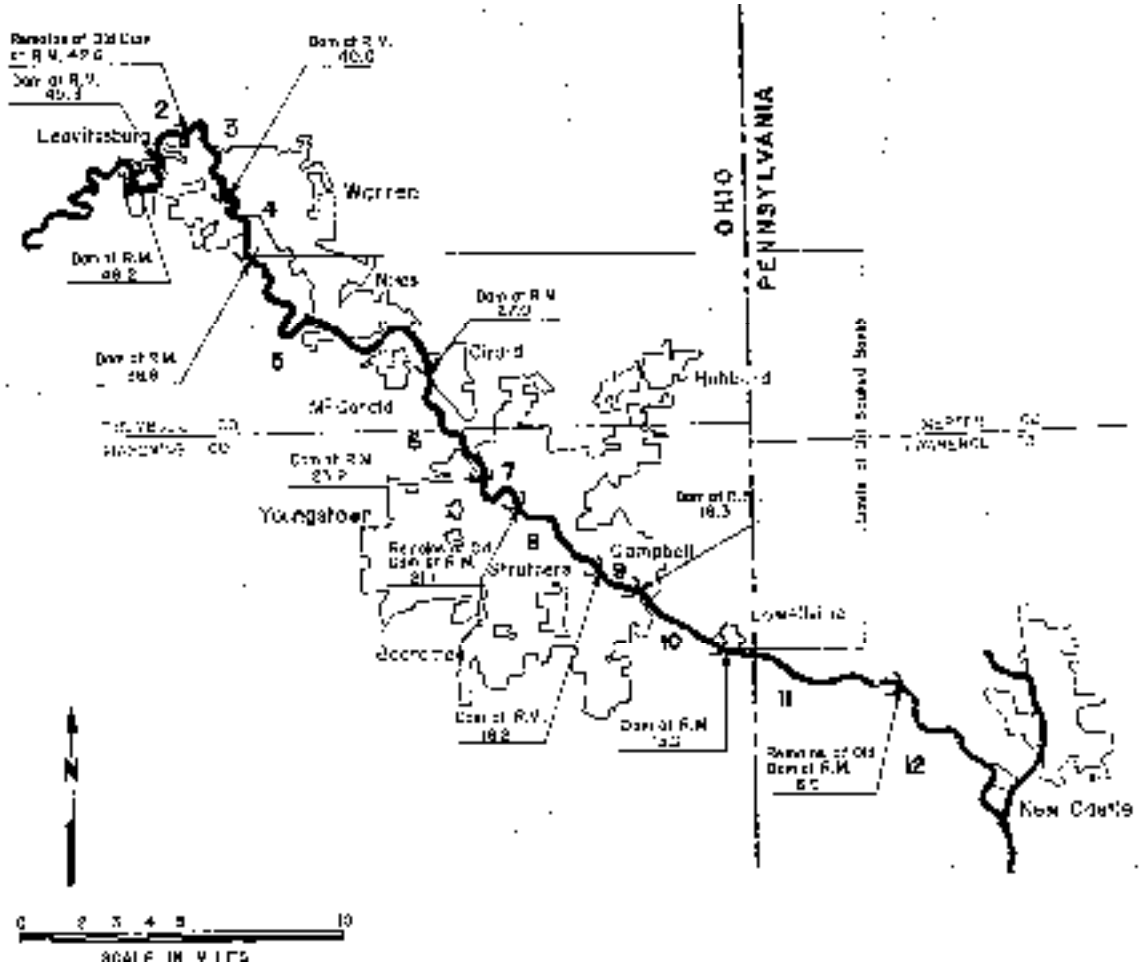

Fig. 1. Map of the lower Mahoning River.

The Ohio EPA and US Army Corps of Engineers have estimated the contamination to a total of 750,000 cubic yards of material spread out over a span of 30 miles (The Mahoning River Education Project). Several river restoration options such as dredging, capping and bioremediation are being introspected upon.

1.2. Polycyclic Aromatic Hydrocarbons:

Polycyclic aromatic hydrocarbons (PAHs) are an important group of organic micropollutants (xenobiotics) due to their widespread distribution in the environment (atmosphere, water and soil) (Berset et al., 1995). These are formed during the incomplete combustion of coal, oil, gas, wood, garbage or other organic substances like tobacco and charbroiled meat or anthropogenic processes (ATSDR, 2004). They are non-polar, hydrophobic compounds that contain fused aromatic rings and do not have heteroatoms or carry substituents. They do not ionize, and possess low water solubility and low volatility with increasing molecular weight (Consuelo Sánchez-Brunete et al., 2007). Because PAHs are hydrophobic, they tend to adsorb tightly to sediment particles and therefore become persistent in the environment.

They can exist in over hundred different combinations but the most common are treated as a group of 15. They may be divided into two groups, low molecular weight PAHs (2-3 ring PAHs) that have a significant acute toxicity and high molecular weight PAHs (4-6 ring PAHs) which show high carcinogenic and mutagenic potentials (Doong et al., 2000). LMW PAHs can be degraded by native bacteria and fungi but HMW PAHs have high hydrophobicity, low water solubility and a tendency to sorb to the organic fraction of soil and sediments. These properties are largely responsible for their low

availability to microorganisms and their persistence in the environment (Hughes et al., 1997) and hence might need the help of more aggressive fungi. This characteristic poses a potential hazard not only to humans but to other life forms (Fang et. al, 1996) and calls out the need for a safe and effective way to remediate PAHs in the Mahoning River sediments (USACE, 1999).

1.2.1. Low molecular weight PAHs found in Mahoning River sediment:

(a) Naphthalene:

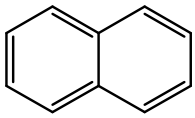

Fig. 2 Naphthalene

Synonyms: Naphthalin, Naphthaline, Napthene, Tar Camphor, White tar, Albocarbon, or Antimite (MSDS).

It is derived from coal tar and a primary ingredient of mothballs. It is used as a tanning agent and in surface active resins and dyes.

Exposure to naphthalene (>2ppb) may cause laryngeal carcinoma, damage of red blood cells (RBC) which leads to the development of hemolytic anaemia. Acute exposure causes cataracts in humans, rats, rabbits, and mice and people have an inherited condition called glucose-6-phosphate dehydrogenase deficiency (USEPA, 1986).

(b) Acenaphthylene:

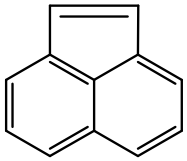

Fig. 3 Acenaphthylene

Synonyms: Acenaphthalene, cyclopenta [*de*]naphthalene (MSDS).

Acenaphthylene is toxic and mostly found in crude oil, coal tar, cigarette smoke, exhaust from automobiles and wood preservatives. It is used to make dyes, plastics and pesticides.

Exposure to acenaphthylene may cause decreased red blood cell, haemoglobin, and hematocrit values and decreased platelet (males) and leukocyte counts (females); hepato cellular hypertrophy; nephropathy and related kidney lesions; decreased ovary weights, decreased ovarian and uterine activity, and smaller and fewer corpora lutea (U.S. EPA, 1989).

(c) Acenaphthene:

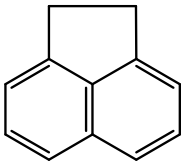

Fig. 4 Acenaphthene

Synonyms: 1, 2-dihydroacenaphthylene, 1,8-ethylenenaphthalene, peri-ethylenenaphthalene, naphthylene ethylene (MSDS).

Acenaphthene is toxic and a constituent of coal tar and is used in preparation of dyes, pesticides and pharmaceuticals.

Exposure to acenaphthene lead to pathological effects reported in rats that inhaled acenaphthene (12 mg/m^3) 4 hours/day, 6 days/week for five months included desquamation of alveolar epithelial cells, focal bronchitis, and widespread cell proliferation of the bronchial epithelium, but no signs of malignancy (U.S. Department of Labor, Occupational Safety and Health Administration).

(d) Fluorene:

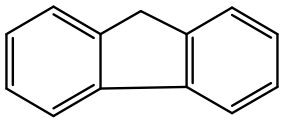

Fig. 5 Fluorene

Synonyms: 9H-fluorene, o-biphenylmethane, diphenylmethane, 2, 3-benzindene (MSDS).

Fluorene is toxic and a tricyclic aromatic hydrocarbon which contains a five-membered ring. It is ranked as one of the most hazardous compounds (worst 10%) to ecosystems and it is more hazardous than most chemicals in 3 out of 5 ranking systems. It is manufactured artificially, although it occurs in the higher boiling fractions of coal tar. Skin, eye and respiratory irritant. It shows evidence of mutagenic properties in laboratory animals. ATSDR minimal risk level ORL 0.04 mg/kg/day (U.S. Department of Labor, Occupational Safety and Health Administration).

(e) Phenanthrene:

Synonyms: Coal tar pitch volatiles, ravatite, phenantrin (MSDS).

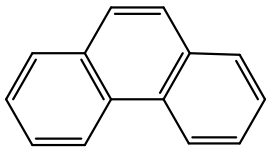

Fig. 6 Phenanthrene

Phenanthrene is a toxic compound and is a polycyclic aromatic hydrocarbon composed of three fused benzene rings and provides a framework for steroids. It is usually found in vehicular emissions, coal and oil burning, wood combustion, coke plants, aluminum plants, iron and steel works, foundries, municipal incinerators, synfuel plants, and oil shale plants (U.S. EPA, 1987). It is one of a number of PAHs on EPA's priority pollutant list (ATSDR, 1990). It is an irritant and causes a photosensitizing effect on skin.

(f) Anthracene:

Synonyms: Paranaphthalene, Anthracin, Green oil (MSDS).

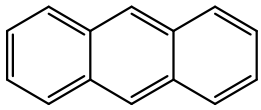

Fig.7 Anthracene

Anthracene is a linear and less stable isomer of phenanthrene and is formed as a product of incomplete combustion of fossil fuels. It is used in the production of dyes and smoke screens. It is also used as an organic semiconductor and plastic scintillator.
Anthracene is toxic and photosensitizing, potentiating skin damage elicited by exposure to ultraviolet (UV) radiation (U.S. EPA, 1987; Dayhaw-Barker et al., 1985; Forbes et al., 1976).

1.2.2. High molecular PAHs found in Mahoning River:

(a) Pyrene:

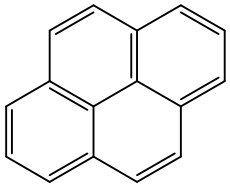

Fig. 8 Pyrene

Synonyms: beta-pyrene, coal tar pitch volatiles (MSDS).

It is produced in a wider range of combustion conditions as it is much more resonance stabilized than its five-member-ring containing isomer fluoranthene.

Skin painting assay evaluations in mice showed complete carcinogenicity in mice (Van Duren and Goldschmidt, 1976). However it has been reported as toxic to humans.

(b) Benzo(a)pyrene:

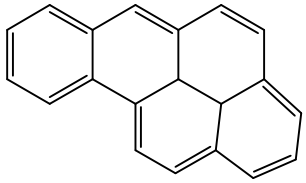

Fig. 9 Benzo(a) pyrene

Synonyms: 3,4-benzopyrene, Benzo (alpha) Pyrene (MSDS).

Benzo[a]pyrene is found in coal tar, in automobile exhaust fumes (especially from diesel engines), tobacco smoke, marijuana smoke, wood smoke, and in charbroiled food.

Benzo(a)pyrene is a probable human carcinogen, (procarcinogen) developmental toxicant, endocrine toxicant, immunotoxicant, respiratory toxicant, skin/sense organ toxicant (USEPA 1994) .

(c) Benzo(a)anthracene:

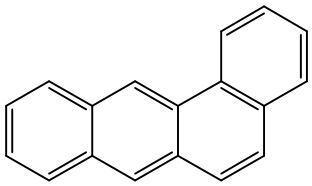

Fig. 10 Benzo(a) anthracene

Syn onyms: Benzo(a)phenanthrene, Tetraphene (MSDS).

It is natural product produced by the incomplete combustion of organic material. The arrangement of the aromatic rings in the benz[*a*]anthracene molecule gives it a "bay region" often correlated with carcinogenic properties (U.S. EPA 1980, Jerina et al., 1977).

(d) Chrysene:

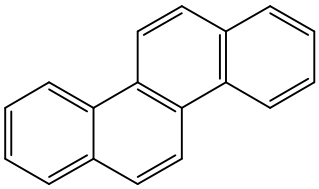

Fig. 11 Chrysene

Synonyms: 1, 2-benzphenanthrene (MSDS).

It is a natural constituent of coal tar. It is formed by combustion of crude oil, garbage, plant and animal material burns, diesel and aircraft exhaust, coke oven emissions and used in the manufacture of artificial dyes.

Chrysene is a probable carcinogen and mutagen and exposure to chrysene (> 0.2 mg/ m^3) for a long time leads to skin cancer (IARC, 1983; ATSDR, 1990).

(e) Dibenz (a,h) anthracene:

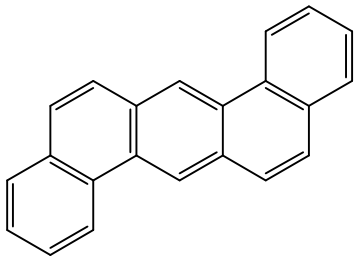

Fig .12 Dibenz (a,h) anthracene

Synonyms: 1, 2:5, 6-Dibenzanthracene (MSDS).

It occurs as a component of coal tars and shale oils. It is found in gasoline engine exhaust, cigarette smoke, vegetation near heavily traveled roads, surface water and soils near hazardous waste sites (ATSDR, 1993; IARC, 1983).

Animal studies have shown the development of pulmonary adenomatosis, alveologenic carcinoma of the lung, hemangio-endotheliomas of the pancreas and mammary carcinomas in females, thus rendering it carcinogenic and mutagenic (ATSDR, 1993; IARC, 1983).

(f) Benzo (ghi) perylene and Benzo (b, k) fluoranthene:

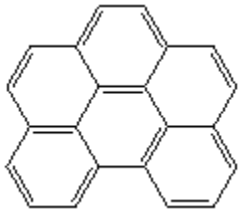

Fig. 13 Benzo(ghi)perylene

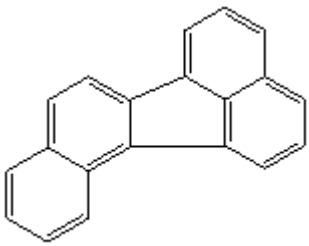

Fig. 14 Benzo (b,k) fluoranthene

Benzo (ghi) perylene:

Synonyms: 1, 12-Benzoperylene; 1, 12-Benzperylene (MSDS).

Benzo (b, k) fluoranthene:

Synonynms: Benzo[b]fluoranthene, benzo[j]fluoranthene (MSDS).

These PAHs most likely result from the incomplete combustion of a variety of fuels including wood and fossil fuels. They are found in mainstream cigarette smoke, urban air, gasoline engine exhaust, emissions from burning coal and from oil-fired heating, broiled and smoked food, oils and margarine (IARC, 1983).

It is classified as probable human carcinogen and exposure may result in tumours (Edmond J. LaVoie et al, 1982).

1.3. Cyclodextrins:

Bioavailability is one main factor that influences the extent of biodegradation of hydrocarbons. The hydrophobic nature of PAHs results in their sorption onto soil matrix, limiting their bioavailability and makes it less available for bioremediation (Baowei Zhao et al., 2005). Surface active compounds have been used in attempts to enhance the bioavailability of PAHs by facilitating their transfer from both solid and nonaqueous phase liquids (NAPL) to associated water phases (Ian J. Allan et al., 2007) enabling the availability of contaminants to microorganisms. They could reduce surface and interfacial tensions by accumulating at interface of immiscible fluids and increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds (Rouse et al., 1994). An important aspect in selecting a solubility enhancing agent is that it should have low or no environmental risk. Application of synthetic surfactants, in several cases gave inconclusive results and in some cases the applied surfactant proved to be toxic (Mónika Molnar et al., 2005). Synthetic surfactants

possess tendencies to be toxic, less biodegradable and form high-viscosity emulsions which are difficult to remove.

Cyclodextrins (CDs) are natural, non-toxic compounds that form soluble inclusion complexes with hydrophobic molecules (like hydrocarbons) and increase their degradation rate *in vitro*. Cyclodextrins possess a low-polarity cavity that is capable of forming inclusion complexes with organic compounds of appropriate shape and size (Badr. T et al., 2004). This property can be exploited for the bioremediation of soils contaminated with hydrocarbons and related chemicals due to their solubility and bioavailability enhancing effect (Mólnar. M et al).

The application of cyclodextrin extraction for prediction of PAH bioavailability was first studied by Reid et al (1998, 1999, 2000). *α, β* and *γ* CDs are non-reducing cyclic glucose oligosaccharides formed by 6, 7or 8 α-1, 4-linked glucose units respectively. Because of their toroidal hydrophobic cavities and a hydrophilic shell, they are water-soluble and form inclusion complexes with hydrophobic molecules of a size compatible with their core. In this way, the aqueous solubility of several compounds is increased through the dynamic equilibrium exchange with guest molecules that dissociate from the complexes becoming available for catabolism (Laura Bardi et al., 2003). The reason why

β- cyclodextrin, inspite of its limited water solubility when compared to α and γ forms, has been preferred over its competitors for remediation is not clearly known. It was found that *β*-cyclodextrin showed a different trait from most of the cyclodextrins that aid in aromatic hydrocarbon degradation, by degrading dodecane, an aliphatic compound (Steffan et al., 2002). This was possible because of the structural favourabilities of

β -cyclodextrin. It might be attributed to the fact that aliphatic and polyaromatic hydrocarbon degradation kinetics was accelerated by *β*-cyclodextrin making them more suitable for *in situ* bioremediation of hydrocarbon-polluted soils or waters. It was also observed that *β*-cyclodextrin reduced PAH leaching that generally occurs during biodegradation ((Laura Bardi et. al, 2007). It was also proved that *β* -cyclodextrin significantly reduced biotoxicities of the low-polarity compounds when compared to the high polarity ones (Song Wen-lu et al., 1999).

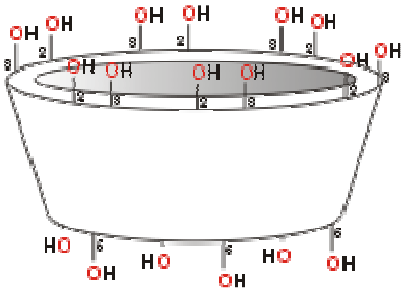

Fig. 15 General structure of *β*-cyclodextrin

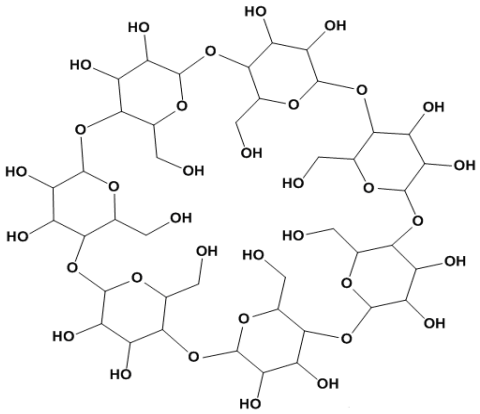

Fig. 16 β -cyclodextrin

However, relatively cheaper industrial mixture of RAMEB (randomly methylated- β -cyclodextrins has been recently tested to enhance the hydrocarbon biodegradation by increasing both the hydrocarbon availability and the availability of specialized bacteria in the soil microcosms when used in the concentrations of 1 – 3% (Fava et al., 2002) s. This can be used as an alternative for β -cyclodextrin due to a better water solubility, non-toxicity and relatively low cost (Fava et al., 2002). Other alternatives like Hydroxypropyl- β -Cyclodextrin which have similar characteristics can prove useful (Fava et al., 1998).

1.4. Bioremediation:

Bioremediation is a managed or spontaneous technique where microbiological processes (bacteria, fungi) are used to degrade or transform contaminants to less toxic or nontoxic forms. Bacteria have been extensively used for use in the degradation of pesticides because of their ease of culture, more rapid growth rates and convenience for genetic manipulation (Kumar, Mukerjii & Lal, 1996). But bacterial remediation has its

own limits in the case that low-molecular-weight PAHs are usually readily degraded, but high-molecular-weight PAHs of five or more rings resist extensive bacterial degradation in soil and sediments. This contumacious behavior can be attributed to the limited bioavailability of PAHs strongly adsorbed onto the soil organic matter (Jim A. Field et al., 1992). Studies have clearly demonstrated that fungi, in particular white rot fungi are capable of degrading vast number of pollutants, including pesticides (Aust, 1990, 1993; Kirk, Lamr& Glaser, 1992; Barr & Aust, 1994a; Paszczynski & Crawford, 1995). Fungi might offer some advantages over bacteria for remediation due to their rapid colonization of substrates and high tolerance of the toxin (Cerniglia et al., 1992, 1993). These fungi display an extracellular degradation system that is capable of cleaving lignin (Kirk and Farrell., 1987), an amorphous and complex biopolymer with an aromatic structure that resembles the aromatic molecular structure of the pollutants like PAHs, pesticides, polychlorinated biphenyls (PCBs), synthetic dyes, etc (Valentin. L et al., 2006). This structural resemblance makes the fungal degradation of PAHs by white rot fungi feasible. Both lignin and PAHs are highly insoluble, hydrophobic and pose similar problems for catalysis by enzymes which tend to be water soluble and usually highly stereospecific. The huge structural diversity of the pollutants degraded by these fungi has fuelled the interest in their use for bioremediation (Barr & Aust, 1994b; Arisoy, 1998).

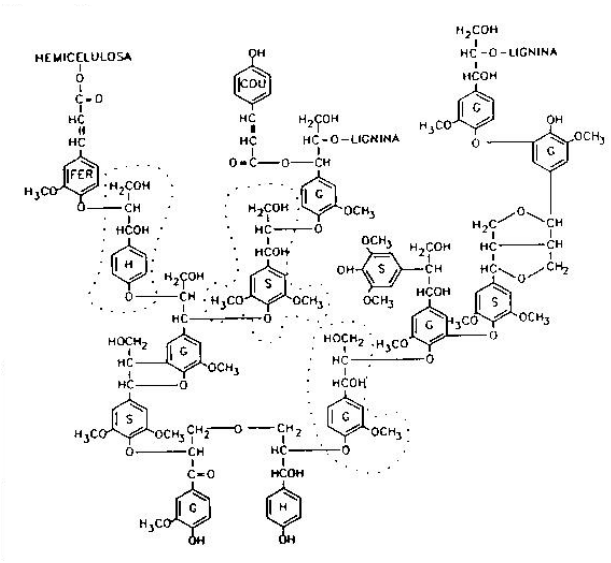

Fig. 17 Structure of Lignin

The lignolytic potential of these fungi can be related to the secretion of oxidative enzymes such as lignin peroxidases, manganese peroxidases and laccases. The principal biochemical reactions in the primarily co-metabolic degradation of pollutants by fungi include oxidation, reduction, hydroxylation, aromatic ring cleavage, hydrolysis, dehalogenation, methylation and demethylation, dehydrogenation, ether cleavage, condensation and conjugate formation.(http://umbbd.msi.umn.edu/flu/flu_map.html), (http://www.genome.ad.jp/kegg/pathway/map/map00628.html), (http://umbbd.msi.umn.edu/pha/pha_map.html)

White-rot fungi require lignocellulosic substrates to survive in soil as their carbon source of energy. Many studies show the use of straw and milled wheat as good substrates for fungal growth (Zadrazil, 1997). Recent findings have proved that the toxicity of organic pollutants was found to be greatly reduced in sawdust-based media than in liquid media (Alleman et al., 1992).

1.5. Objectives:

The objectives of this research were to (1) determine the effects of β -cyclodextrin in mobilizing PAHs during bioremediation in the historically contaminated sediment. This might present an idea about the ability of cyclodextrins to enhance PAH degradation by *Pleurotus ostreatus* (2) compare two PAH extraction methods and (3) determine the effect of β- cyclodextrin on PAH extraction.

Chapter 2: Methods

2.1. Sample Collection:

Mahoning River sediment from Lowellville, Ohio was collected directly into plastic tubes and stored at 4 ^{o}C until use. Sediment was weighed out and the PAHs were extracted using both sonication and lipid extraction techniques.

2.2.1. Experimental Treatments:

One liter of the sediment was taken in 2 liter glass containers ("fish bowls"). Nineteen experimental treatments were tested on the whole in nine bowls during a 6 week period. Initially, nine treatments were tested. After 3 weeks (21 days), half of each treatment was mixed to determine the tilling effect on the PAH degradation. Sawdust (60% by volume) and shredded paper (60% by volume) were added to the sediment samples as substrates for fungal growth. The fungus was grown as described in Bosiljcic's (2008).

To the treatments with sawdust, fungi (*Pleurotus ostreatus*) (10% by volume) was added and an additional nitrogen source (10% by volume), to stimulate fungal growth.

A treatment-free sediment sample was taken as control. *β*-cyclodextrin (1g/100g sediment) (1%) was added to some sediment samples to determine its effect on PAH extraction and bioavailability. The incubations were set up at 25^{o}C. Sampling was done on days 0, 21, and 42 days and extracted via sonication. Triplicate incubations (runs 1, 2 and 3) were set up. The treatments are listed in Table 1

Table 1. Sample treatments

1.	Control
2.	Control (mix)
3.	Sediment+cyclodextrin(25 ^{o}C)
4.	Sediment+cyclodextrin(25 ^{o}C) (Mix)
5.	Sawdust only (60%)
6.	Sawdust (Mix)
7.	Sawdust+ N
8.	Sawdust+cyclodextrin
9.	Sawdust+cyclodextrin(Mix)
10.	Sawdust (60%) + Fungi (10%)
11.	Sawdust+Fungi (Mix)
12.	Sawdust+Fungi+N
13.	Shredded Paper only (60%)
14.	Shredded Paper (Mix)
15.	Paper (60%) +Fungi (10%)
16.	Paper+Fungi (Mix)
17.	Sawdust+cyclodextrin+Fungi
18.	Sawdust+cyclodextrin+Fungi (Mix)
19.	Sawdust+cyclodextrin+Fungi+N

2.2.2. Procedures for PAH extraction:

Lipid extraction procedure:

All the samples were run in triplicate. PAHs were extracted using a modified lipid extraction method for simultaneous recovery of organic pollutants and microbial lipids from sediments (Fang and Findlay, 1996) based on the Bligh and Dyer method (figure 2). A mixture of dichloromethane (DCM), methanol, phosphate buffer (pH 7.4) and a surrogate solution were added to the samples, mixed well and extracted (Detailed procedure in Appendix 1 and 3).

The organic phase (DCM) containing PAHs was collected and purified over sodium sulfate columns. The sample was further concentrated by evaporating the solvent.

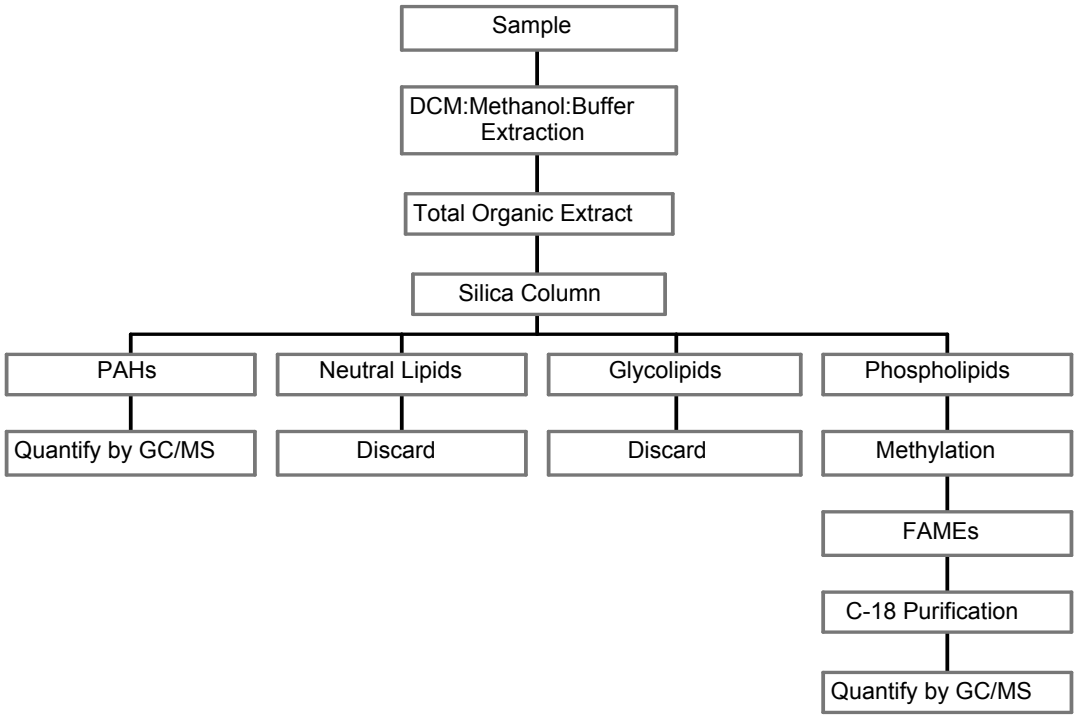

Fig.18 PAH extraction procedure flow chart (based on Fang and Findlay, 1996)

Sonication :

All the runs were done in triplicate for comparision of extraction efficiencies of the two methods. Anhydrous sodium sulfate was added to the samples to drive the moisture out and a mixture of surrogate and DCM were added. The samples were further sonicated thrice at room temperature for every 20 minutes (Detailed extraction procedure in Appendix 1H. The samples were purified over sodium sulfate columns and were concentrated by evaporating the solvent.

Purification:

Both the samples from sonication and lipid extraction procedure were concentrated and passed over silica columns. Any unwanted sediment waste adsorbs onto the activated silica thus purifying the samples. The samples were further purified twice

over the amino propyl columns and analyzed over the GC-MS after the internal standard was added.

Analysis of PAHs:

The PAH fractions were analyzed on a Hewlett Packard 5890 Gas Chromatograph/ 5989A Mass Spectrometer equipped with a DB-5 column (30 m, 0.32 mm ID, and a HP 6890 Series Injector.

Program for PAH analysis:

The injector and the detector temperatures were set at 250 ^{0}C and 300 ^{0}C respectively. The oven temperature was held at 45°C for 2 minutes, and then ramped at 20 ^{0}C per minute to 310°C. The final temperature was held for 5.5 minutes.

2.2 Soil characteristics:

Particle Size Distribution:

A hydrometer method (Fisher Environmental) was used to analyze the particle size (Acharya, 2008).

Total Organic Carbon:

Sediments dried at 105°C were weighed into aluminum boats and combusted at 550°C for 24 hours then weighed again (Acharya, 2008; Tiessen and Moir, 1993).

$$\frac{\text{Dry Sediment Weight-Combusted Sediment Weight}}{\text{Dry Sediment Weight}} \times 100$$

Percent Moisture:

Sediments were weighed into aluminum boats and dried in an oven at 105°C for 24 hours, then weighed again (Acharya, 2008).

Percent moisture was determined by:

$$\frac{\text{Wet Sediment Weight-Dry Sediment Weight}}{\text{Wet Sediment Weight}} \times 100$$

pH

USEPA Method's 9040C and 9045C were followed (USEPA, 1996). Student t-test with unequal variance ($p=0.05$) was used to determine differences between sites (Acharya, 2008).

Water Holding Capacity

Dried sediments (50mL) were place into a 600 mL beaker and Millipore water was added until saturation (Acharya, 2008).

Water holding capacity was calculated by:

$$\frac{\text{volume water}}{\text{sediment volume}} \times 100 = \%\ \text{water holding capacity}$$

Chapter 3: Results and Discussion

3.1. Comparision of extraction methods of sonication and lipid extraction

Twelve PAHs were extracted by two methods, sonication and lipid extraction. Lipid extraction appeared to be more efficient but sonication was preferred as it is less time consuming and does not require many solvents. Dichloromethane was used as the solvent for extraction but based on recent findings, it was observed that sonication when performed with an efficient solvent system (1:1 DCM: Acetone: Hexane) could bring out productive results for PAH extraction (Simpson S.L. et al., 2006).

3.2. Choosing β -Cyclodextrin concentration for incubation:

β - cyclodextrin was chosen as the solubility-enhancing agent for PAH removal from the contaminated sediment. Three concentrations (1%, 1.8%, 2. 0%) were chosen and extracted with the sediment at time zero to test their effects.

Most of the studies done on the cyclodextrins showed that they had a positive effect on PAH degradation after an incubation period (Fenyvesi E, et al., 2005). So, the differences in the PAH concentrations seen in table 2 which were run at time zero can be considered as variations within the samples. Most of the literature claimed a concentration range of 0.8 % - 2.0% to possess solubility-enhancing capabilities (Fabio Fava et al., 1998), so we chose a lower concentration (1 %) in this range for practical reasons.

Table 2. Effect of different concentrations of *β*- cyclodextrin on PAH extraction

Target Compounds	Conc (ug/gm) Control (without cyclodextrin)	Conc (ug/gm) 1% cyclodextrin	Conc (ug/gm) 1.8% cyclodextrin	Conc (ug/gm) 2.4% cyclodextrin
Naphthalene	19.01	9.12	7.39	8.97
Acenaphthylene	4.14	2.68	2.35	3.30
Acenaphthene	8.19	0.00	0.00	0.00
Fluorene	13.50	7.31	5.51	7.97
Phenanthrene	36.41	21.94	17.26	18.30
Anthracene	14.12	8.45	7.19	7.52
Fluoranthene	91.47	55.42	45.99	54.09
Pyrene	74.25	41.99	32.12	36.37
Benzo(a) anthracene	7.89	5.22	4.60	5.33
Chrysene	7.92	5.20	4.56	5.29
Benzo(b&k) fluoranthene	9.78	8.01	7.52	8.10
Benzo(a) pyrene	13.05	4.56	10.18	10.31
Dibenz(ah) anthracene	3.55	4.05	5.58	4.69
Indeno(1,2,3-cd) pyrene	2.99	3.01	0.00	0.00
Benzo(ghi) perylene	5.56	5.02	4.83	5.53

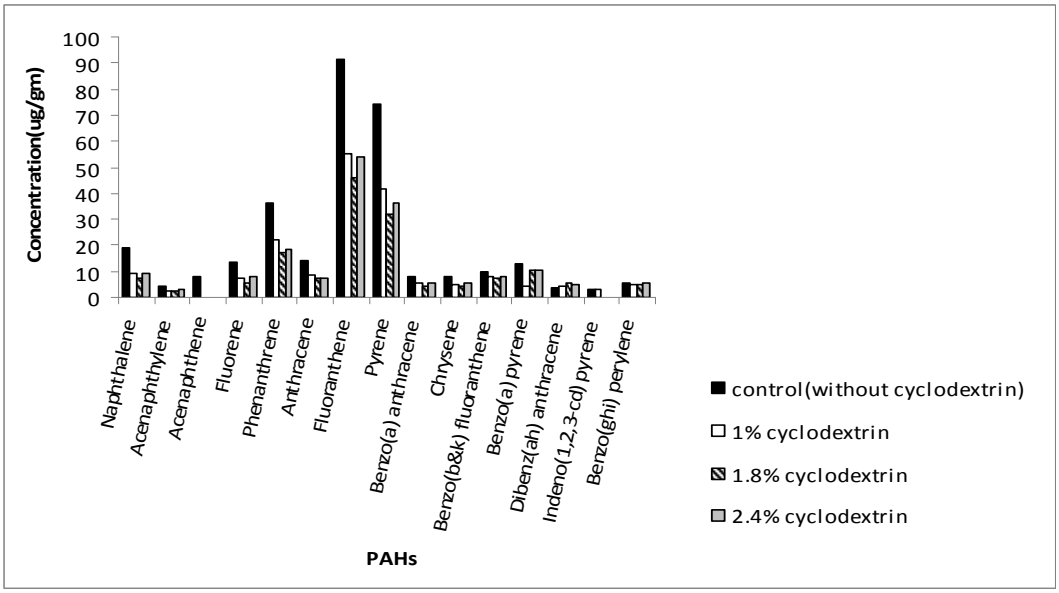

Fig.19 Effect of β-cyclodextrin concentrations on PAH extraction

3.3. Biodegradation of PAHs:

Incubations were set up over a period of 42 days at 25 ^{o}C and three runs were performed to confirm the PAH degradation patterns. The sediment samples were treated with certain

amendments which are believed to improve the biodegradation rates. β - cyclodextrin was added to improve the transfer of PAHs from organic to aqueous phases thus making them accessible to white rot fungi. Saw dust substrate and nitrogen supplements were added for fungal growth. A treatment-free control was also included in the runs to check the results without any amendments.

The data is shown as a comparision of degradation patterns of low and high molecular weight PAHs (ug PAHs/ gm sediment dry weight).

PAHs can be classified based on differences in their molecular weights.

Table 3. Low and high molecular weight PAHs

LOW MOLECULAR WEIGHT PAHs	HIGH MOLECULAR WEIGHT PAHs
Naphthalene – 2 rings	Chrysene – 4 rings
Acenaphthylene – 3 rings	Pyrene – 4 rings
Acenaphthene – 3 rings	Benzo(a)anthracene – 4 rings
Fluorene – 3 rings	Fluoranthene – 5 rings
Phenanthrene – 3 rings	Benzo(b&k)fluoranthene – 5 rings
Anthracene – 3 rings	

3.3.1. Low Molecular Weight PAHs (LMW PAHs):

The LMW PAHs are 2/3 ring organic compounds which are hydrophobic and can be degraded by bacteria (Bouchez M, et al., 1999). Napthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene can be classified under LMW category.

3.3.1.1. Biodegradation of total low molecular weight PAHs (LMW):

Run 1:

On day 21 the degradation of the total LMW PAHs was negative in some of the treatments (range -21% to -31%), and positive in others (range 1% to 36%). On day 42 most of them showed negative (range -63% to -154%) and few of the PAHs showed positive value (range 20% to 63%). These negative numbers could be explained by sample variation.

The control showed negative degradation initially (-21 %) and showed no change by the 42nd day (0%) which could be attributed to the activity of the indigenous microbes. The same pattern was followed by β -cyclodextrin treated samples which could be explained by the low initial availability (slow desorption rates) of PAHs. (Allan I. J, et al., 2007) or by the limited solubility of β- cyclodextrin (Hanna K. et al., 2003), or a combination of both.

The total low molecular weight PAHs in the sawdust-treated samples showed negative degradation after 21 days (-32%), but a positive degradation (41%-55%) after 42 days. Amendments with nitrogen brought about 53 % degradation by the end of incubation (day 42). Sawdust-treated samples with the addition of β -cyclodextrin showed a PAH degradation of 20%-38%.

The paper-treated samples showed an increase from -63% to 25% on mixing and amendments with fungi led to a degradation of 40% - 59% in the paper-treated sample augmented with fungi. Proper mixing of the sediment can improve PAH degradation by indigenous aerobes and, fungi, in case of fungi-augmented samples.

The LMW PAHs showed a degradation of 39%-50% in treatments amended with

sawdust and fungi. On further amendment with nitrogen the degradation increased to 63%. This is in agreement with the conclusion of Boyle et al (2006) that even low nitrogen levels amplify the degradative activities of white rot fungi. The LMW PAHs degraded in a similar way with both carbon substrates (paper and sawdust). This could be attributed to the synergistic action of fungi and indigenous microbes. The PAH degradation by fungi is assumed to be initiated by extracellular lignolytic enzymes (Hammel et al., 1986 and Barr and Aust 1994). The partially oxidized hydrocarbon intermediates are believed to be available in the extracellular space and may be further degraded by the soil microorganisms. Tilling increased the degradation from 40% to 59% in this treatment, resulting in the highest degradation of the total LMW PAHs. The effect of mixing boosted the PAH degradation by an additional 15% in the samples sawdust, sawdust + β -cyclodextrin, paper and paper-treatment augmented with fungi. Tilling effect could have produced a positive impact on the degradation of fungi-augmented treatments by allowing proper fungal growth.

Table 4. Run 1: Biodegradation of total low molecular weight PAHs

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run1 (%Biodegradation) 21days	Conc (ug/gm) 42 days	Run1 (% Biodegradation) 42 days
Control	84	102	-21	84	0
Control (mix)	84	102	-21	213	-154
Sediment+cyclodextrin(25 ^{o}C)	101	133	-32	156	-54
Sediment+cyclodextrin(25 ^{o}C) (Mix)	101	133	-32	177	-75
Sawdust only	95	124	-31	43	55
Sawdust (Mix)	95	124	-31	56	41
Sawdust+N	95	75	20	45	53
Sawdust+cyclodextrin	116	115	1	93	20
Sawdust+cyclodextrin(Mix)	116	115	1	72	38
Sawdust+Fungi	104	87	16	64	39
Sawdust+Fungi (Mix)	104	87	16	51	50
Sawdust+Fungi+N	104	103	1	39	63
Paper only	110	86	22	179	-63
Paper (Mix)	110	86	22	83	25
Paper+Fungi	127	110	14	76	40
Paper+Fungi (Mix)	127	110	14	52	59
Sawdust+cyclodextrin+Fungi	127	81	36	65	49
Sawdust+cyclodextrin+Fungi (Mix)	104	87	16	52	50
Sawdust+cyclodextrin+Fungi+N	127	88	31	65	49

Run 2:

On day 21 the degradation of the total LMW PAHs was negative in some of the treatments (-4% to -69%), and positive in others (1% to 45%). On day 42 the control showed degradation of 42%. A similar degradation pattern was seen in samples amended with β -cyclodextrin. Tilling had a positive impact on the PAH degradation by 8% - 21%.

The sawdust-treated sediments showed an increase of the total LMW PAH degradation from 1% after 21 days to 35% after 42 days. Nitrogen amendments brought about an additional increase of 13% bringing the total to 47% degradation.

β -cyclodextrin additions gave similar results like in the control, again with a positive effect of tilling. 47% of LMW PAHs were degraded in sawdust-treated sediment which was augmented with fungi. Nitrogen addition brought the degradation to 64%. No

difference was observed between the two carbon substrates paper and sawdust as both of them show similar degradation rates.

Sawdust-treated sediments amended with β -cyclodextrin, fungi and nitrogen did not show a difference in the degradation of the various LMW PAHs.

The highest degradation rates of LMW PAHs (64%) for this run were observed in samples amended with sawdust, fungi and nitrogen.

Table 5. Run 2: Biodegradation of total low molecular weight PAHs

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run2 (%Biodegradation) 21 days	Conc (ug/gm) 42 days	Run2 (%Biodegradation) 42 days
Control	111	116	-4	64	42
Control (mix)	111	116	-4	61	46
Sediment+cyclodextrin(25 ^{o}C)	77	131	-69	71	8
Sediment+cyclodextrin(25 ^{o}C) (Mix)	77	131	-69	61	21
Sawdust only	96	95	1	63	35
Sawdust (Mix)	96	95	1	64	34
Sawdust+N	96	67	31	51	47
Sawdust+cyclodextrin	82	107	-30	46	44
Sawdust+cyclodextrin(Mix)	82	107	-30	42	49
Sawdust+Fungi	110	98	11	58	47
Sawdust+Fungi (Mix)	110	98	11	66	39
Sawdust+Fungi+N	110	60	45	39	64
Paper only	104	96	8	66	36
Paper (Mix)	104	96	8	70	32
Paper+Fungi	109	88	19	63	42
Paper+Fungi (Mix)	109	88	19	57	48
Sawdust+cyclodextrin+Fungi	98	91	8	54	45
Sawdust+cyclodextrin+Fungi (Mix)	98	91	8	61	38
Sawdust+cyclodextrin+Fungi+N	98	92	7	54	45

RUN 3:

On day 21 the degradation of the total LMW PAHs was negative in some of the treatments (-18% to - 43%) and positive in others (6% to 60%). The highest degradation (60%) in this run was shown in the sawdust-treated samples amended with β -cyclodextrin which is different from runs 1 and 2.

For Run 3 there is no data available for day 42 due to technical difficulties with the GC-MS.

Table 6. Run 3: Biodegradation of total low molecular weight PAHs

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run3 (% Biodegradation) 21 days
Control	123	96	22
Control (mix)	123	96	22
Sediment+cyclodextrin(25 ^{o}C)	127	119	6
Sediment+cyclodextrin(25 ^{o}C) (Mix)	127	119	6
Sawdust only	79	NA*	NA*
Sawdust (Mix)	79	NA*	NA*
Sawdust+N	79	75	5
Sawdust+cyclodextrin	135	54	60
Sawdust+cyclodextrin(Mix)	135	54	60
Sawdust+Fungi	91	111	-22
Sawdust+Fungi (Mix)	91	111	-22
Sawdust+Fungi+N	91	89	3
Paper only	72	104	-43
Paper (Mix)	72	104	-43
Paper+Fungi	115	95	17
Paper+Fungi (Mix)	115	95	17
Sawdust+cyclodextrin+Fungi	71	84	-18
Sawdust+cyclodextrin+Fungi (Mix)	71	84	-18
Sawdust+cyclodextrin+Fungi+N	71	NA*	NA*

NA* - data not available due to technical difficulties.

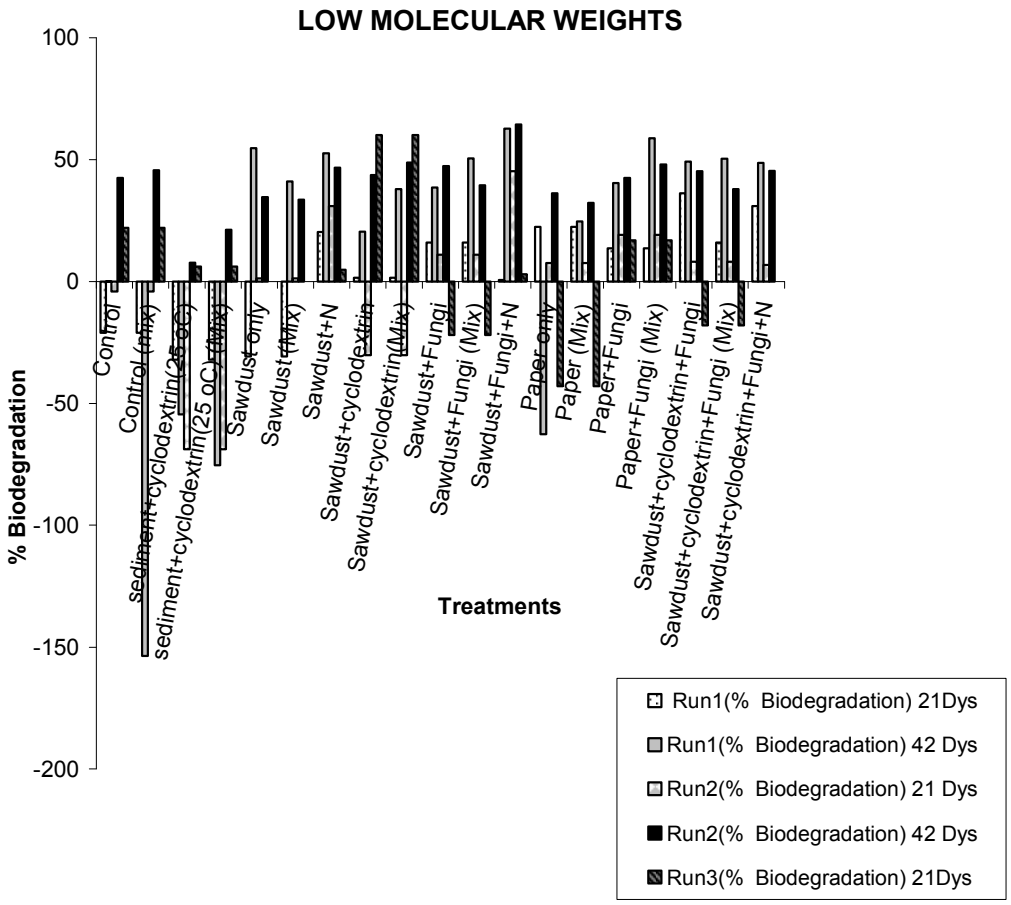

Fig.20 Degradation of total low molecular weight PAHs based on treatment

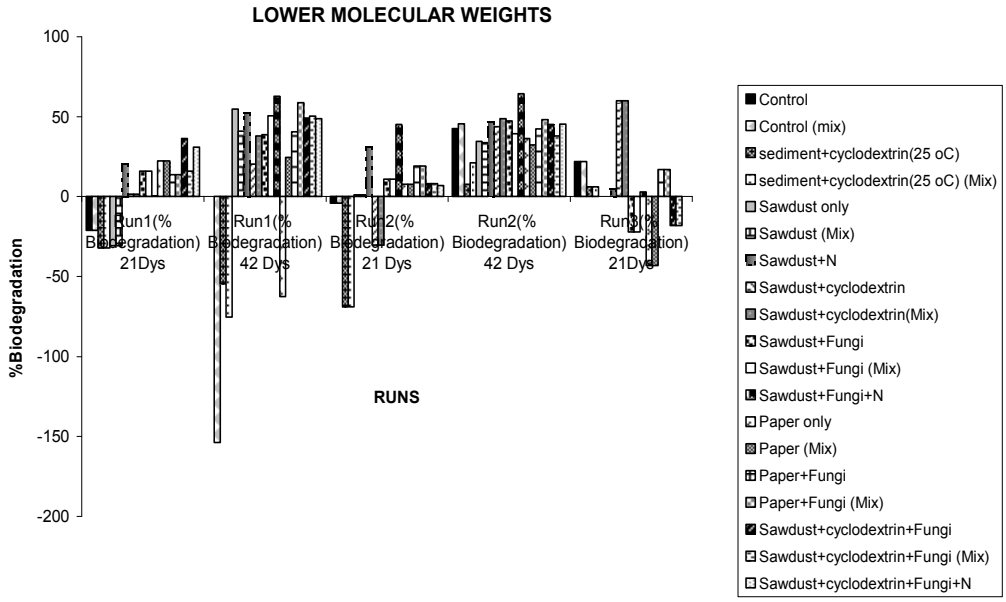

Fig. 21 Degradation of total low molecular weight PAHs based on run

3.3.1.2. Biodegradation of Naphthalene:

Naphthalene was chosen as a representative compound to show the degradation of the LMW PAHs.

Run 1:

On day 21 the degradation of naphthalene was negative in some of the treatments (-7% to -267%), and positive in others (5% to 54%). On day 42 naphthalene degradation followed negative for some treatments(-15% to -267%) and positive in others (5%-54%). The highest degradation (54%) was shown in the sawdust-treated sample amended with nitrogen and in the sawdust-treated sample amended with β -cyclodextrin, fungi and nitrogen.

By day 42, positive degradation was observed in the control, the sawdust-only and all the nitrogen and fungi amended samples. However, the highest naphthalene removal (56%) was seen in sample treated with sawdust, β -cyclodextrin, fungi and nitrogen.

Table 7. Run1: Biodegradation of naphthalene

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run1 (% Biodegradation) 21 days	Conc (ug/gm) 42 days	Run1 (% Biodegradation) 42 days
Control	21	30	-46	20	5
Control (mix)	21	30	-46	76	-267
Sediment+cyclodextrin(25 ^{o}C)	24	45	-90	48	-100
Sediment+cyclodextrin(25 ^{o}C) (Mix)	24	45	-90	54	-128
Sawdust only	24	45	-92	16	30
Sawdust+N	24	22	6	11	54
Sawdust+cyclodextrin	32	30	5	24	24
Sawdust+cyclodextrin(Mix)	32	30	5	36	-15
Sawdust+Fungi	25	27	-7	14	44
Sawdust+Fungi (Mix)	25	27	-7	19	26
Sawdust+Fungi+N	25	33	-30	16	34
Sawdust+cyclodextrin+Fungi	32	24	24	14	54
Sawdust+cyclodextrin+Fungi+N	32	28	11	14	56

Run 2:

On day 21 the degradation of naphthalene was negative in some of the treatments (-14% to -164%), and positive in others (7% to 48%). On day 42 naphthalene followd a negative value in some treatments (-16% to 0%) and positive in others (7% to 43%). The highest degradation (43%) was observed in sawdust-treated samples amended with nitrogen and in the sawdust-treated samples amended with fungi and nitrogen. A negative degradation was observed in the other treatments.

Table 8. Run 2: Biodegradation of naphthalene

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run2 (% Biodegradation) 21 days	Conc (ug/gm) 42 days	Run 2 (% Biodegradation) 42 days
Control	24	34	-39	13	48
Control (mix)	24	34	-39	15	39
Sediment+cyclodextrin(25 ^{o}C)	15	40	-164	14	7
Sediment+cyclodextrin(25 ^{o}C) (Mix)	15	40	-164	15	0
Sawdust only	21	25	-20	13	39
Sawdust+N	21	17	16	12	43
Sawdust+cyclodextrin	14	20	-40	13	8
Sawdust+cyclodextrin(Mix)	14	20	-40	12	17
Sawdust+Fungi	15	19	-29	14	10
Sawdust+Fungi (Mix)	15	19	-29	18	-16
Sawdust+Fungi+N	15	12	18	11	24
Sawdust+cyclodextrin+Fungi	16	18	-15	13	16
Sawdust+cyclodextrin+Fungi+N	16	18	-14	13	18

Run 3:

On day 21 the degradation of naphtalene was negative in some of the treatments (-44% to -174%), and positive in others (37% to 67%). The highest degradation (67%) was observed in sawdust-treated samples amended with β -cyclodextrin, followed by (60%) degradation (highest) of naphthalene for sawdust-treated samples amended with β-cyclodextrin, 55% in sawdust-treated samples amended with nitrogen, and 48% in the control and sawdust-treated samples amended with β -cyclodextrin and fungi.

Table 9. Run 3: Biodegradation of naphthalene

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run 3 (%Biodegradation) 21 days
Control	50	31	37
Control (mix)	50	31	37
Sediment+cyclodextrin(25 ^{o}C)	32	53	-66
Sediment+cyclodextrin(25 ^{o}C) (Mix)	32	53	-66
Sawdust only	30	NA*	NA*
Sawdust+N	30	13	57
Sawdust+cyclodextrin	42	14	67
Sawdust+cyclodextrin(Mix)	42	14	67
Sawdust+Fungi	17	47	-174
Sawdust+Fungi (Mix)	17	47	-174
Sawdust+Fungi+N	17	28	-64
Sawdust+cyclodextrin+Fungi	19	27	-44
Sawdust+cyclodextrin+Fungi+N	19	12	37

NA* - data not available due to technical difficulties.

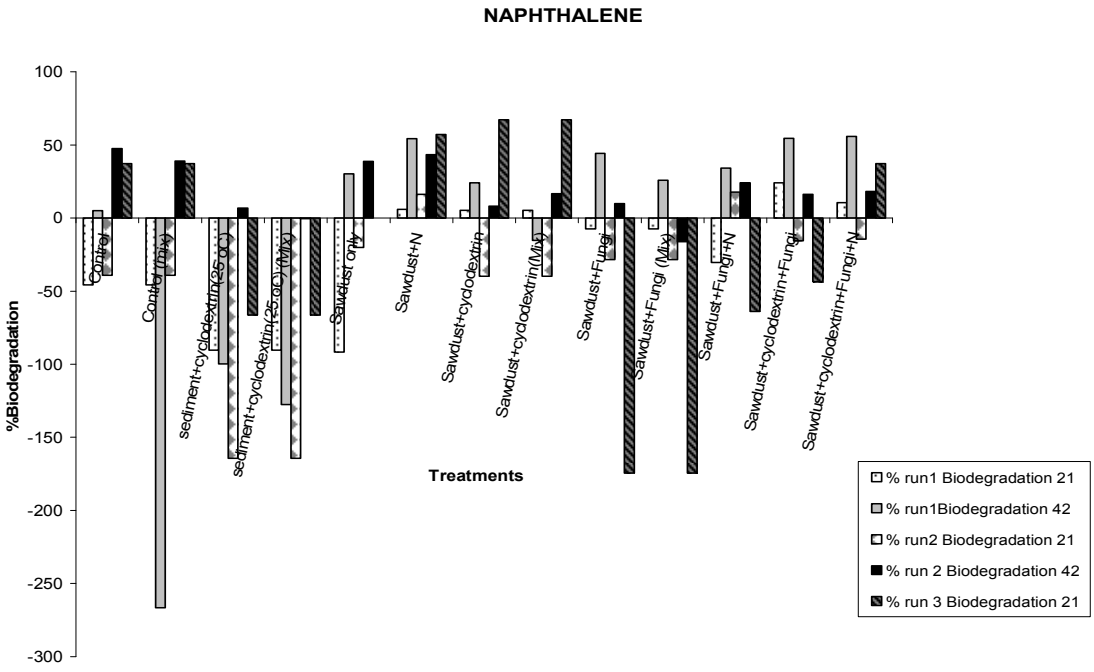

Fig.22 Degradation of naphthalene based on treatment

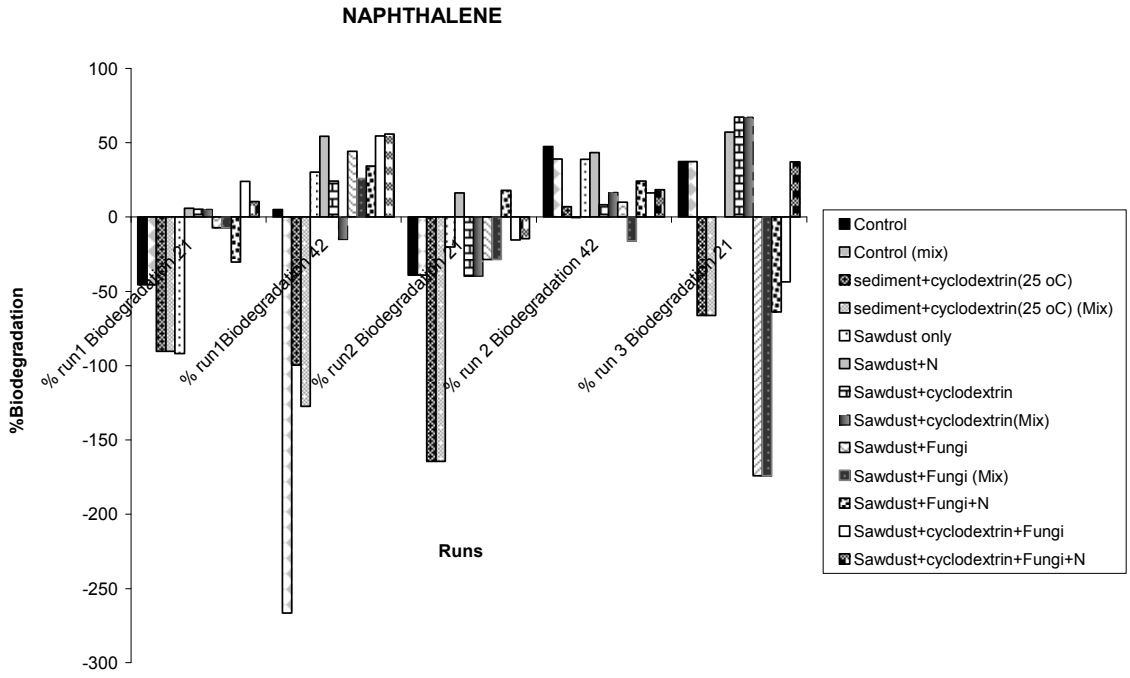

Fig. 23 Degradation of naphthalene based on run

3.3.1.3. Biodegradation of Fluorene:

Fluorene was chosen as a representative compound to show the degradation of the LMW PAHs.

<u>Run 1</u>:

On day 21 the degradation of fluorene was negative in some of the treatments (-1% to -112%), and positive in others (8% to 78%). On day 42 fluorene followed a negative value (-4% to -112%) and a positive value (42% to 78%).The highest degradation (78%) was observed in sawdust-treated samples, their amendments with nitrogen (76%) and augmentations with fungi and β -cyclodextrin (76%).

Table 10. Run 1: Biodegradation of fluorene

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run1 (%Biodegradation) 21days	Conc (ug/gm) 42 days	Run1 (%Biodegradation) 42 days
Control	12	13	-7	13	-7
Control (mix)	12	13	-7	26	-112
Sediment+cyclodextrin(25 ^{o}C)	13	20	-46	22	-61
Sediment+cyclodextrin(25 ^{o}C) (Mix)	13	20	-46	26	-97
Sawdust only	13	13	-1	3	78
Sawdust+N	13	9	33	6	55
Sawdust+cyclodextrin	14	19	-32	15	-4
Sawdust+cyclodextrin(Mix)	14	19	-32	4	76
Sawdust+Fungi	14	10	29	8	42
Sawdust+Fungi (Mix)	14	10	29	4	70
Sawdust+Fungi+N	14	13	8	3	76
Sawdust+cyclodextrin+Fungi	17	10	42	9	45
Sawdust+cyclodextrin+Fungi+N	17	11	33	9	49

Run 2:

On day 21 the degradation of fluorene was negative in some of the treatments (-1% to -49%), and positive in others (3% to 39%). Fluorene showed highest degradation (39%) in sawdust-treated samples augmented with fungi and nitrogen, and sawdust-treated samples amended with nitrogen (28%), and sawdust, β -cyclodextrin, fungi and nitrogen (15%).

By day 42, fluorene showed a positive degradation in all the treatments. The highest degradation (60%) was observed for the sawdust-treated sample augmented with fungi and nitrogen and the sawdust-treated sample amended with β -cyclodextrin (58%).

Table 11. Run 2: Biodegradation of fluorene

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run2 (% Biodegradation) 21 days	Conc (ug/gm) 42 days	Run2 (% Biodegradation) 42 days
Control	20	19	6	13	37
Control (mix)	20	19	6	9	54
Sediment+cyclodextrin(25 ^{o}C)	13	20	-49	11	16
Sediment+cyclodextrin(25 ^{o}C) (Mix)	13	20	-49	9	31
Sawdust only	16	16	-1	11	34
Sawdust+N	16	11	28	6	62
Sawdust+cyclodextrin	14	18	-33	6	57
Sawdust+cyclodextrin(Mix)	14	18	-33	5	67
Sawdust+Fungi	17	16	3	9	48
Sawdust+Fungi (Mix)	17	16	3	8	52
Sawdust+Fungi+N	17	10	39	5	71
Sawdust+cyclodextrin+Fungi	16	17	-2	8	53
Sawdust+cyclodextrin+Fungi+N	16	15	10	7	58

Run 3:

In Run 3, fluorene showed a positive degradation in all the treatments except the control which was unchanged. The highest degradation (98%) was observed in the sawdust-treated sample and in the sawdust-treated samples amended with β -cyclodextrin (60%) and with fungi and nitrogen (40%).

Table 12. Run 3: Biodegradation of fluorene

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run3 (% Biodegradation) 21days
Control	14	14	0
Control (mix)	14	14	0
Sediment+cyclodextrin(25 ^{o}C)	17	13	25
Sediment+cyclodextrin(25 ^{o}C) (Mix)	17	13	25
Sawdust only	11	NA*	NA*
Sawdust+N	11	10	8
Sawdust+cyclodextrin	19	6	66
Sawdust+cyclodextrin(Mix)	19	6	66
Sawdust+Fungi	14	11	22
Sawdust+Fungi (Mix)	14	11	22
Sawdust+Fungi+N	14	9	34
Sawdust+cyclodextrin+Fungi	11	11	0
Sawdust+cyclodextrin+Fungi+N	11	8	24

NA* - data not available due to technical difficulties.

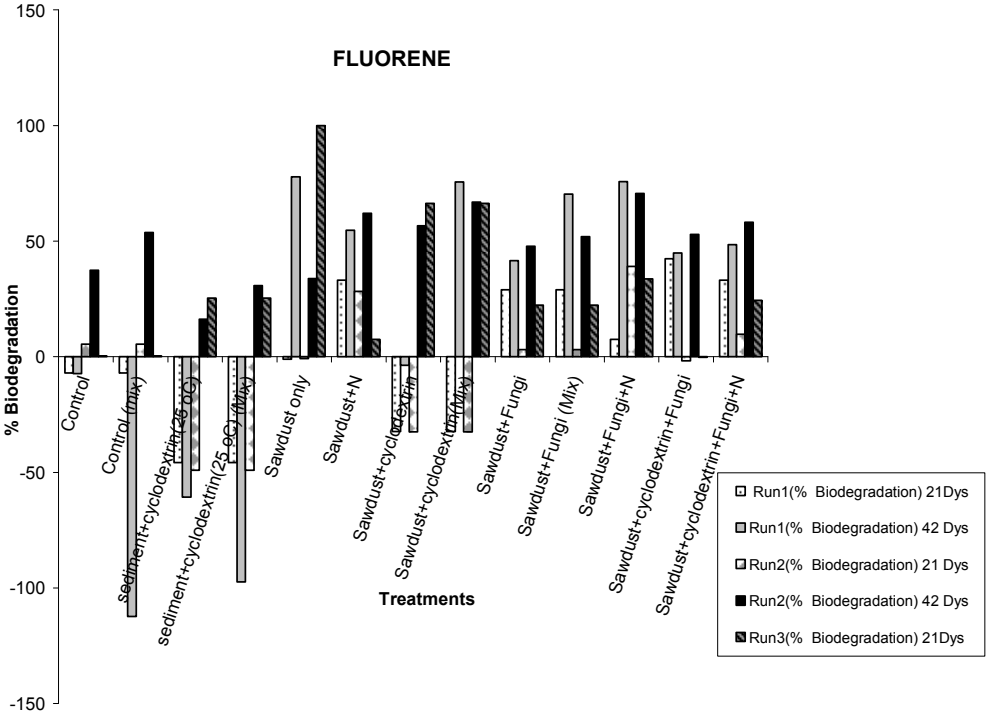

Fig. 24 Degradation of fluorene based on treatment

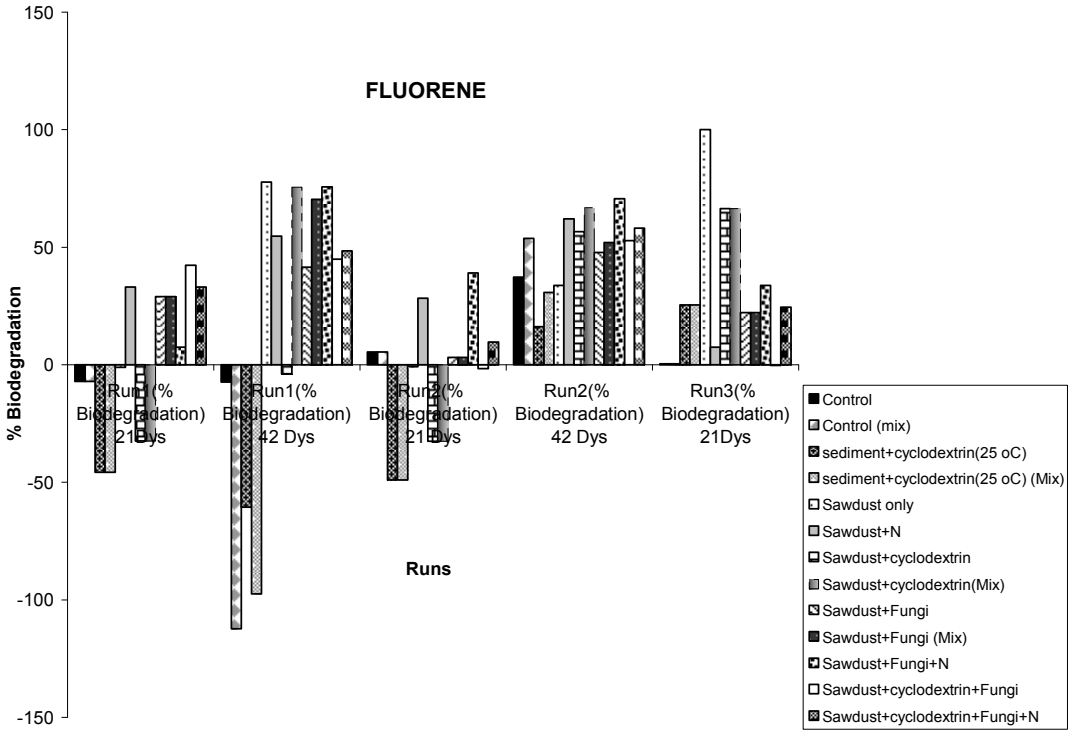

Fig. 25 Degradation of fluorene based on run

3.3.2. High Molecular Weight PAHs (HMW PAHs):

The HMW PAHs are organic compounds with 4 or more aromatic rings which makes them strongly hydrophobic. Pyrene, fluoranthene, chrysene, benzo(a)anthracene, benzo(b,k)fluoranthene are classified in this category.

3.3.2.1. Biodegradation of total high molecular weight PAHs (HMW):

<u>Run 1</u>:

On day 21 the degradation of the total HMW PAHs was negative in some of the treatments (-13% to -70%), and positive in others (11% to 29%). On day 42 it showed a negative value (-2% to -138%) for some treatments and a positive value (5% to 47%).

There was no degradation in the control and the *β*-cyclodextrin amended samples which could be explained by the fact that these compounds are very hydrophobic and therefore less available for microbial degradation.

In contrast to the LMW PAHs, the HMW PAHs showed little or no degradation (-18 to 7%) in sawdust-treated samples. Nitrogen supplementation did not have an effect on the degradation. These limited biodegradation rates can be attributed to low availability (i.e., slow desorption rates) in the soil rather than a lack of microorganisms capable of their degradation (Huesemann, M. H. et al., 2003). The sawdust-treated samples amended with β-cyclodextrin exhibited a high degradation of 45% which could be due to the increased availability of PAHs.

Sawdust-treated samples augmented with fungi showed no change on day 21 but with tilling, a degradation of 47% was observed on day 42. Amendments with nitrogen gave more or less the same result (31%).

The treatments with paper as substrate for fungal growth showed positive degradation (29%) by day 21, but negative degradation (-98%) by day 42.

Sawdust-treated samples augmented with *β*-cyclodextrin and fungi showed a positive degradation of 31% by day 42 which increased to 47% with mixing. Nitrogen additions however did not result in any greater degradation.

Table 13. Run 1: Biodegradation of high molecular weight PAHs

Treatments	Conc (ug/gm) 0 days	Conc (ug/gm) 21 days	Run1 (%Biodegradation) 21days	Conc (ug/gm) 42 days	Run1 (%Biodegradation) 42 days
Control	137	188	-38	179	-31
Control (mix)	137	188	-38	325	-138
Sediment+cyclodextrin(25 ^{o}C)	181	232	-28	249	-38
Sediment+cyclodextrin(25 ^{o}C) (Mix)	181	232	-28	290	-60
Sawdust only	159	187	-18	147	7
Sawdust (Mix)	159	187	-18	151	5
Sawdust+N	159	132	17	163	-2
Sawdust+cyclodextrin	192	234	-22	183	5
Sawdust+cyclodextrin(Mix)	192	234	-22	105	45
Sawdust+Fungi	169	192	-13	161	5
Sawdust+Fungi (Mix)	169	192	-13	90	47
Sawdust+Fungi+N	169	211	-25	118	31
Paper only	192	136	29	379	-98
Paper (Mix)	192	136	29	140	27
Paper+Fungi	124	211	-70	171	-38
Paper+Fungi (Mix)	124	211	-70	154	-25
Sawdust+cyclodextrin+Fungi	220	197	11	152	31
Sawdust+cyclodextrin+Fungi (Mix)	169	192	-13	90	47
Sawdust+cyclodextrin+Fungi+N	220	180	18	153	31

.

Run 2:

On day 21 the degradation of the total HMW PAHs was negative in some of the treatments (-31% to -43%) and positive in others (6% to 42%). On day 42, it showed a negative value (-2%) for just one treatment- control amended with β -cyclodextrin and positive value (18% to 54%) for the other treatments.The degradation was greater in most treatments compared to Run 1. The highest degradation (54%) was observed in sawdust-treated samples and their nitrogen amendments.

The control with and without β -cyclodextrin showed a positive degradation (20%). Tilling increased the degradation to 35 %.

Nitrogen amendments did not have an effect of the HMW PAH degradation rates.

Sawdust treatments amended with β-cyclodextrin enhanced PAH removal from

-31% to 38%. Sawdust treated samples augmented with fungi gave a 50% degradation of HMW PAHs. Nitrogen amendments did not further increase the degradation. These results support the thesis that biodegradation of HMW PAHs can be increased in the presence of substrates like sawdust, white rot fungi (*Pleurotus ostreatus*) and nitrogen (Pignatello, Li, J. et al., 2005). No final conclusions can be drawn for paper as substrate as there were too many variations between runs.

Table 14. Run 2: Biodegradation of total high molecular weight PAHs

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run2 (% Biodegradation) 21 days	Conc (ug/mg) 42 days	Run2 (%Biodegradation) 42 days
Control	249	248	0	189	24
Control (mix)	249	248	0	163	35
Sediment+cyclodextrin(25 ^{o}C)	182	261	-43	186	-2
Sediment+cyclodextrin(25 ^{o}C) (Mix)	182	261	-43	145	20
Sawdust only	216	199	8	153	30
Sawdust (Mix)	216	199	8	159	26
Sawdust+N	216	148	31	153	29
Sawdust+cyclodextrin	189	249	-31	118	38
Sawdust+cyclodextrin(Mix)	189	249	-31	124	35
Sawdust+Fungi	252	237	6	125	50
Sawdust+Fungi (Mix)	252	237	6	168	33
Sawdust+Fungi+N	252	145	42	116	54
Paper only	250	201	19	162	35
Paper (Mix)	250	201	19	204	18
Paper+Fungi	255	192	25	156	39
Paper+Fungi (Mix)	255	192	25	156	39
Sawdust+cyclodextrin+Fungi	246	210	15	134	45
Sawdust+cyclodextrin+Fungi (Mix)	246	210	15	164	33
Sawdust+cyclodextrin+Fungi+N	246	216	12	156	36

Run 3:

On day 21 the degradation of the total HMW PAHs was negative in some of the treatments (-2% to -8%), and positive in others (3% to 53%). The highest degradation (53%) was observed in the sawdust-treated samples amended with β -cyclodextrin. Most HMW PAHs showed very little or no degradation in this run. However, no final conclusions can be drawn from this run since no data from day 42 are available.

Table 15. Run 3: Biodegradation of total high molecular weight PAHs

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run3 (% Biodegradation) 21days
Control	123	96	22
Control (mix)	123	96	22
Sediment+cyclodextrin(25 ^{o}C)	127	119	6
Sediment+cyclodextrin(25 ^{o}C) (Mix)	127	119	6
Sawdust only	79	NA*	NA*
Sawdust (Mix)	79	NA*	NA*
Sawdust+N	79	75	5
Sawdust+cyclodextrin	135	54	60
Sawdust+cyclodextrin(Mix)	135	54	60
Sawdust+Fungi	91	111	-22
Sawdust+Fungi (Mix)	91	111	-22
Sawdust+Fungi+N	91	89	3
Paper only	72	104	-43
Paper (Mix)	72	104	-43
Paper+Fungi	115	95	17
Paper+Fungi (Mix)	115	95	17
Sawdust+cyclodextrin+Fungi	71	84	-18
Sawdust+cyclodextrin+Fungi (Mix)	71	84	-18
Sawdust+cyclodextrin+Fungi+N	71	NA*	NA*

NA* - data not available due to technical difficulties.

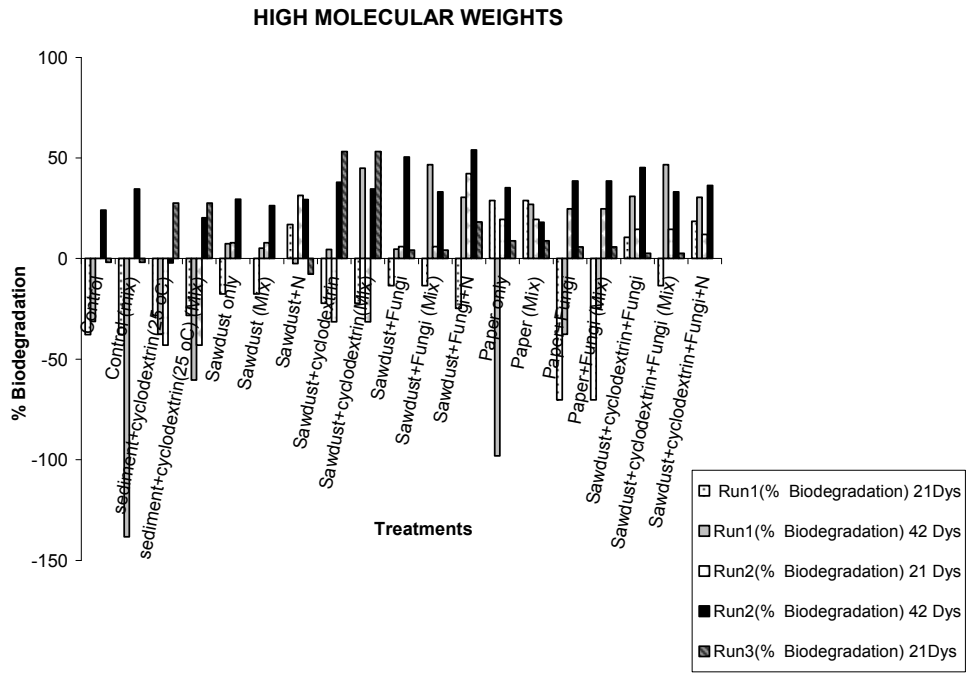

Fig. 26 Degradation of total high molecular weight PAHs based on treatment

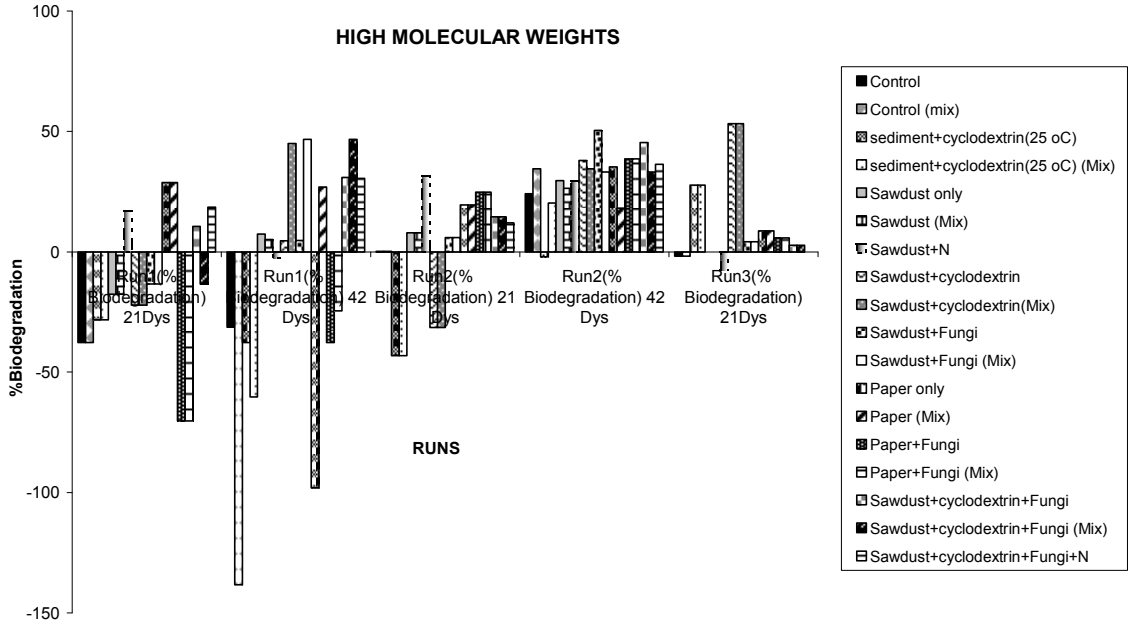

Fig. 27 Degradation of total high molecular weight PAHs based on run

3.3.2.2. Biodegradation of Fluoranthene:

Fluoranthene is a high molecular weight PAH with five rings. Fluoranthene was chosen as a representative compound to show the degradation of the HMW PAHs. Pyrene behaved in a similar fashion.

<u>Run 1</u>:

The highest degradation (52%) was observed in the sawdust-treated sample amended with nitrogen and in the sawdust-treated sample amended with β -cyclodextrin, fungi and nitrogen (50% and 51%, respectively).

Table 16. Run 1: Biodegradation of fluoranthene

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run1 (% Biodegradation) 21days	Conc (ug/mg) 42 days	Run1 (% Biodegradation) 42 days
Control	62	82	-31	66	-7
Control (mix)	62	82	-31	140	-124
Sediment+cyclodextrin(25 ^{o}C)	82	96	-18	104	-27
Sediment+cyclodextrin(25 ^{o}C) (Mix)	82	96	-18	122	-49
Sawdust only	70	78	-12	34	52
Sawdust+N	70	56	21	37	47
Sawdust+cyclodextrin	90	92	-2	68	24
Sawdust+cyclodextrin(Mix)	90	92	-2	46	49
Sawdust+Fungi	80	73	10	59	26
Sawdust+Fungi (Mix)	80	73	10	40	51
Sawdust+Fungi+N	80	77	4	40	50
Sawdust+cyclodextrin+Fungi	107	73	32	55	49
Sawdust+cyclodextrin+Fungi+N	107	71	34	56	48

Run 2:

On day 21 the degradation of fluoranthene was negative in some of the treatments (-31% to -38%), and positive in others (1% to 48%). On day 42, it did not show any degradation (0%) and positive in other treatments (28% to 64%). The highest degradation (64%) was seen in the sawdust-treated sample augmented with fungi and nitrogen.

By day 42, fluoranthene showed a degradation of more than 50% in sawdust-treated sample amended with nitrogen and β-cyclodextrin and in the sawdust-treated sample amended with β -cyclodextrin, fungi and nitrogen.

Table 17. Run 2: Biodegradation of fluoranthene

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run2 (% Biodegradation) 21 days	Conc (ug/mg) 42 days	Run2 (% Biodegradation) 42 days
Control	120	119	1	85	29
Control (mix)	120	119	1	71	41
Sediment+cyclodextrin(25 ^{o}C)	89	123	-38	88	0
Sediment+cyclodextrin(25 ^{o}C) (Mix)	89	123	-38	64	28
Sawdust only	103	90	12	66	36
Sawdust+N	103	65	37	67	35
Sawdust+cyclodextrin	90	118	-31	49	46
Sawdust+cyclodextrin(Mix)	90	118	-31	51	43
Sawdust+Fungi	124	114	8	53	57
Sawdust+Fungi (Mix)	124	114	8	78	37
Sawdust+Fungi+N	124	65	48	45	64
Sawdust+cyclodextrin+Fungi	127	99	22	59	54
Sawdust+cyclodextrin+Fungi+N	127	108	15	69	45

Run 3:

On day 21 the degradation of fluoranthene was negative in some of the treatments (-3% to -23%), and positive in others (1% to 54%). The highest degradation (54%) was observed in the sawdust-treated sample amended with β-cyclodextrin.

Table 18. Run 3: Biodegradation of fluoranthene

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run3 (% Biodegradation) 21days
Control	82	81	1
Control (mix)	82	81	1
Sediment+cyclodextrin(25 ^{o}C)	103	65	37
Sediment+cyclodextrin(25 ^{o}C) (Mix)	103	65	37
Sawdust only	55	NA*	NA*
Sawdust+N	55	68	-23
Sawdust+cyclodextrin	108	49	54
Sawdust+cyclodextrin(Mix)	108	49	54
Sawdust+Fungi	80	82	-3
Sawdust+Fungi (Mix)	80	82	-3
Sawdust+Fungi+N	80	64	20
Sawdust+cyclodextrin+Fungi	74	67	10
Sawdust+cyclodextrin+Fungi+N	74	50	33

NA* - data not available due to technical difficulties.

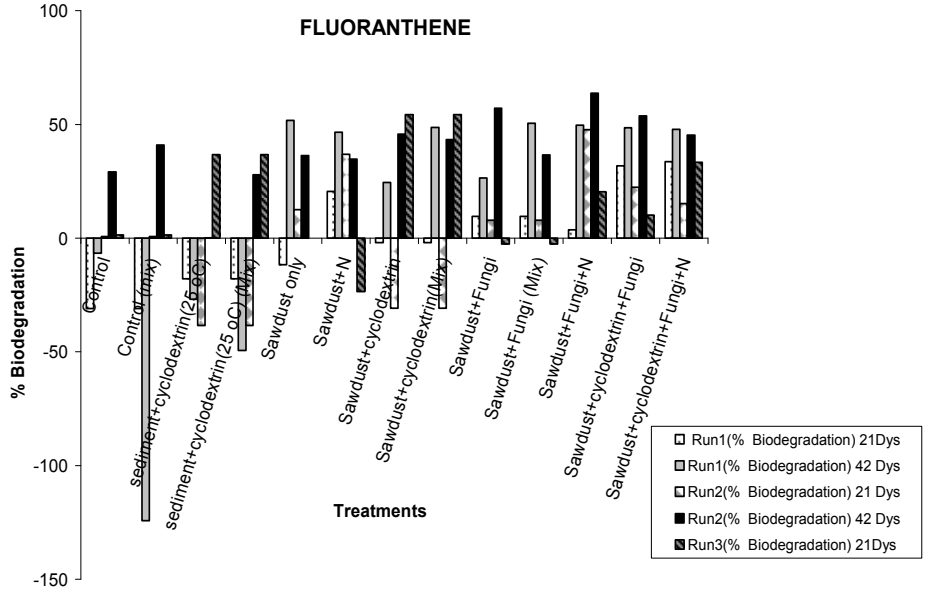

Fig. 28 Degradation of fluoranthene based on treatment

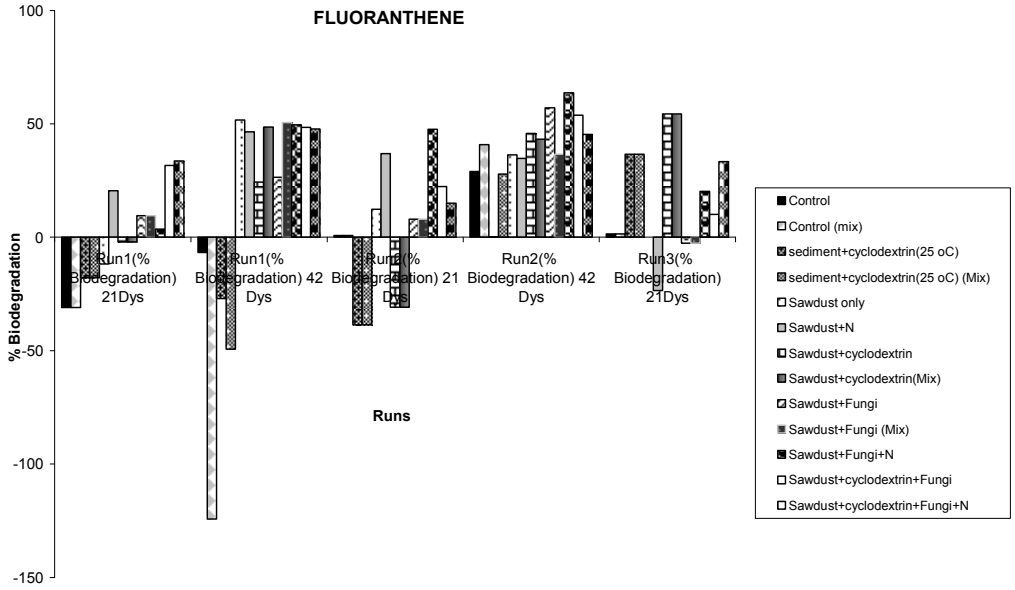

Fig. 29 Degradation of fluoranthene based on run

3.3.2.3. Biodegradation of Chrysene:

Chrysene is an organic compound with four aromatic rings. Chrysene was chosen as a representative compound to show the degradation of the HMW PAHs. Benz(a)anthracene behaved in a similar fashion..

Run 1:

On day 21 the degradation of chrysene was negative in some of the treatments (-31% to -294%), and no positive degradation. On day 42, it showed negative degradation in some treatments (-154% to -371%). The highest degradation (50%) was observed in the sawdust-treated sample augmented with fungi. The remaining treatments showed no degradation.

Table 19. Run 1: Biodegradation of chrysene

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run1 (% Biodegradation) 21days	Conc (ug/mg) 42 days	Run1 (% Biodegradation) 42 days
Control	8	11	-49	25	-230
Control (mix)	8	11	-49	29	-275
sediment+cyclodextrin(25 ^{o}C)	10	23	-143	31	-225
sediment+cyclodextrin(25 ^{o}C) (Mix)	10	23	-143	31	-225
Sawdust only	9	18	-89	39	-311
Sawdust+N	9	12	-31	45	-371
Sawdust+cyclodextrin	9	24	-174	25	-179
Sawdust+cyclodextrin(Mix)	9	24	-174	0	100
Sawdust+Fungi	8	23	-211	27	-265
Sawdust+Fungi (Mix)	8	23	-211	4	50
Sawdust+Fungi+N	8	30	-294	19	-158
Sawdust+cyclodextrin+Fungi	8	22	-161	24	-184
Sawdust+cyclodextrin+Fungi+N	8	22	-167	21	-154

Run 2:

On day 21 the degradation of chrysene was negative in some of the treatments (-2% to -50%), and positive in others (1% to 12%). On day 42, chrysene showed negative degradation in some treatments (-2% to -13%). The samples amended with sawdust and β-cyclodextrin showed positive degradation (24%).

Table 20. Run 2: Biodegradation of chrysene

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run2 (% Biodegradation) 21 days	Conc (ug/mg) 42 days	Run2 (%Biodegradation) 42 days
Control	9	7	12	10	-13
Control (mix)	9	7	12	6	27
Sediment+cyclodextrin(25 ^{o}C)	6	9	-50	7	-12
Sediment+cyclodextrin(25 ^{o}C) (Mix)	6	9	-50	7	-10
Sawdust only	7	8	-19	7	2
Sawdust+N	7	8	-13	7	2
Sawdust+cyclodextrin	7	8	-4	6	24
Sawdust+cyclodextrin(Mix)	7	8	-4	6	16
Sawdust+Fungi	7	7	1	7	-2
Sawdust+Fungi (Mix)	7	7	1	7	10
Sawdust+Fungi+N	7	6	12	7	3
Sawdust+cyclodextrin+Fungi	7	8	-16	6	8
Sawdust+cyclodextrin+Fungi+N	7	7	-2	7	-7

Run 3:

On day 21 the degradation of chrysene was negative in some of the treatments (-2% to -64%), and positive in others (7% to 29%). The samples amended with sawdust and β-cyclodextrin showed positive degradation (29%).

Table 21. Run 3: Biodegradation of chrysene

Treatments	Conc (ug/mg) 0 days	Conc (ug/mg) 21 days	Run3 (% Biodegradation) 21days
Control	8	13	-55
Control (mix)	8	13	-55
Sediment+cyclodextrin(25 ^{o}C)	8	8	-2
Sediment+cyclodextrin(25 ^{o}C) (Mix)	8	8	-2
Sawdust only	7	NA*	NA*
Sawdust+N	7	7	7
Sawdust+cyclodextrin	9	6	29
Sawdust+cyclodextrin(Mix)	9	6	29
Sawdust+Fungi	4	4	6
Sawdust+Fungi (Mix)	4	4	6
Sawdust+Fungi+N	4	7	-64
Sawdust+cyclodextrin+Fungi	8	8	-7
Sawdust+cyclodextrin+Fungi+N	8	6	25

NA* - data not available due to technical difficulties.

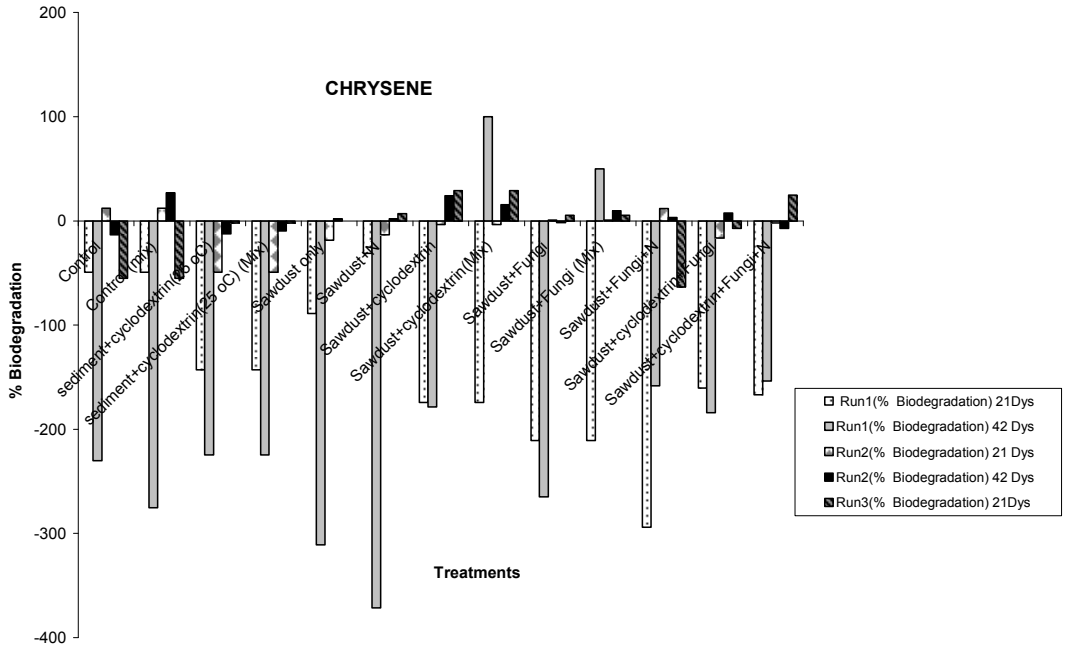

Fig. 30 Degradation of chrysene based on treatment

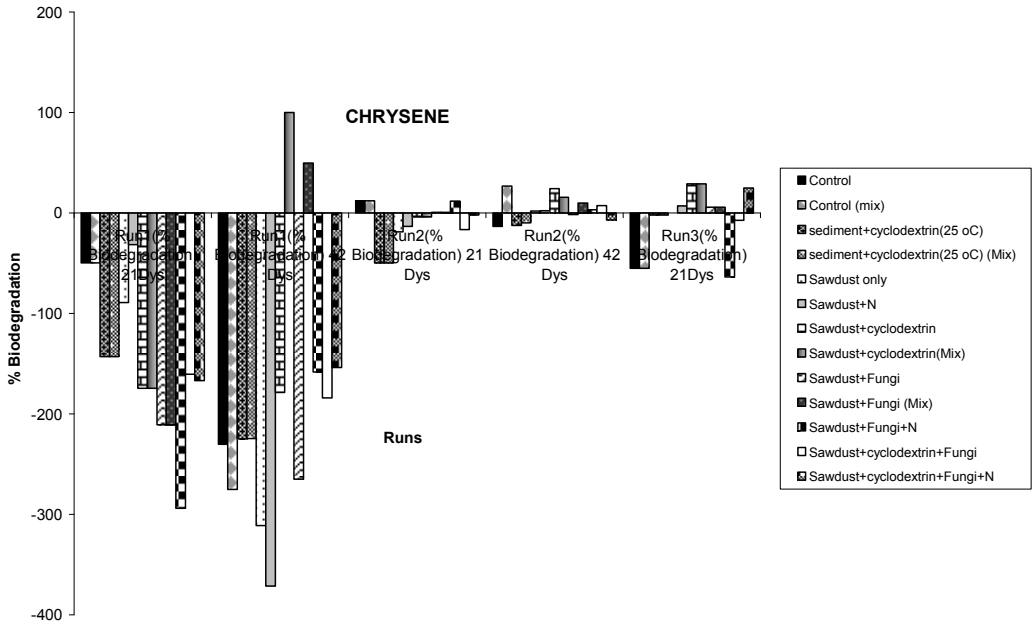

Fig. 31 Degradation of chrysene based on run

3.4. Extraction efficiency:

The efficiency of extraction was calculated.

Table 22. Percent recovery of the surrogate compounds

	Nitrobenzene-d5	Standard deviation	2-fluorobiphenyl	Standard deviation	Terephenyl-dl4	Standard deviation
Run 1	38	17	78	17	148	43
Run 2	42	10	75	12	182	16
Run 3	31	NA*	80	NA*	171	NA*

NA* - Data not available and yet to be analyzed.

The percent recovery for run 3 could not be calculated as the samples for 42nd day are yet to be analyzed.

Chapter 4: Summary and Conclusions

The white rot fungi, *Pleurotus ostreatus* was able to degrade almost all low molecular weight and a few high molecular weight PAHs to a certain degree. The surfactant effects of β-cyclodextrin were not consistently positive, possibly due to its low water solubility and the slow desorption rates of PAHs into the aqueous phase making them less available to β-cyclodextrin for removal. Sawdust-treated samples amended with fungi and nitrogen on average showed consistently the greatest PAH degradation. This is in agreement with the literature that reports maximum PAH degradation when the sample is provided with proper fungal substrate and nutrient supplements.

Low molecular weight PAHs showed more consistently positive and higher degradation rates than high molecular weight PAHs. Some high molecular weight PAHs such as fluoranthene and pyrene, however, showed good degradation for sawdust treatments amended with nitrogen and sawdust treatments amended with fungi, β-cyclodextrin and nitrogen.

There was high sample variability (within and between runs) which lead to variations in PAH degradation results and is the most likely the explanation for observation of negative degradation.

The sonication method for the extraction of the PAHs was simpler, less laborious and time-consuming than the lipid extraction method, and was therefore chosen for this research.

Chapter 5: Recommendations

1. The sonication method can be optimized by using a different solvent combination such as (1:1:1 acetone: DCM: hexane).
2. Triplicate extractions of each treatment would accommodate the inherently large sample variation better and improve the statistics.
3. For triplicate incubations, sediments from the same site and the same spot sampled at same time are recommended.
4. Homogenous mixing of each treatment with a mechanical device is also recommended to overcome sample heterogeneity.
5. The effects of surfactant activities of *β*-cyclodextrin and other cyclodextrins on the PAH bioavailability needs further investigation.
6. The effective concentrations of *β*-cyclodextrin are to be optimized.
7. Due to the limited water solubility of *β*-cyclodextrin, more water-soluble forms (methyl-cyclodextrin, hydroxyl propyl *β*-cyclodextrin) are recommended for further research.
8. *Pleurotus ostreatus* was found to be effective in removing PAHs from the contaminated sediment. It is recommend to repeat the study with the most effective treatments.
9. A controlled study with *Pleurotus ostreatus* that can be done by incubating the fungi in a solution containing known amounts of different PAHs is strongly recommended.
10. Optimization of the effective nitrogen concentrations favorable for fungal growth.
11. Scale up the technology.

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Appendices

Appendix 1:
PAH extraction

A) Reagents for PAH extraction

1. Dichloromethane(DCM) optima grade
2. Methanol optima grade
3. 50 mM phosphate buffer (add 8.7 g of KH_2PO_4 to 700 ml milli-Q water, stir and adjust he pH to 7.4 with 1 N HCl, complete to 1000 ml with milli-Q water)
4. Sodium chloride
5. Chloroform optima grade preserved with 0.75% ethanol
6. Anhydrous sodium sulfate for preparation of sodium sulfate columns
7. Unisil (activated silicilic acid, 100-200 mesh size) for construction of silica columns
8. Aminopropyl columns
9. Copper filings

B) Preparation of Sodium Sulfate columns

1. Use 6 ml glass columns with Teflon frits in the bottom.
2. Prepare the columns just before the run, so that the DCM does not dry out.
3. Load the columns with 1 g of anhydrous sodium sulfate.
4. Saturate the columns with DCM.
5. Add 2 ml of DCM to the assembled columns.
6. Allow DCM to drip through, stopping when the meniscus is just above the Na_2SO_4.

7. Discard the DCM and the waste collection tubes and replace the waste tubes with clean round bottom evaporating flasks.

C) Preparation of silica columns.

1. Weigh 0.5 g of Unisil in the glass columns with frits.
2. Heat the columns with Unisil at 100 ^{0}C for 2 hours to activate Unisil (to get rid of any moisture present in the Unisil).
3. Place glass columns in Visiprep apparatus and close valves.
4. Add 4 ml of chloroform to Unisil in the glass columns.
5. Open valves and let chloroform drip through at 1 drop per second, do not let the column dry.
6. Rinse the glass column with 2 ml of chloroform.
7. Stop the flow when the meniscus is just above the silica.
8. Add copper filings (20-30) per column- This is done to get rid of any sulfur present within the glass column.(cleaned in 2 rinses of 1N HCl, 2 rinses methanol, 2 rinses of DCM, 2 rinses of hexane and dried under nitrogen).

D) Preparation of Aminopropyl Columns

1. Use 3 ml aminopropyl columns (prepacked).
2. Rinse columns with 1 ml chloroform, rinse again with 2 ml and pull through with vacuum one drop per second.
3. Rinse with 2 ml of hexane pull through with vacuum 1 drop per second but do not let the column dry.

E) Extraction of Polycyclic Aromatic Hydrocarbons by Lipid Extraction:

1. Take 0.65g of sediment and 0.5 ml milli-Q water in a 50 ml glass tube.

2. At this point a treatment can be done.
3. Add 7.5 ml of dichloromethane (DCM) and 15 ml of methanol, followed by 5.3 ml of phosphate buffer.
4. Mix the contents by shaking and venting, and check for any leaks.
5. Place samples on platform shaker for about 2 hours at 320 rpm and cover them with an aluminum foil to protect them from light exposure.
6. Remove the samples from the shaker, add 7.5 ml of DCM, 7.5 ml phosphate buffer, shake and vent again.
7. Add a pinch of sodium chloride, shake and vent again.
8. The samples should be placed in the dark at 4 ^{0}C for 24 hours.

After 24 hours,

9. The sample shows 2 distinct phases.
10. Remove the upper water/methanol phase with a pipette and discard.
11. Remove the lower phase using another clean pipette to a 15 ml conical tube.
12. The amount of sample recovered should be recorded at this point.
13. To recover more sample, add 1 ml of DCM to original tube, vortex and wait for 5 minutes, see if any organic phase can be recovered.
14. Repeat 2 more times with out vortex.(if sample cannot be seen in organic phase discard it)
15. Transfer all organic phase from the conical tubes to sodium sulfate columns on the Supelco visiprep apparatus.
16. Samples should be collected in 50/100 ml round bottom evaporating flasks under the Supelco visiprep apparatus.

17. Rinse conical tubes three times using 3 ml of DCM and transfer the wash to the sodium sulfate columns.
18. Rinse column with two1 ml aliquots of DCM, then pull to dryness using vacuum.
19. Use evaporating flasks to rotovap and concentrate the sample to around 1 ml.
20. Transfer sample from evaporating flask to conical tube using a clean pipette.
21. Rinse evaporating flask with two 1 ml aliquots of DCM and add to the conical tube.
22. Concentrate the sample to one drop under nitrogen at 37 ^{0}C, but don't let the sample dry.
23. Bring volume of the sample to 1 ml- 1.5 ml using chloroform.
24. Record the amount of sample
25. Samples can be stored at -20 ^{0}C for short term storage or at -70 ^{0}C for long term storage. The sample at this stage can be used for the extraction of PAHs.

F) PAH Fraction

1. Silica columns should be prepared as outlined in appendix 1 (c)
2. Transfer the sample (1.0 – 1.5 ml) in chloroform to 200 µl hexane using solvent exchange.(do not let the sample dry as this will reduce PAH recovery).
3. Concentrate sample to 100 µl under nitrogen evaporator, then add 1 ml hexane. Concentrate sample to 100 µl again. Dope sample with one drop of chloroform, vortex and transfer to silica column. Draw sample through, but do not let column dry.
4. Repeat step 3 two more times using two aliquots 100 µl hexane

5. Rinse conical tube with 1 ml, 2ml and 2ml aliquots of hexane to recover more of the sample. Use this hexane to rinse sides of the silica column after rinsing conical tubes. Draw between each aliquot but do not let the column dry.
6. Rinse silica column once more with 100 µl hexane to finish recovery of PAH fraction.
7. Store PAH fraction in hexane without drying at -20 ^{0}C for short term storage or at -70 ^{0}C for long term storage until ready to clean with aminopropyl columns

G) Cleanup of PAH Fraction on Aminopropyl Columns

1. Aminopropyl columns should be prepared prior to cleanup.
2. Concentrate PAH fraction to one drop under nitrogen evaporator.
3. Bring volume to 200 µl with hexane.
4. If water is present in the sample add methanol until clear. Pipette off top PAH fraction and discard bottom methanol fraction.
5. Dope with one drop of chloroform, vortex sample and add to column.
6. Rinse conical tube three more times using 300 µl hexane in three aliquots of 100 µl, dope with one drop of chloroform, vortex and add to column each time.
7. Draw sample through column
8. Wash PAH fraction from column using 5 ml hexane in three aliquots: 1 ml, 2ml, 3 ml and let go to dryness.
9. The sample is then concentrated to 1.0 ml under nitrogen evaporator.
10. If the sample evaporates more than that accidentally, make up the volume to the 1.0 mL mark with DCM and then transfer to an auto sampler vial.
11. Label the vial appropriately.

12. Add 20 µl internal standard before being read on the GC-MS.

H) Extraction of Polycyclic Aromatic Hydrocarbons by Sonication:

1) All the extractions were done in triplicate.
2) 10.0 g of sediment to be massed out using a Denver Instrument M-120 into a 150 mL beaker and the sample mass accurately recorded.
3) Sonicate the surrogate mixture and add 50 uL of it to each sample.
4) Add Anhydrous Sodium sulfate in double the amount of the samples weight.
5) Add 50 mL of Optima grade Dichloromethane (DCM) to the samples and mix well.
6) Cover the beakers with aluminium foil and seal them with parafilm.
7) The samples were extracted in the Sonicator for 1 hour at 20 min intervals and 22^{o} C.
8) Filter the samples over DCM treated Sodium sulfate columns into clean evaporating flasks.
9) Add 40 mL of DCM to the sediment again, mix well and seal the beaker with aluminium foil and parafilm.
10) Repeat the steps 8 and 9 two more times.
11) Rinse column with two1 ml aliquots of DCM, then pull to dryness using vacuum
12) Concentrate the sample in the evaporating flask at 39 ^{o}C to 1 mL.
13) Transfer sample from evaporating flask to conical tube using a clean pipette.

14) Rinse evaporating flask with two 1 ml aliquots of DCM and add to the conical tube.

15) Concentrate the sample to one drop under nitrogen at 37 ^{0}C, but don't let the sample dry.

16) Bring volume of the sample to 1 ml- 1.5 ml using chloroform.

17) Record the amount of sample.

18) Samples can be stored at -20 ^{0}C for short term storage or at -70 ^{0}C for long term storage. The sample at this stage can be used for the extraction of PAHs.

19) Follow appendix-1 (f) and (g) for clean up.

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Appendix 2:
Solutions for the extraction of PAHs

Optima Grade Chloroform (Fisher): preserved with 0.75% ethanol.

Optima Grade Methylene chloride (DCM)

50 mM Phosphate buffer: add 8.7 g of K_2HPO_4 (Sigma) to approximately 950 ml of Millipore water. Adjust pH to 7.4 with 1N hydrochloric acid (HCl). Adjust to 1000 ml final volume in 1L volumetric flask with Millipore water.

0.2 N KOH: One pellet potassium hydroxide pellet (Fisher). Add 5 ml optima grade methanol and multiply weight of pellet by 89.29. That is total amount of methanol needed.

Saturated potassium persulfate solution: Add 10 g of $K_2S_2O_8$ (Sigma) and 2 ml of conc. sulfuric acid (Fisher) to 200 ml volumetric flask and fill to 200 ml with Milli-Q water. This mixture is light sensitive and must be stored in the refrigerator until use. Before use, it must be warmed up to room temperature.

Sodium sulfate (Na_2SO_4) columns: Add 1 g of dry Na_2SO_4 (Fisher) to clean 6 ml glass column. The columns were then packed with 2 ml of DCM without letting the packing go dry.

Solvent exchange: samples in DCM were concentrated to 100 µl using a nitrogen evaporator. Hexane in the amount of 1 ml was added and the samples again concentrated to 100 µl. This was repeated two more times.

Unisil (100 – 200 mesh) activated silicic columns (Clarkson Chromatography): 0.5 g of unisil were placed into 10 ml tubes and heated at 100°C for 2 hours to activate. The activated unisil was dissolved in 2 ml of chloroform and was transferred to the glass column. The tube was rinsed 4 times with 1 ml of chloroform and the solution transferred to the column. The chloroform was pulled through at 1 drop/second without the unisil go dry. The sides of the column were rinsed with two 1 ml aliquots of chloroform and 2 ml of hexane. Copper filings (cleaned in 2 rinses of 1 N HCl, methanol, DCM, and hexane and dried under nitrogen) were added to the column. The columns were then ready to use.

Aminopropyl (NH_2) columns (VWR): 1 ml of optima grade chloroform, then another 2 ml were added to the column before pressurizing it and letting it drip. Hexane in the amount of 2 ml was added and pulled through at 1 drop per second, without letting the packing go dry.

Appendix 3: Standard Curve for PAHs

A standard curve was performed using 10.0, 20.0, 30.0, 40.0 and 50.0 ug/mL concentrations of the calibration mix, 20 uL of internal standard, and 50 uL of surrogate solution were added and the volume adjusted to 1.0 mL with hexane. 2 mL autosampler vials were used.

Table 23. Concentrations for standard curve- PAH analysis

Surrogate mix (µL)	PAH mix (µL)	Internal Standard (µL)	Solvent (Hexane) (µL)
50µL	10 µL	20 µL	920 µL
50 µL	20 µL	20 µL	910 µL
50 µL	30 µL	20 µL	900 µL
50 µL	40 µL	20 µL	890 µL
50 µL	50 µL	20 µL	880 µL

A) Nitrobenzene standard curve:

$y = 1612x - 985.95$

$R^2 = 0.9994$

Table 24. Nitrobenzene standard curve

Concentration (ug/mL)	Response
5	5955
10	16683
20	31205
40	62331
60	96993
80	127507

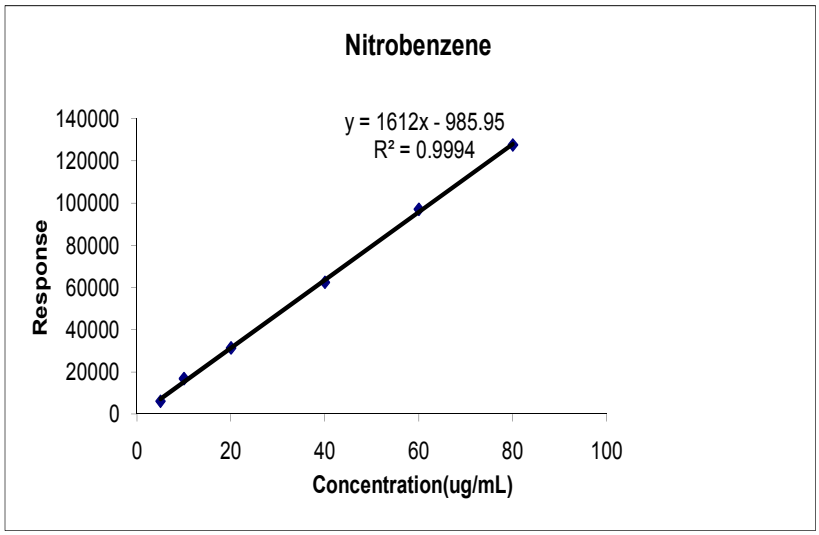

Fig. 32 Nitrobenzene standard curve

B) Naphthalene standard curve:

$y = 3135.4x + 8274.8$

$R^2 = 0.9946$

Table 25. Naphthalene standard curve

Concentration (ug/mL)	Response
5	17430
10	36626
20	75901
40	140733
60	202517
80	250545

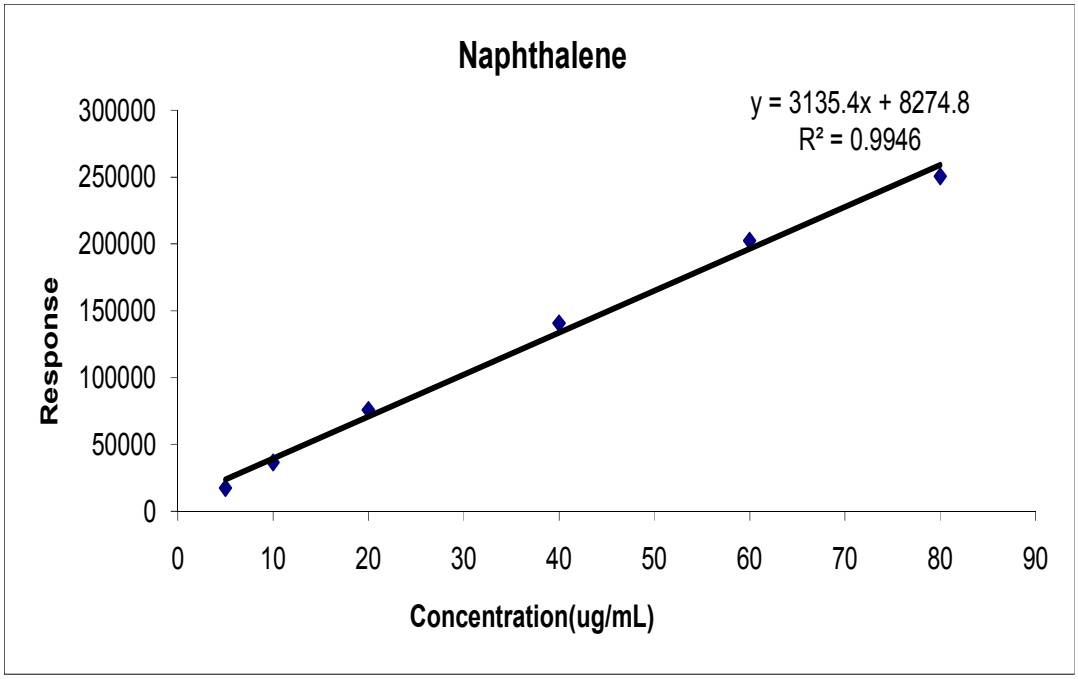

Fig. 33 Naphthalene standard curve

C) 2-fluorobiphenyl standard curve

$y = 4137.8x + 3330.8$

$R^2 = 0.9966$

Table 26. 2-fluorobiphenyl standard curve

Concentration (ug/mL)	Response
5	17728
10	41596
20	93801
40	170098
60	260436
80	325954

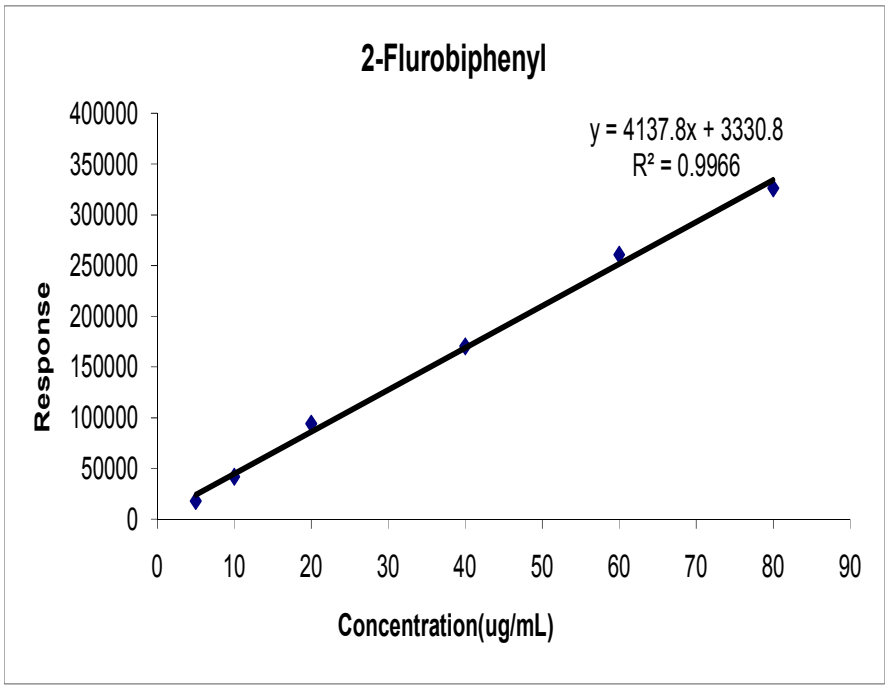

Fig. 34 2-fluorobiphenyl standard curve

D) Acenaphthylene standard curve:

$y = 2598.6x - 875.5$

$R^2 = 0.9986$

Table 27. Acenaphthylene standard curve:

Concentration (ug/mL)	Response
5	9088
10	23486
20	55424
40	105979
60	153277
80	206187

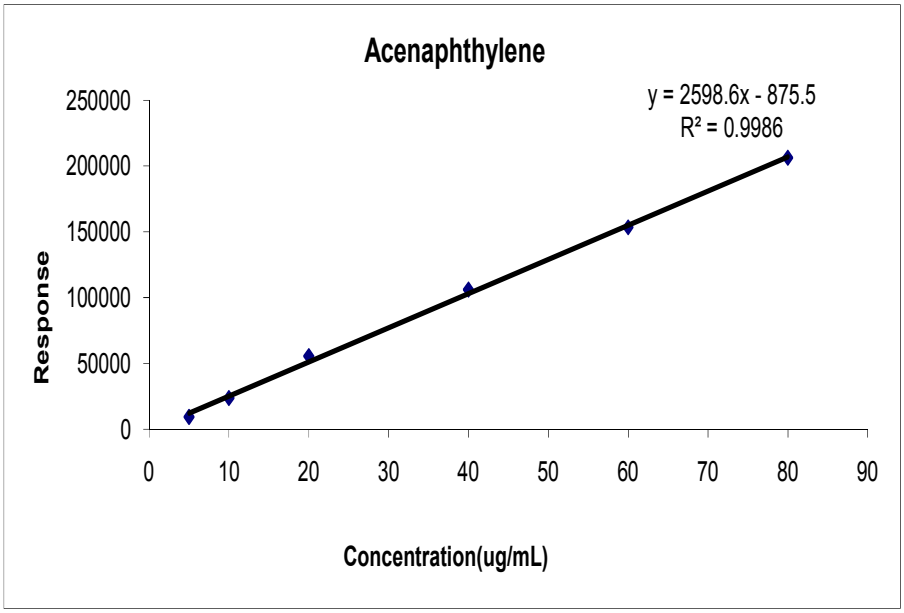

Fig 35 Acenaphthylene standard curve:

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E) Acenaphthene standard curve:

$y = 1676.6x + 1255.1$

$R^2 = 0.9961$

Table 28. Acenaphthene standard curve

Concentration (ug/mL)	Response
5	5824.253
10	16837.52
20	39817.7
40	70573.15
60	100702
80	134250.6

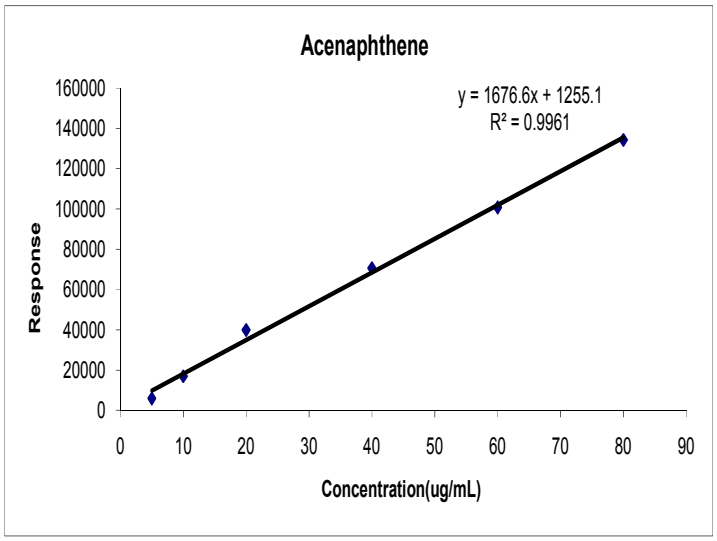

Fig. 36 Acenaphthene standard curve

F) Fluorene standard curve:

$y = 1801.5x - 1551.5$

$R^2 = 0.9984$

Table 29. Fluorene standard curve

Concentration (ug/mL)	Response
5	6136.31
10	14220.36
20	37614.66
40	72658.17
60	105633.5
80	141753.3

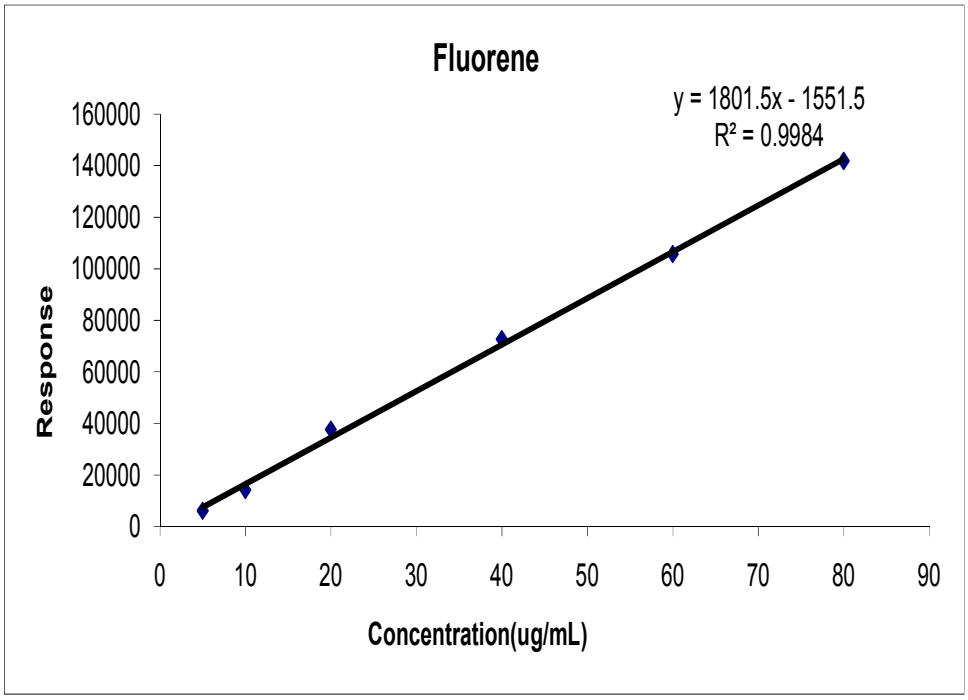

Fig. 37 Fluorene standard curve:

G) Phenanthrene standard curve:

$y = 2505.2x - 1422.5$

$R^2 = 0.9988$

Table 30. Phenanthrene standard curve

Concentration (ug/mL)	Response
5	9310.828
10	23914.74
20	47338.98
40	102703.9
60	150806.8
80	196009.9

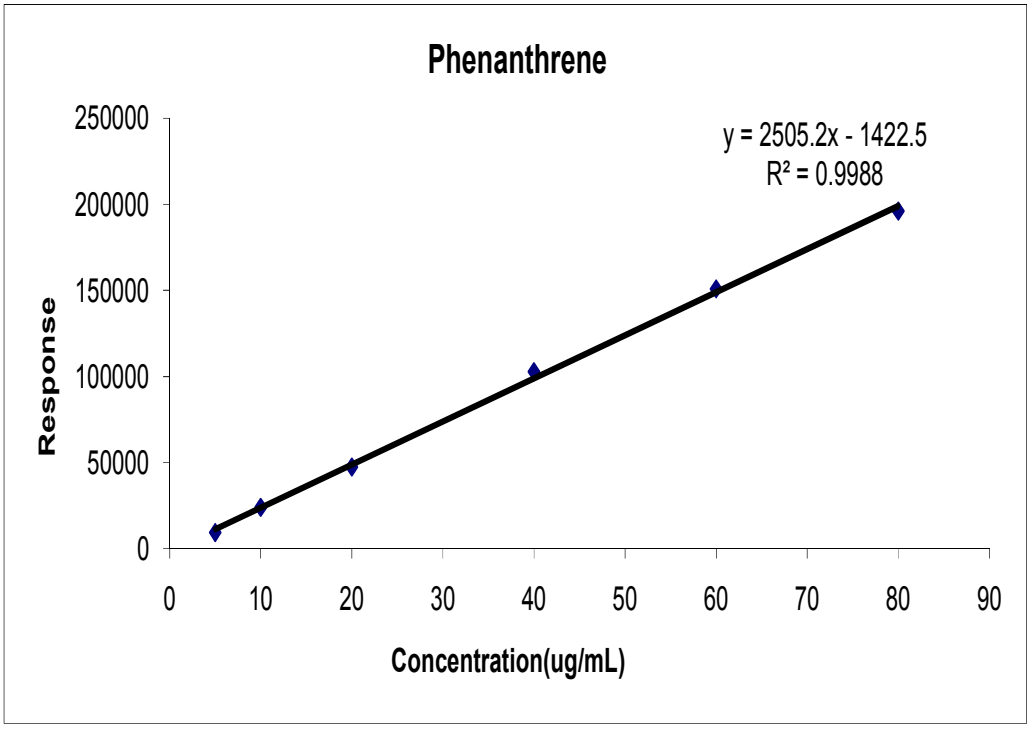

Fig. 38 Phenanthrene standard curve

H) Anthracene standard curve:

$y = 2137.1x - 2933.4$

$R^2 = 0.9959$

Table 31. Anthracene standard curve

Concentration (ug/mL)	Response
5	6707.934
10	13936.54
20	47517.15
40	81382.22
60	124167
80	168160.4

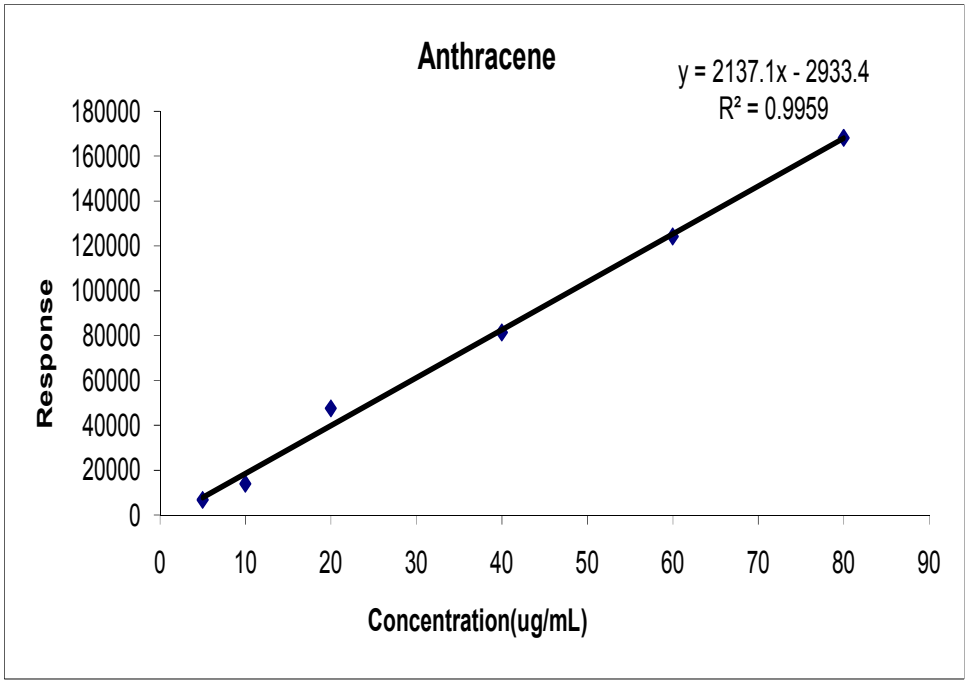

Fig. 39 Anthracene standard curve

I) Fluoranthene standard curve:

$y = 1722x - 4640.6$

$R^2 = 0.9992$

Table 32. Fluoranthene standard curve

Concentration (ug/mL)	Response
5	4778.385
10	11014.09
20	31730.52
40	62528.12
60	98754.64
80	133581.8

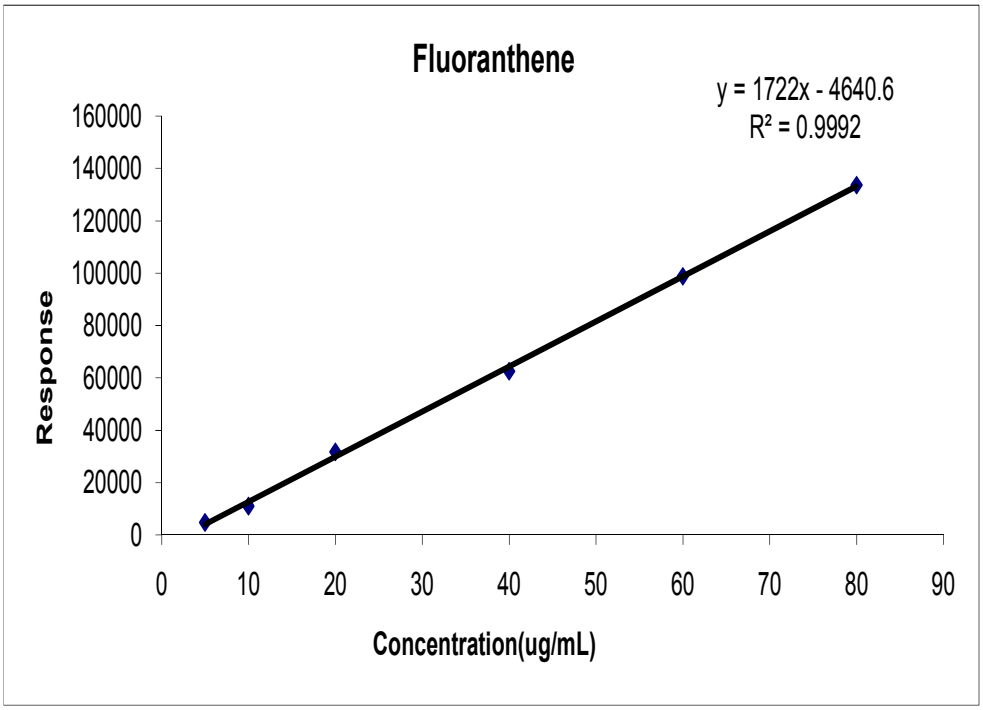

Fig. 40 Fluoranthene standard curve

J) Pyrene standard curve:

$y = 1818.3x - 5651.3$

$R^2 = 0.9991$

Table 33. Pyrene standard curve

Concentration (ug/mL)	Response
5	3234.24
10	12186.36
20	32921.96
40	64346.19
60	104284.7
80	140061.6

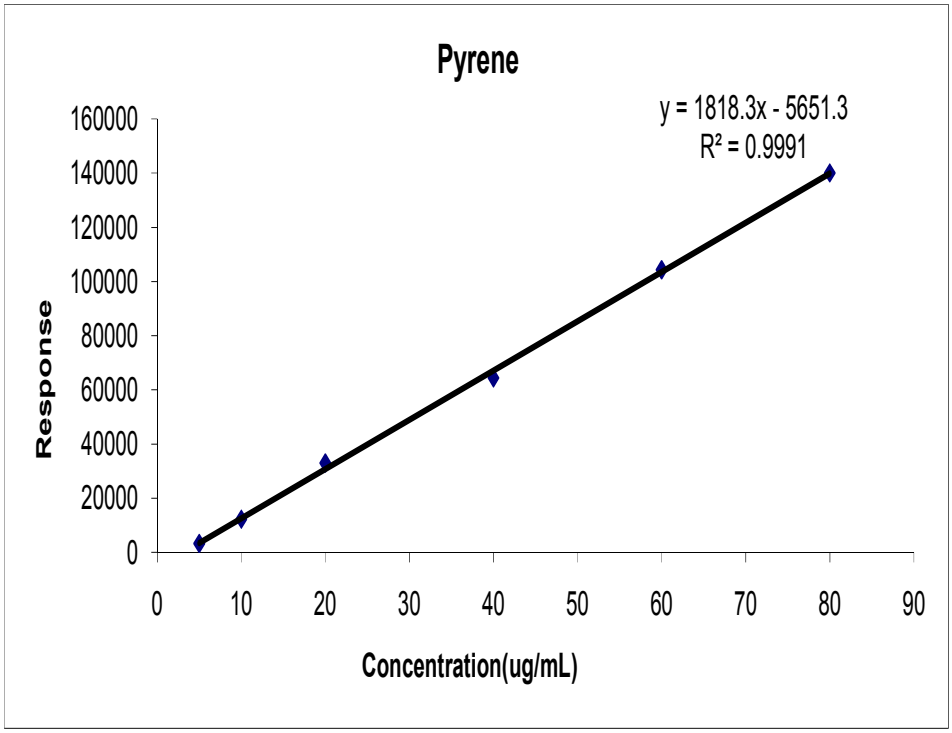

Fig. 41 Pyrene standard curve

K) Terephenyl-d14 standard curve:

$y = 2439.3x - 8537.4$

$R^2 = 0.9987$

Table 34. Terephenyl-d14 standard curve

Concentration (ug/mL)	Response
5	5500.581
10	14733.87
20	42057.27
40	84210.08
60	139055.7
80	187660.2

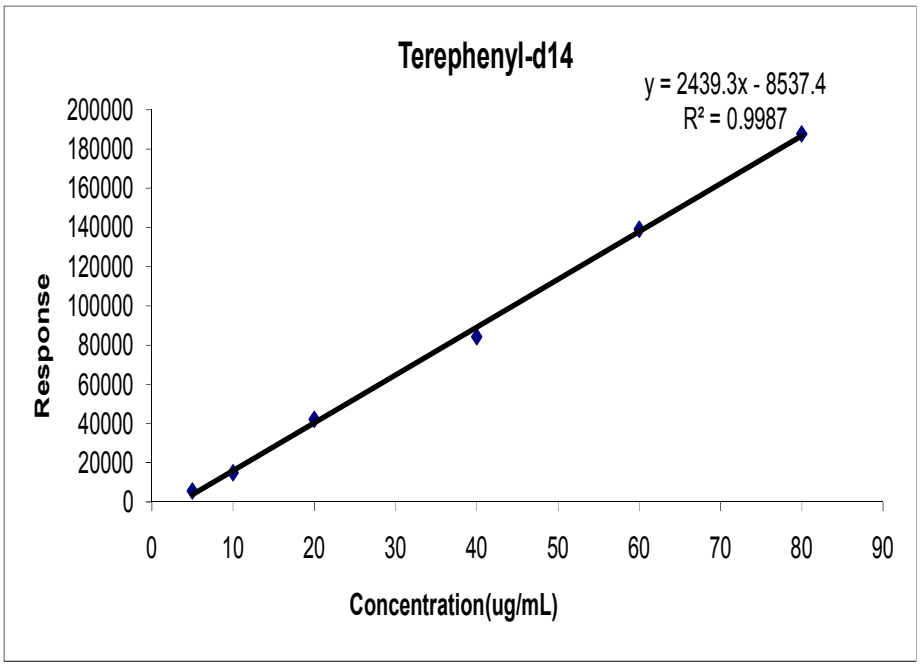

Fig. 42 Terephenyl-d14 standard curve

L) Benzo(a)anthracene standard curve:

$y = 1436.6x - 8780$

$R^2 = 0.9958$

Table 35. Benzo(a)anthracene standard curve

Concentration (ug/mL)	Response
5	622.015
10	6639.906
20	17074.04
40	48863.85
60	73674.57
80	109309.6

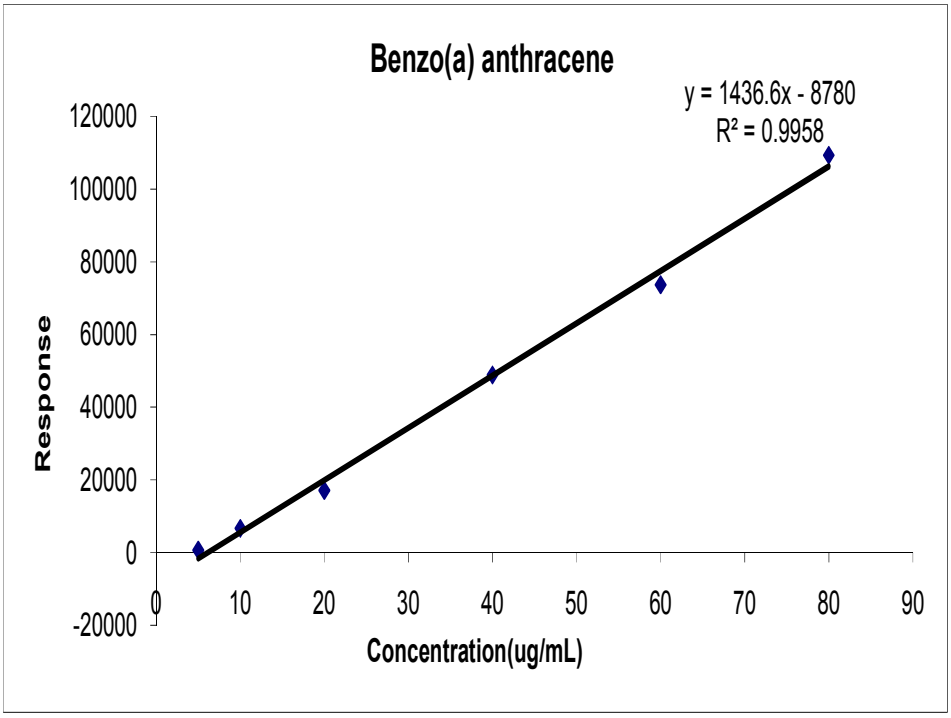

Fig. 43 Benzo(a)anthracene standard curve

M) Chrysene standard curve:

y = 1412.7x - 7048

R^2 = 0.9966

Table 36. Chrysene standard curve

Concentration (ug/mL)	Response
5	1036.64
10	8006.664
20	20545.23
40	48939.44
60	73660.66
80	109253.1

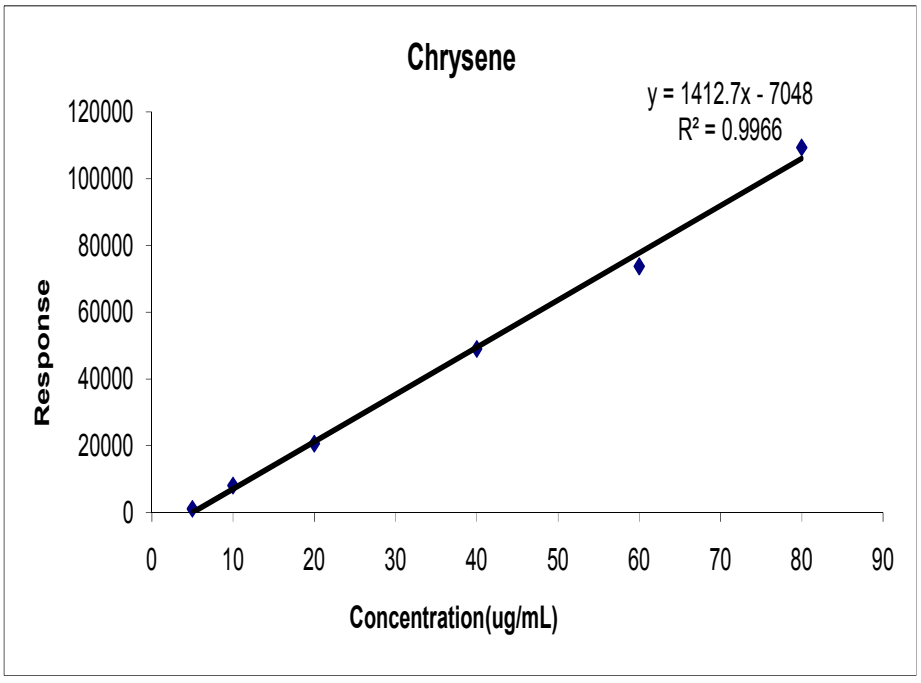

Fig. 44 Chrysene standard curve

N) Benzo(b&k)fluoranthene standard curve:

y = 1547.2x - 11710

R^2 = 0.9972

Table 37. Benzo(b&k)fluoranthene standard curve

Concentration (ug/mL)	Response
5	0
10	727.247
20	18921.41
40	49062.6
60	79997.89
80	113670.1

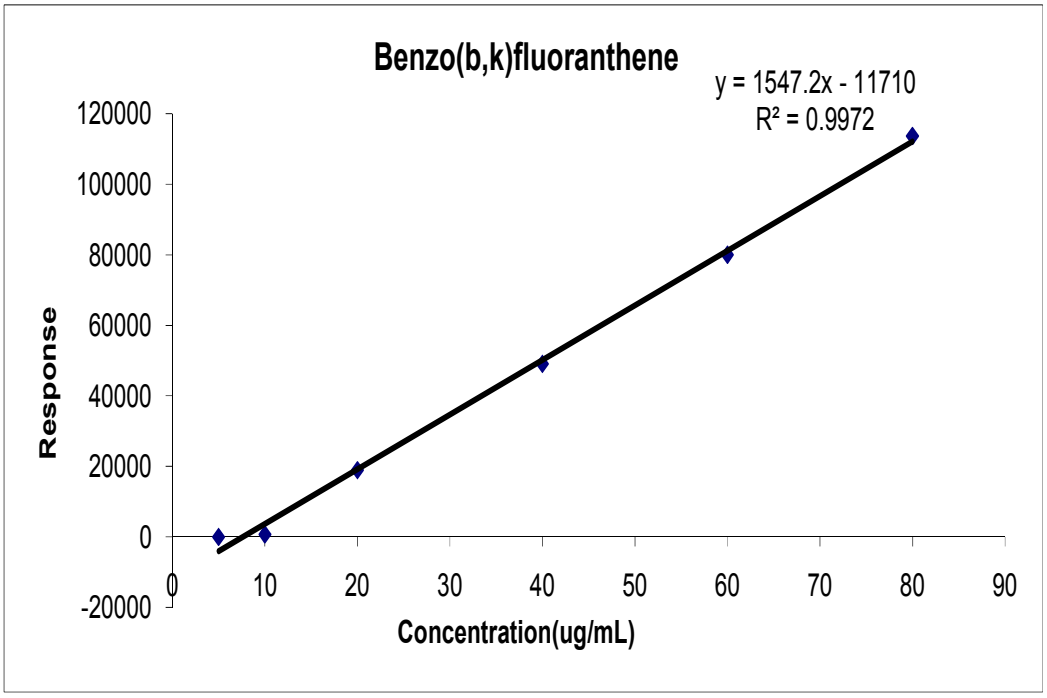

Fig.45 Benzo(b&k)fluoranthene standard curve

Appendix 4: Standards for PAHs and Lipids

Surrogate Solution: Restek B/N surrogate mix

2-fluorobiphenyl

nitrobenzene-d5

p-terphenyl-d14

1,000 µg/ml each in methylene chloride, 1ml/ampul

Calibration Mix: Restek SV Calibration Mix #5 / 610 PAH Mix

acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorine, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene

2,000 µg/ml each in methylene chloride, 1ml/ampul

Internal Standards: Restek SV Internal Standard Mixes

acenaphthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12, phenanthrene-d10

2,000 µg/ml each in methylene chloride, 1ml/ampul

Appendix 5: Correlated Internal Standards with PAHs and Surrogates

Internal Standards:	Correlating PAHs and Surrogates
Napthalene-d8:	Nitrobenzene-d5 (surrogate), Napthalene
Acenaphthene-d10:	2-fluorobiphenyl (surrogate), Acenaphthylene, Acenaphthene, Fluorene
Phenanthrene-d10:	Phenanthrene, Anthracene, Fluoranthene, Pyrene,
Chrysene-d12:	Terephenyl-d14 (surrogate), Benzo(a)anthracene, Chrysene, Benzo(b,k)fluoranthene, Benzo(a)pyrene
Perylene-d12:	Dibenz(ah)anthracene, Ideno(1,2,3-cd)pyrene, Benzo(ghi)perylene

Appendix 6: Standard Curve for PAHs

A standard curve was performed using 10.0, 20.0, 30.0, 40.0, and 50.0 µg/ml concentrations of the PAH mix, 20 µl of internal standard, and 50 µl of surrogate solution were added and the volume adjusted to 1.0 ml with hexane. Two ml autosampler vials were used.

Table 38. Standard curve for PAHs

Surrogate mix (µL)	PAH mix (µL)	Internal Standard (µL)	Solvent (Hexane) (µL)
50µL	10 µL	20 µL	920 µL
50 µL	20 µL	20 µL	910 µL
50 µL	30 µL	20 µL	900 µL
50 µL	40 µL	20 µL	890 µL
50 µL	50 µL	20 µL	880 µL

B/N Surrogate mix, PAH mix and Internal Standard mix solutions are warmed and sonicated prior to use. They are stored at 4^0C after use.

Internal Standard: An *internal standard* is a chemical substance that is added in constant amounts to samples, the blank and calibration standards in a chemical analysis, to correct the loss of analyte during sample preparation or sample inlet. The internal standard is a compound that matches in many aspects, with the chemical species of interest in the samples, as the effects of sample preparation should, relative to the amount of each species, be the same for the signal from the internal standard as for the signal(s) from the species of interest in the ideal case.

Surrogate: *Surrogate compounds* are organic compounds which are similar to the analytes of interest chemically, but which are not normally found in environmental samples. Surrogates are added to samples to monitor the effect of the specific sample matrix on the accuracy of the analysis.

Matrix Spike: *Matrix Spike Samples* are aliquots of client-supplied environmental samples that are spiked with target compounds representative of the method analytes, and carried through every aspect of the procedure, including preparation, clean-up, and analysis. Matrix spike samples are analyzed to evaluate the effect of the sample matrix on the accuracy of the analytical procedure.

Reference:
http://www.groundwateranalytical.com/qual_quality_assur.htm

Appendix 7: Comparision between sonication and lipid extraction

The data were reported on the basis of their wet weights.

Table 39. Comparision of extraction efficiencies of sonication and lipid extraction

Target compounds	Sonication	Lipid extraction	Standard deviation (Sonication)	Standard deviation (Lipid extraction)
Naphthalene	0.47	0.12	0.24	0.08
Acenaphthylene	0.06	0.32	0.18	0.10
Acenaphthene	0.10	2.11	1.43	0.49
Fluorene	1.73	0.86	0.61	0.18
Phenanthrene	0.82	3.67	2.02	1.17
Anthracene	5.28	2.75	1.79	0.67
Fluoranthene	4.93	3.52	1.00	1.78
Pyrene	1.95	4.97	2.13	2.01
benzo (a) anthracene	3.50	0.10	2.41	0.10
Chrysene	2.69	0.10	1.83	0.10
benzo (b&k) fluoranthene	1.59	0.10	1.05	0.10
benzo (a) pyrene	0.10	0.10	0.10	0.10

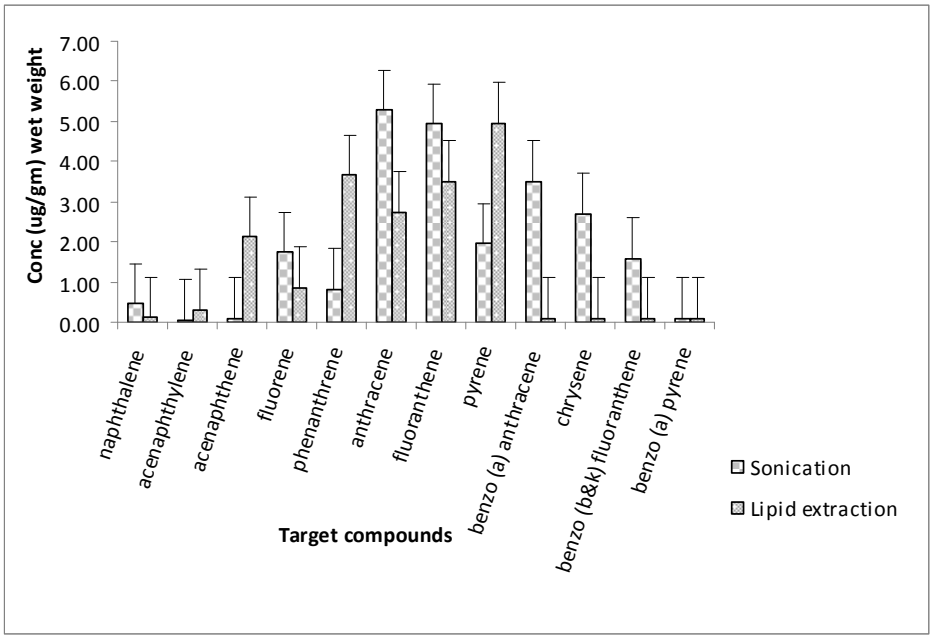

Fig. 46 Sonication vs lipid extraction

Appendix 8: Percent moisture content and dry matter content of the sediment versus time in triplicate runs. (Acharya, 2008)).

Table 40. Percent moisture content and dry matter content of the sediment versus time in triplicate runs for 0 days.

0 days								
Sample	% Moisture Content			Average	% Dry matter			Average
	Run1	Run2	Run3		Run1	Run2	Run3	
Paper	45.31	44.49	43.29	44.36	54.69	55.51	56.71	55.64
Control	44.65	43.01	44.73	44.13	55.35	56.99	55.27	55.87
Sawdust+ fungi+ cyclodextrin	43.82	42	38.08	41.3	56.18	58	61.92	58.7
Sawdust+ cyclodextrin	43.23	38.92	40.69	40.95	56.77	61.08	59.31	59.05
Sawdust	42.77	39.56	40.23	40.85	57.23	60.44	59.77	59.15
Paper +fungi	46.55	42.28	42.91	43.91	53.45	57.72	57.09	56.09
Sawdust +fungi	41.54	40.95	40.06	40.85	58.46	59.05	59.94	59.15
Sediment+ cyclodextrin (25^0 C)	47.73	44.1	43.14	44.99	52.27	55.9	56.86	55.01

Table 41. Percent moisture content and dry matter content of the sediment versus time in triplicate runs for 21 days.

21 days								
Sample	% Moisture Content			Average	% Dry matter			Average
	Run1	Run2	Run3		Run1	Run2	Run3	
Paper	45.1	37.08	45.32	42.5	54.9	62.92	54.68	57.5
Control	45.08	42.51	53.44	47.01	54.92	57.49	46.56	52.99
Sawdust +fungi+ cyclodextrin	43.5	40.88	43.94	42.77	56.5	59.12	56.06	57.23
Sawdust+ fungi+ cyclodextrin +N	41.76	39.54	41.26	40.85	58.24	60.46	58.74	59.15
Sawdust+ cyclodextrin	42.78	39.68	39.82	40.76	57.22	60.32	60.18	59.24
Sawdust+ N	41.03	39.16	38.41	39.53	58.97	60.84	61.59	60.47
Sawdust	39.36	38.5	45.24	41.03	60.64	61.5	54.76	58.97
Paper +Fungi	45.9	41.75	45.61	44.42	54.1	58.25	54.39	55.58
Sawdust+ Fungi+ N	41.26	30.73	42	38	58.74	69.27	58	62
Sawdust +Fungi	39.39	38.15	39.34	38.96	60.61	61.85	60.66	61.04
Sediment+ cyclodextrin (25^0 C)	46.57	44.87	46.32	45.92	53.43	55.13	53.68	54.08

Table 42. Percent Moisture content and Dry Matter content of the sediment versus time in triplicate runs for 42 days.

42 days								
Sample	% Moisture Content			Average	% Dry matter			Average
	Run1	Run2	Run3		Run1	Run2	Run3	
SawDust (mix)	39.08	40.94	39.07	39.7	60.92	59.06	60.93	60.3
Paper+Fungi (mix)	45.69	41.63	45.01	44.11	54.31	58.37	54.99	55.89
SawDust+N	42.69	42.69	40.83	42.07	57.31	57.31	59.17	57.93
Paper+Fungi	44.69	41.28	44.48	43.48	55.31	58.72	55.52	56.51
SawDust	40.25	39.08	39.78	39.7	59.75	60.92	60.22	60.29
Paper	43.8	41.15	42.81	42.59	56.2	58.85	57.19	57.14
Paper (mix)	46.05	41.17	42.57	43.26	53.95	58.83	57.43	56.73
Sediment+cyclodextrin (25^0C) (mix)	45.9	43.28	45.78	44.99	54.1	56.72	54.22	55.01
Sediment+cyclodextrin (25^0 C)	46.12	43.82	43.95	44.63	53.88	56.18	56.05	55.37
SawDust+cyclodextrin (mix)	41.35	40.52	38.46	40.11	58.65	59.48	61.54	59.89
SawDust+cyclodextrin	41.9	38.19	39.86	39.98	58.1	61.81	60.14	60.01
SawDust+Fungi(mix)	40.62	37.82	42.09	40.18	59.38	62.18	57.91	59.82
SawDust+Fungi+N	41.67	35.76	41.7	39.71	58.33	64.24	58.3	60.29
Fungi+SawDust+cyclodextrin	41.87	40	41.44	41.1	58.13	60	58.56	58.89
Fungi+SawDust+cyclodextrin +N	46.61	44.33	39.93	43.62	53.39	55.67	60.07	56.37
Fungi+SawDust+cyclodextrin (mix)	41.48	39.81	42.55	41.28	58.52	60.19	57.45	58.72
Control (mix)	46.16	44.21	40.28	42.24	53.84	55.79	59.72	56.45
Control	44.47	41.97	40.23	39.28	55.53	58.03	59.77	57.77
SawDust+Fungi	39.78	37.84	39.07	39.7	60.22	62.16	60.93	61.1