

Effect of *Pleurotus ostreatus* on Bioremediation of PAH Contaminated River Sediment

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THESIS APPROVAL AND RELEASE FORM

Effect of *Pleurotus ostreatus* on Bioremediation of PAH Contaminated River Sediment

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Abstract

The purpose of this study was to optimize bioremediation of Mahoning River sediment historically contaminated with polycyclic aromatic hydrocarbons (PAHs) using white rot fungi. *Pleurotus ostreatus* grown on grain (10% v/v) was added to contaminated sediment amended with sawdust (80% v/v), with and without fungal specific nutritional nitrogen (to enhance fungal growth), and with cyclodextrin (to increase PAH availability). Sediment mixtures were incubated in the dark at 25°C for 6 weeks. Sawdust made the sediment more porous, allowed better colonization by fungi, and did not greatly increase volume. Fungal biomass, determined using fluorescent microscopy, indicated initial fungal colonization but then fungal growth was inhibited, likely by toxic metals or high moisture content in the sediment. Growth of unidentified fungi was observed, especially in treatments amended with nitrogen. Total PAH concentrations (in the order of 100 ppm), analyzed using a gas chromatograph mass spectrometer (GCMS), and significantly decreased ~ 50-60% in all treatments, including sediment only controls within the first two weeks. Thus, aerobic degradation by native bacteria and volatilization were likely responsible for most of the observed decreases in PAH concentrations. High heterogeneity of PAHs in this historically contaminated sediment led to high variance between replicates. There was a slight decrease in 5 ring PAHs associated with sediment inoculated with *P. ostreatus* and also a slight decrease in total PAH concentrations associated with sediment amended with sawdust and cyclodextrin (with or without *P. ostreatus*). Increased nitrogen did not enhance PAH degradation. Sediment inoculated with *P. ostreatus* after two weeks, rather than initially, showed better fungal growth and colonization, but PAH data was not yet available. These data indicate there is great potential for bioremediation of PAH contaminated sediment conditions by

stimulating indigenous bacteria under aerobic conditions followed by the addition of white rot fungi. However, further testing and optimization is still required.

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1. Introduction

1.1 Mahoning River and Watershed

The Mahoning River (**figure 1**) is located in Northeastern Ohio and Western Pennsylvania. The Mahoning River is approximately 182 km long and has a watershed with an area of approximately 1821 km². The river and the area around it are home to many species of birds, aquatic mammals, macro invertebrates and fish. Along with being a home for wildlife, the Mahoning River is also an important historic waterway that has supported industry. Approximately 500,000 people and several cities are along its banks, including Youngstown OH. It was and still is a very valuable aspect for the economy and life of the surrounding areas.

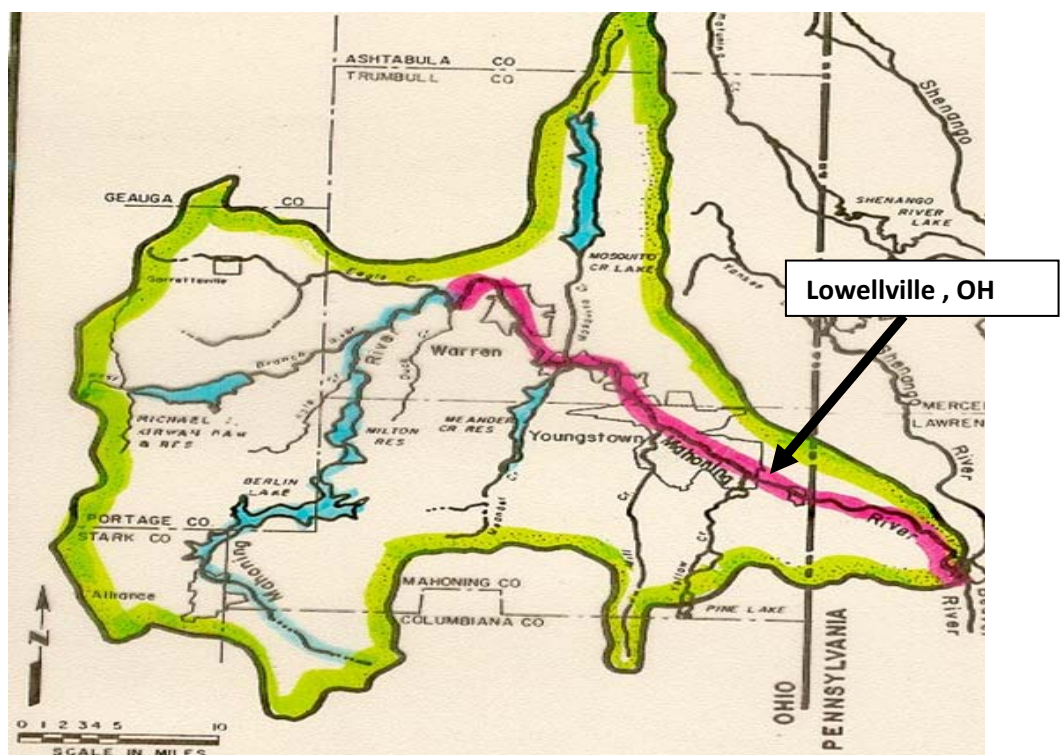


Figure 1. Mahoning River and its watershed (picture modified from http://www.lrp.usace.army.mil/pm/mah_watershd.jpg)

Industrial activity has caused extensive environmental contamination to the Mahoning River and its surrounding area. While industry was at its height in the area surrounding the river, roughly between the early 1900s and 1977, massive amounts of organic and metal contaminants were released into the river. Industrial cooling also caused thermal pollution, greatly increasing river water temperature year round. This activity resulted in the populations of many native organisms in the river being greatly impacted and/or reduced.

The legacy of this industrial activity and contamination is still present in the river, decades after the collapse of the steel industry. While contamination of the water is negligible, high concentrations of toxic compounds remain in the sediment and still impact the environment. A black, tar like sludge is still commonly found along the river bottom and its banks in the area (**figure 2**). PAHs are the contaminants of concern in this toxic “sludge”. The Ohio Environmental Protection Agency has issued a contact ban for such sediment in the 51 km stretch of the river from Warren OH to Lowellville OH. In this same area, a fish consumption advisory has been implemented, by the Ohio EPA, due to the risk of contamination. This contamination of fish can readily be seen in specimens taken from the area. There is evidence that the diversity and abundance of benthic microorganisms in the area have also been reduced by the contamination (**Xu and Leff 2004**).



Figure 2. PAH contaminated sediment from Lowelville, OH.

1.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are organic compounds, made of several aromatic rings. PAHs are mainly produced from the incomplete combustion of organic substances, such as coal, gas and oil. They are also intentionally produced for the use in some products, such as dyes and pesticides. This group is diverse in structure and toxicity; some examples can be seen in **figure 3**. They are a widespread, common industrial contaminant found in many locations around the world. In the U.S. alone PAHs have been found in 600 of the National Priorities List sites identified by the Environmental Protection Agency.

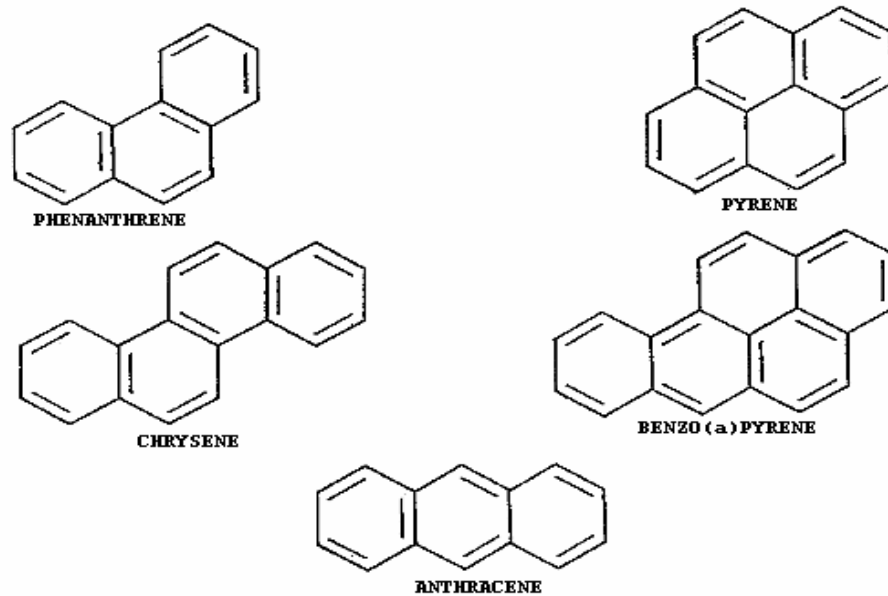


Figure 3. Examples of PAHs found in the Mahoning River (picture taken from <http://www.osha.gov/dts/sltc/methods/organic/org058/org058.html>)

PAHs have been found to cause many negative health and environmental effects. According to the Agency for Toxic Substances and Disease Registry, mice exposed to PAHs over a long period of time demonstrated increased risk of birth defects, skin irritation and an inhibition of the immune system. Benthic fish and other benthic organisms suffer large amounts of negative physiological effects when in contact with PAHs (**Myers et al. 1998 and Nacci et al. 2002**). More importantly however, PAHs are suspected carcinogens. In several studies performed it has been found that PAH exposure correlates with increased risks of cancer (**Mastrangelo et al. 1996 and Boffetta et al. 1997**). Several other studies have demonstrated that PAH exposure in the environment has correlated with an increase risk of reproductive damage (**Hsu et al. 2006 and Miller et al. 2004**) and immune system suppression/damage (**Sul et al. 2003**).

PAHs persist in the environment for long stretches of time because they adsorb to sediment and are resistant to biodegradation. They can be very difficult and costly to remove. PAHs with more ring structures and thus a higher molecular weight are typically more resistant to degradation. The key to breaking down these compounds is through the cleavage of these rings, allowing for increased bioavailability.

1.3 White rot fungi

White rot fungi are a genetically diverse group of wood degrading fungi that are found worldwide. Examples of white rot fungi include: *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Trametes versicolor* and *P. ostreatus*. They are grouped together because they all have the ability to degrade lignin. Lignin is an important chemical compound for plants because it provides the structural integrity of their cell walls. Like PAHs, lignin is a complex organic polymer that is aromatic in structure, making it recalcitrant to degradation. White rot fungi are unique in nature because they are capable of breaking down lignin and PAHs.

Lignin peroxidases, manganese peroxidases and laccases are the three main types of enzymes produced by white rot fungi to degrade lignin. Lignin peroxidases are monomeric hemoproteins, found in many white rot fungi (**Hammel and Cullen 2008**). These enzymes work when they are oxidized by H_2O_2 . Upon oxidation, lignin peroxidase releases free radicals capable of breaking apart other compounds. Manganese peroxidases contain manganese and are found in many species of white rot fungi and may be an alternative to lignin peroxidases in lignin degradation (**Hammel and Cullen 2008**). The mechanism for the degradation of organic compounds by manganese peroxidase focuses on the manganese contained within the enzyme. The enzyme is capable of converting the manganese contained within the enzyme from Mn^{2+} to

Mn^{3+} . This Mn^{3+} can then oxidize lignin molecules. Laccases are glycoproteins found throughout nature, and are multicopper enzymes (**Claus 2004**). The copper centers of these enzymes are able to produce highly reactive free radicals. These free radicals are capable of breaking apart covalent bonds found in many complex organic polymers, such as lignin and organic pollutants (**Mayer and Staples 2002**).

Fortuitously these lignin degrading enzymes can also degrade a wide range of organic contaminants. Contaminants that have been successfully degraded by white rot fungi include explosives (**Sheremata and Hawari 2000 and Axtell et al. 2000**), PCBs (**Dierich et al. 1995 and Fernandez-Sanchez et al. 2001**), oil (**Isikhuemhen et al. 2003**) and PAHs (**Pickard et al. 1999, Levin et al. 2003 and Zheng et al. 2001**). In the case of PAHs these enzymes are effective since PAHs and lignin have similar chemical structure (**figure 3 & 4**). Lignin degrading enzymes work by producing free radicals that cleave the ringed structures of PAHs. This causes PAHs to become more water soluble and more bioavailable to indigenous microbes, which can then also use these compounds as a carbon source (**Boochan et al. 2000**).

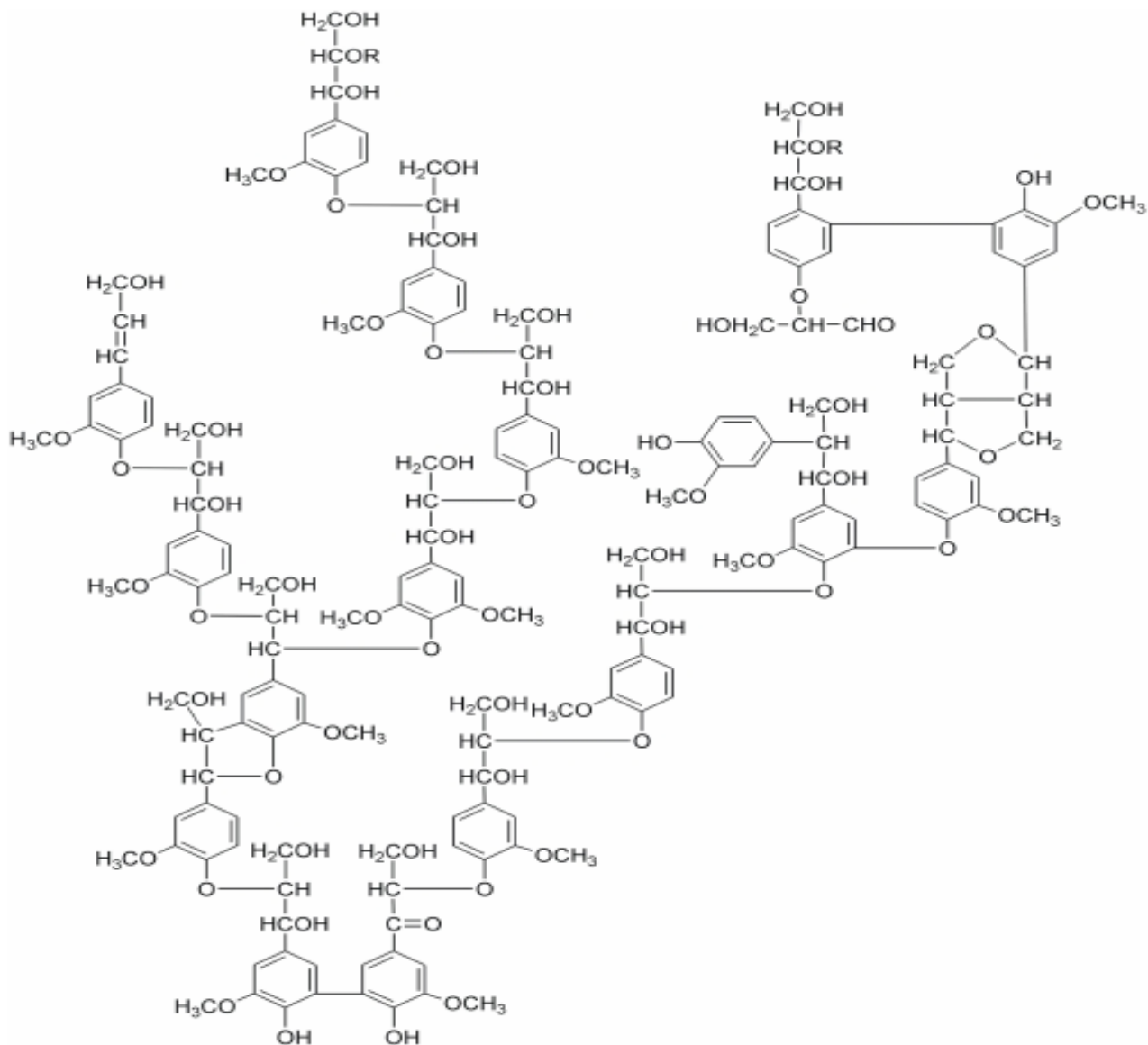


Figure 4. Chemical structure of lignin. (Picture taken from

http://www.ibwf.de/env&enz_index.htm)

1.4 *Pleurotus ostreatus*

The organism used in this study was *Pleurotus ostreatus*, an edible, white rot fungus, commonly referred to as the oyster mushroom. It is useful in bioremediation because it can easily colonize contaminated soil and break down PAHs (Novotný et al. 1999 and Eggen 1999). *P.*

ostreatus has been found to produce high levels of the extracellular enzyme laccase (**Hou et al. 2004**) and has the potential to completely mineralize PAHs (**Bezalel et al 1996**). Since *P. ostreatus* is a noninvasive and non pathogenic fungus, commonly grown and eaten, it may be more readily accepted by the public for bioremedial applications.

1.5 Role of Nitrogen in Fungal Bioremediation

Nitrogen is a key component in many compounds necessary for life, such as amino acids and nucleic acids. However, the effect of available nitrogen has shown mixed results on white rot fungi's ability to express lignin degrading enzymes. It has been shown that the addition of nitrogen may actually be inhibitory toward the production of extracellular enzymes in white rot fungi. One such studied showed that high nitrogen was inhibitory towards Poly R 478 dye decolorization in several species of white rot fungi on solid culture (**Leung and Pointing 2002**). Another study also showed that the white rot fungi *Lentinula edodes* was inhibited in its ability to decolorize poly R-478 and orange II (**Hatvani and Mecs 2002**). In contrast, other studies have shown that increased nitrogen may actually increase levels of extracellular enzymes produced by certain types of white rot fungi. A study performed in liquid culture using the fungi *L. edodes*, found increasing the level of nitrogen increased the level of laccase produced while decreasing MNP activity (**Buswell et al. 1995**). Another study showed that the white rot fungi *Irpex lacteus* was inhibited in its ability to produce LIP, MNP and laccase when in nitrogen limited liquid culture (**Novotny et al. 2000**). *P.ostreatus* and several other white rot fungi were found to produce increased levels of lignolytic enzymes in the presence of nitrogen rich media in another study (**Kaal et al. 1995**).

There is much debate in the scientific community about how nitrogen levels impact the bioremediation potential of white rot fungi. Studies have shown that white rot fungi growing in liquid culture with increased levels of nitrogen were able to successfully degrade TNT (**Kim and Song 2000**) and the PAH, chrysene (**Hadibarata et al. 2009**). In contrast a study performed using *I. lacteus* showed that increased nitrogen levels had no impact on its ability to degrade PAHs in liquid culture (**Cajthaml et al. 2008**). It has also been shown that growth under nitrogen limiting conditions increases the white rot fungi's ability to break down contaminants. For example *Phanerochaete chrysosporium* was able to rapidly degrade PCP in nitrogen limited liquid culture (**Reddy and Gold 2000**).

1.6 Cyclodextrins

One of the main problems with the degradation of PAHs is their low bioavailability and low water solubility. A way to increase both of these aspects of PAHs is through the use of surfactants. These compounds can be produced naturally or synthetically. Surfactants have been used in the past to increase the availability of certain toxins and increase their degradation in the environment. Surfactants can increase PAH bioavailability (**Cuyppers et al. 2002**) and also increase rates of degradation (**Kim et al. 2001**). Unfortunately, many types of surfactants are toxic and their addition to the environment may be detrimental.

Cyclodextrins are non toxic, biodegradable, naturally occurring, oligosaccharides capable of forming complexes with hydrophobic compounds, such as hydrocarbons. These complexes allow for increased solubility and bioavailability of the hydrophobic compounds. The addition of cyclodextrins has been shown to increase the biodegradation of hydrocarbons by microorganisms in liquid culture (**Bardi et al. 2000**) and in soil (**Bardi et al. 2002**). Studies

performed using cyclodextrin in conjunction with fungi have demonstrated increased degradation rates of fluorene (**Garon et al. 2004**) and decabromodiphenyl ether (**Zhou et al. 2007**).

1.7 Internal Transcribed Spacer Region

The region of DNA often used to identify fungi is the internal transcribed spacer (ITS) region (**figure 5**). This DNA is repeating, has non-coding sequences and is found in all eukaryotic organisms. The non-coding DNA sequences allow this region to be highly variable and allows for determination of species through restriction fragment length polymorphism analysis and other downstream applications. The multiple repeating nature of this region allows it to be easily amplified. Many studies have been performed using this region for species identification. For example, one study was able to identify 33 species of wine yeast using RFLP analysis of the ITS region from cultures (**Guillamon et al. 1998**). Another study was able to successfully identify several different species of fungi from agricultural soil samples using RFLP analysis of the ITS region (**Viaud et al. 2000**). Previous research has shown that white rot fungi species identification in environmental samples can be determined through RFLP analyses (**Johnston and Aust 1994**).

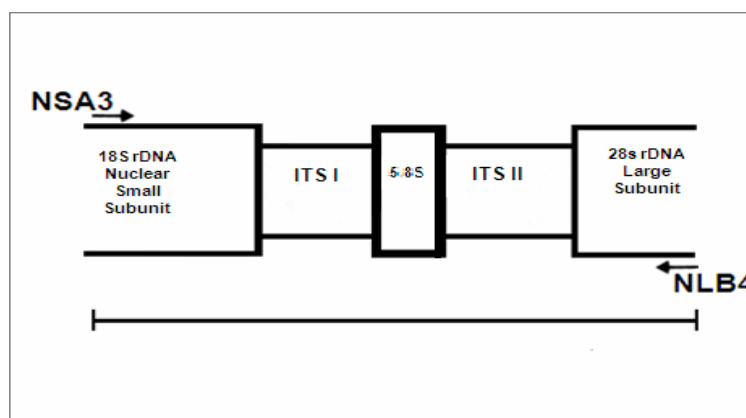


Figure 5. ITS region and primers used for PCR amplification and RFLP analysis

1.8 Goals

The goal for this study is to determine an optimal aerobic PAH degradation treatment using *P. ostreatus* in historically contaminated river sediment. Nitrogen and cyclodextrin amendments were used to test their effects on *P.ostreatus*'s ability to degrade PAHs. Most fungal bioremediation research studies have been conducted in liquid (**Levin et al. 2003**) or PAH spiked soils (**Garon et al. 2004**). Very few studies on bioremediation with white rot fungi use historically contaminated soils (**Axtell et al. 2000**). It has been found that historically PAH contaminated sediment is much more difficult to remediate due to increased adsorption of PAHs to sediment over time and decreased bioavailability (**Alexander 2000**). This current study is unique in that *P. ostreatus* is added as a solid culture to a heterogeneous and historically PAH contaminated river sediment. The addition of *P. ostreatus*, sawdust, nitrogen and cyclodextrin would be an environmentally friendly and relatively cost effective means of degrading PAH contaminants in dredged river sediment.

1.9 Hypotheses

The addition of *P.ostreatus* will increase the rate of PAH degradation through the production of extracellular lignin degrading enzymes. The addition of a fungal specific nitrogen source will increase the biomass of *P.ostreatus* and increase the rate of PAH degradation. The addition of cyclodextrin will increase the solubility and bioavailability of PAHs contained within the sediment and thus increase the rate of PAH degradation.

2. Methods

2.1 Sediment Collection and Characterization

PAH contaminated sediment was collected from Lowellville, OH. This is an area of historically high concentrations of contamination on the Mahoning River (**figure 1**). The reason for this high contamination is due to its location downstream of industry and upstream of a low head dam, which allowed for the settling of contaminated sediment. Sediment was collected by inserting plexiglass tubes into the river bank, just below the water line. Contaminated sediment was placed into buckets and stored at 4°C until use.

2.2 Fungal Culturing

Pleurotus ostreatus strain Florida F6 (**ATCC Product # 58053**) was used in this study. Spores were stored at -80°C in sterile water. Spores were taken from the starting culture and streaked onto potato dextrose agar (**PDA, Sigma Aldrich Product # 70139-500**) plates and incubated at 25°C for 1 week and then stored at 4°C until use. Inoculated agar plates were sliced and placed into 125 ml of potato dextrose broth (**PD, Sigma Aldrich Product # P6685-250g**). The broths were then placed onto a shaker and incubated at 25°C for 1 week. Cultures showing sufficient growth and no evidence of contamination were stored at 4°C until use.

Grain (1000 ml) and 350 ml of water were placed into a spawn bag (**Fungi Perfecti**) and then sealed using an impulse sealer. The sealed spawn bag containing the grain was then autoclaved at 121°C for 20 minutes. Fungal inoculated potato dextrose broth was then used to inoculate the grain. The inoculated grain was incubated for approximately 1 week at 25°C and then stored at 4°C until use.

Fungal selective media was prepared by adding 19.5 g Potato Dextrose Agar into 500 ml of water and autoclaving for 20 min at 121°C. After the mix had sufficiently cooled 0.275 g of streptomycin (**Sigma Aldrich S6501-100g**) and 0.0008 g of benomyl (**Sigma Aldrich Product # 381586-5g**) (dissolved into 5 ml of acetone) were added (Dietrich and Lamar 1990).

2.3 Incubation Set Up

The first set of incubations was performed to determine the effect of the level of nitrogen on *P. ostreatus*'s ability to biodegrade PAHs. A list of the treatments involved in the first incubation can be seen in **table 1**. Contaminated river sediment (1 L) was removed from storage and placed into a DCM washed stainless steel bowl. Sawdust (800 ml), obtained from a local wood mill, was added to treatments involving sawdust. Sawdust was used to bulk up sediment, making it more porous, more like a soil. A commercially available fungal nitrogen source was added at 25 ml for low nitrogen and 50 ml for high nitrogen as nutritional nitrogen supplements. Sediment and all amendments were added and then thoroughly homogenized by hand.

Following mixing, aliquots of approximately 5 grams were taken and placed into 60 ml VOA vials. Fungi were added to each vial by placing three grains of fungal inoculated barley for fungal treatments. Moistened paper towel was used to keep samples moistened. Treatment set up in VOA vials can be seen in **figure 6**. All vials were opened to the atmosphere every three days. All treatments for biomass and PAH analysis were performed in triplicate. PAHs concentrations, biomass and moisture content were analyzed at time 0, week 2 and week 6.

A second set of incubations were performed at larger scale and done to determine the effect of cyclodextrin on the ability of *P. ostreatus*'s to degrade PAHs. The treatments are listed

in **table 2**. Contaminated sediment (500 ml) was placed into a DCM washed stainless steel bowl. Sawdust (400 ml) was added to treatments containing sawdust. Fungal inoculated grain (50 ml) was added for all fungal treatments. β -cyclodextrin (**Fisher Scientific Product# AC22728-1000**) was added to the sediment in a 0.8% (wt/v) concentration to the sediment. After all amendments were added to the sediment, treatments were homogenized by hand. Autoclaved gravel was placed at the bottom of DCM cleaned 2 L glass bowls to keep sediment evenly moist. Amended sediment was then placed into 2 L glass bowls. A Petri dish containing moistened paper towel was suspended in the bowls to keep samples moist. An example of the set up can be seen in **figure 7**. After 2 weeks, fungus was added to one treatment by adding 50 ml of fungal inoculated grain and mixing thoroughly by hand.

Table 1. List of Sediment Treatments (Fungi/Nitrogen Incubation)

Sediment Only (control)
Sediment and Sawdust
Sediment, Sawdust and Fungi
Sediment, Sawdust, Fungi and Low Level Nitrogen
Sediment, Sawdust, Fungi and High Level Nitrogen

Table 2. List of Sediment Treatments (Fungi/Cyclodextrin Incubation)

Sediment only (control)
Sediment and Sawdust
Sediment, Sawdust and Cyclodextrin
Sediment, Sawdust, Cyclodextrin and Fungi
Sediment, Sawdust, Cyclodextrin and Fungi after 2 weeks

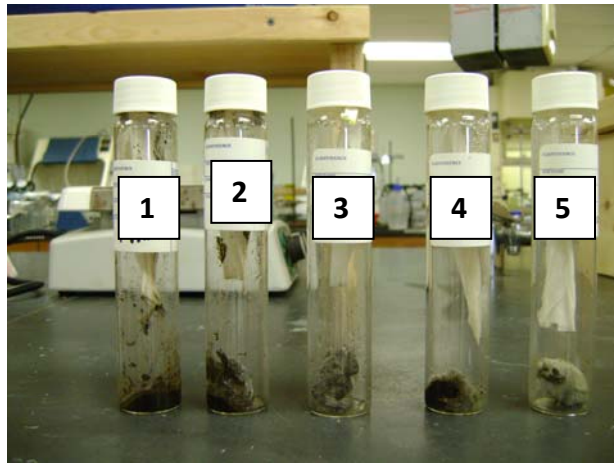


Figure 6. Incubation vials are shown from the first smaller scale Mahoning River incubation after 2 weeks. Vial 1= Sediment only, Vial 2 = Sediment and sawdust, Vial 3 = Sediment, sawdust and fungi, Vial 4 = Sediment, sawdust, low nitrogen and fungi, Vial 5 = Sediment, sawdust, high nitrogen and fungi

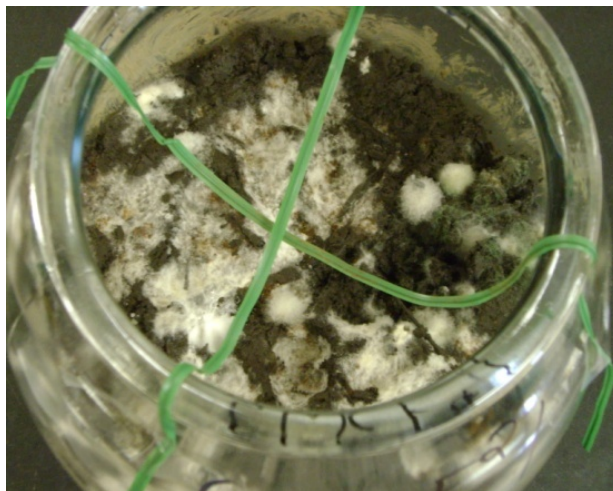


Figure 7. Top view of an incubation bowl from the second larger scale incubation of Mahoning River sediment. This treatment consisted of cyclodextrin added at time 0 and fungi added after 2 weeks. The photo was taken at 4 weeks.

2.4 Moisture Content

Moisture content was taken by removing approximately 1 gram of sediment from the treatments at each sampling time and placing them onto pre heated (550°C for 2 hours) and pre weighed aluminum dishes. The 1 gram sample was then placed into an oven at a temperature of 105°C until its weight stabilized and recorded. The sediment was then placed into an incinerator at 550°C until its weight had also stabilized. Moisture content was then calculated using the following calculation:

$$\text{((Wet Weight-Dry Weight)/Wet Weight) X 100 = Moisture Content}$$

2.5 Fungal Biomass

For total fungal hypha (**figure 8**) quantification, a modified version of the fluorescent microscopy method was used (**Stahl et al. 1996**). Approximately 1 gram of sediment was removed from the treatment and added into 100 ml of sterile water, inside of a sterile blender

head. The water and sediment were then mixed at low speed for 1 minute. An aliquot (1 ml) of the sample was then removed and passed through a 0.22 μm black mesh polycarbonate filter (**Fisher Scientific Product # GTBP02500**). CalcofluorTM solution was allowed to sit on the filter for 30 seconds. The filter was then placed onto a glass microscope slide and allowed to dry. Two drops of immersion oil were placed onto the dry filter and a glass cover slip was placed on top. Ten random fields of view were then taken using Ax70 fluorescent microscope at 100x magnification using a wavelength of 432 nm. All observed hypha on the slides were measured and recorded using the program Microsuite Special Edition. The amount of fungal mycelia found on the slides was then converted into meters of mycelia per gram of sediment (m/g).

For the analysis of metabolically active fungal hypha (**figure 9**) a modified fluorescent microscopy method was used (**Stahl et al. 1996**). Sterile Milli-Q water (200 ml) was placed into a sterilized Waring blender head. Sediment (1 g) was added to the 200ml of sterile water and mixed at low speed for 1 minute. A Fluorescein diacetate (FDA) solution was added to the 200ml sediment water solution. The solution was then allowed to sit for 3 minutes, with occasional mixing at low speed. The solution was then mixed again at low speed for 1 minute. A 2ml aliquot was removed from the blender head and passed through a 0.22 μm black mesh polycarbonate filter. The polycarbonate filter was then removed, placed onto a microscope slide and allowed to dry. Two drops of immersion oil were placed onto the dry filter and a glass cover slip was placed on top. Ten random fields of view were then analyzed using the Ax70 fluorescent microscope at 100x magnification, with a wavelength of 524 nm. All hypha that were detected on the slides were measured and recorded using the program Microsuite Special Edition. All values were converted to meters per gram of sediment.

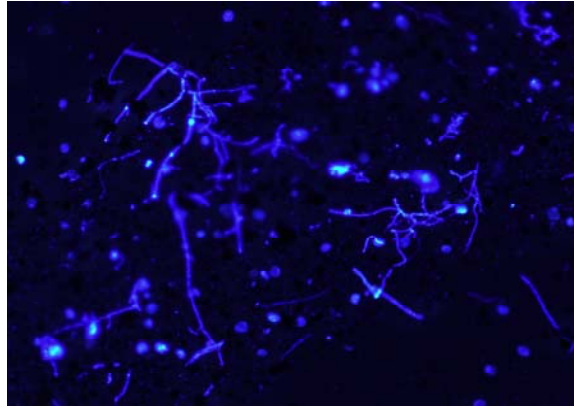


Figure 8. CalcofluorTM stain showing total fungal hypha in a sawdust and fungi treatment after incubating for 2 weeks (100x magnification) using a fluorescence microscope.

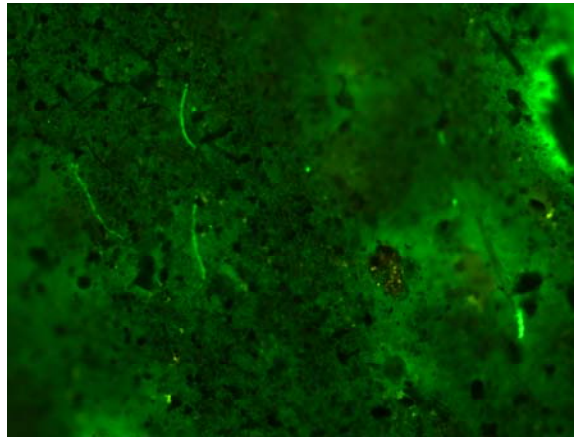


Figure 9. Fluorescein diacetate stain showing metabolically active fungal hypha in a sawdust and fungi treatment after incubating for 2 weeks (100x magnification) using a fluorescence microscope.

2.6 Fungal Isolation from Treatments

Fungal colonies were isolated from treatments by removing 1 gram of sediment from the homogenized sample from the treatments. The sediment was then placed into a sterile blender head containing 100ml of sterile water. The sediment, water mix was then blended for approximately 1 min at maximum setting. Dilutions (100 ul) were removed and plated onto

white rot fungal selective media. The plates were then allowed to grow at 25°C for 5 days (**figure 10**).



Figure 10. Fungal selective media, containing colonies of *P. ostreatus*

2.7 Molecular Analysis

DNA was extracted using the MasterPure Yeast DNA Purification Kit (**Epicentre Product # MPY2-70420**) from fungal colonies on selective media, isolated from treated sediment or pure cultures. PCR of the ITS region of DNA was performed with primers targeting the ITS region of basidiomycetes specific fungi DNA (Martin et al. 2005). The primers and DNA were mixed with GoTaqTM reaction mix (**Promega Product # M5132**) as detailed by Promega. The sequences for the primers used were as follows: 5'**NSA3** AACTCTGTCGTGCTGGGGATA3' and **NLB4** 5'GGATTCTCACCTCTATGAC3' (**Martin and Rygiewicz 2005**).

A THERMO Px2 thermocycler was used with the following program: 2 min at 95°C, 95°C for 1 min, 60°C for 1 min, 72°C for 1 min. Steps 2-4 were repeated 35 times and then 72°C

for 5 min. PCR amplified ITS DNA was digested with restriction enzymes. The restriction enzymes used were *Hae*III and *Taq*I (**New England Bio labs**). A .1-10kb DNA ladder (**New England Biolabs Product#N3270S**) was used as a reference for band sizes.

2.8 PAH Extraction and Analysis

The extraction protocol was modified from EPA method 3550c. PAHs were extracted using dichloromethane (DCM) as detailed in **appendix 3**.

3. Results and Discussion

Studies have shown that fluorescence microscopy can be effectively used to indicate the levels of fungi present within soil (Morris et al. 1997). Total and active mycelia were detected within all samples using fluorescence microscopy using the stains fluorescein diacetate for active fungi (figure 11A and 12A) and CalcofluorTM for total fungi (figure 11B and 12B). Total biomass was always much higher than active biomass in all treatments. The reason for this increase is that CalcofluorTM dye binds with chitin and stains all fungi, living or dead. Fluorescein diacetate must be absorbed by living cells and metabolized to produce fluorescein and thus to fluoresce.

In the nitrogen/fungi incubation, both active and total fungal mycelia increased after 2 weeks in all samples except for the control sediment (figure 11). Active and total mycelia were at their highest levels in treatments where the fungal nitrogen had been added. The low nitrogen treatment showed a total biomass of 18.94 ± 6.86 m/g of mycelia and an active biomass of $2.43 \pm .47$ m/g of mycelia after 2 weeks. The high nitrogen treatment showed a total biomass of 49.85 ± 12.13 m/g of mycelia and an active biomass of $3.25 \pm .49$ m/g of mycelia after 2 weeks. Sediment amended with only sawdust added showed an increase in fungal biomass suggesting the presence of native fungi on the sawdust. Control sediment showed only a minor increase in total fungal biomass. By week 6, all sediment treatments inoculated with fungi and with or without nitrogen showed a decrease in levels of active mycelia. This suggests that fungal growth peaked earlier in these incubations. One possible problem could be the presence of materials toxic to the fungi. High concentrations of metals such as cadmium and mercury have been shown

to be toxic to *P.ostreatus* and limit colonization in solid culture (Baldrian et al 2000). Another possibility is that the sediment may have been too wet.

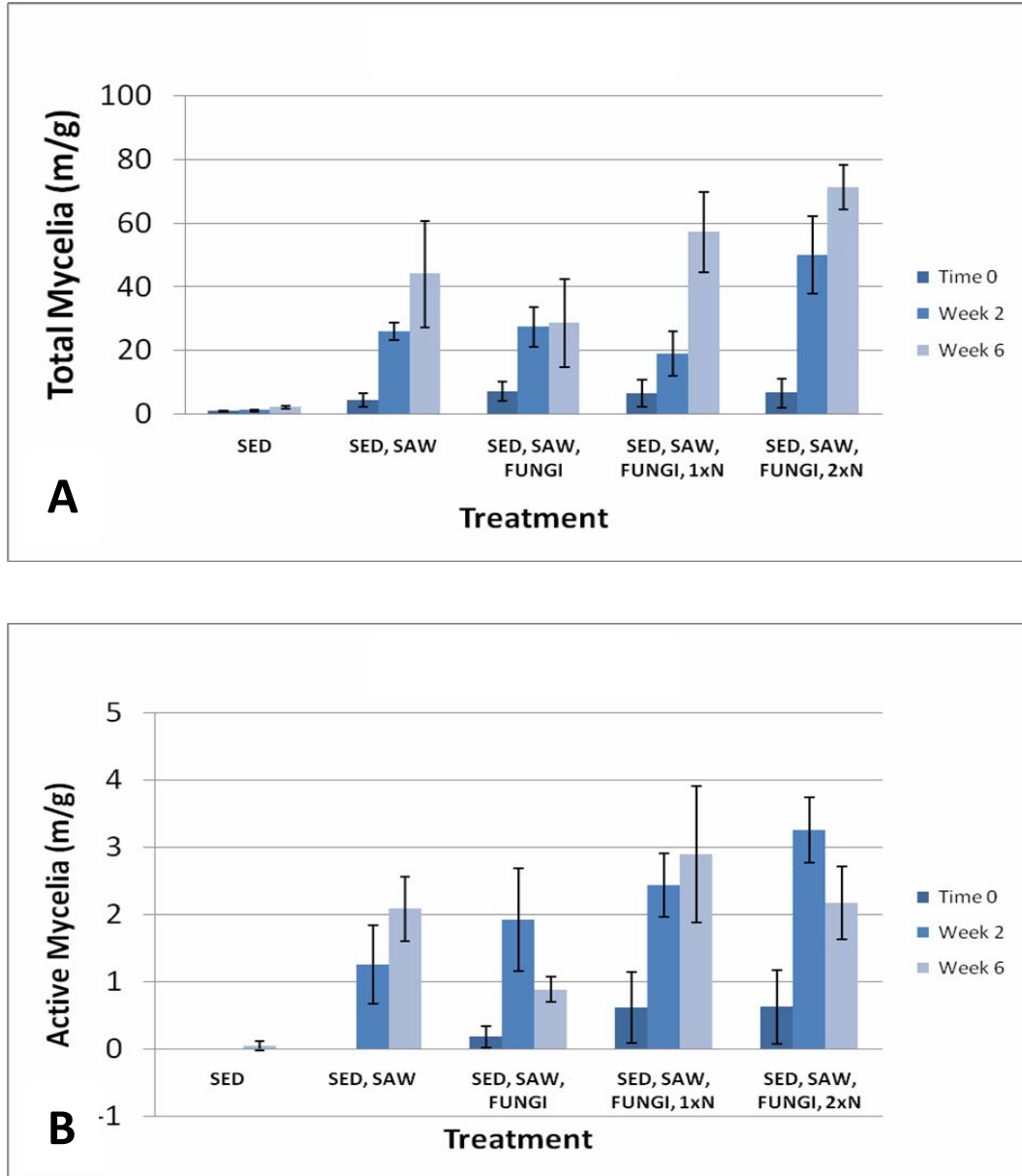


Figure 11. Nitrogen/fungi incubation data for total fungal biomass (A) and active fungal biomass (B) at time 0 (black), week 1 (dark grey) and week 2 (light grey). Standard deviation is shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen

Visual evidence correlates with biomass data in the nitrogen/fungi incubations. Treatments inoculated with *P. ostreatus* showed visible fungal growth. Sediment amended with the fungal nitrogen source and or only sawdust also showed visible fungal growth. This was expected since the sawdust was not sterile. Sediment only controls showed no visible fungal growth. After approximately 2 weeks sediment inoculated with fungi and amended with nitrogen, were covered by fungal mycelia, some of which appeared to be *P. ostreatus*. At two weeks, sediment inoculated with *P.ostreatus*, but without nitrogen, showed the most visible fungal mycelia that appeared to be *P. ostreatus*. Between two and six weeks no new growth visually appeared to have occurred on any of the samples.

Time 0 levels of fungal biomass in the larger scale cyclodextrin/fungi incubation (**figure 12**) were similar to those found in the nitrogen/fungi incubation (**figure 11**). Sediment inoculated with fungi showed higher initial fungal biomass values as expected. However, levels of active fungi did not appear to increase between time 0 and week 2 in sediment inoculated with fungi. The active biomass value at time 0 was 6.95 ± 0.92 m/g of mycelia and 6.10 ± 0.15 m/g of mycelia at 2 weeks. Total fungal biomass did increase from 25.36 ± 7.16 m/g of mycelia at time 0 to 114.48 ± 37.58 m/g of mycelia at week 2 in this treatment, indicating that fungi had in fact grown. Fungal mycelia was detected in sediment that had fungi added to it at 2 weeks (**figure 12**). The amount detected (active biomass of 7.50 ± 1.70 m/g of mycelia) was similar to what was seen for the treatment that had fungi added to it at time 0.

Visual evidence correlates with biomass data in the cyclodextrin/fungi incubation (**figure 8**). However, by week 2, fungal growth appeared to have stopped in these treatments; only

sporadic colonies were visible. When fungi had been added to sediment after 2 weeks, noticeable visible fungal growth with the appearance of *P. ostreatus* appeared in treatments within 3 days.

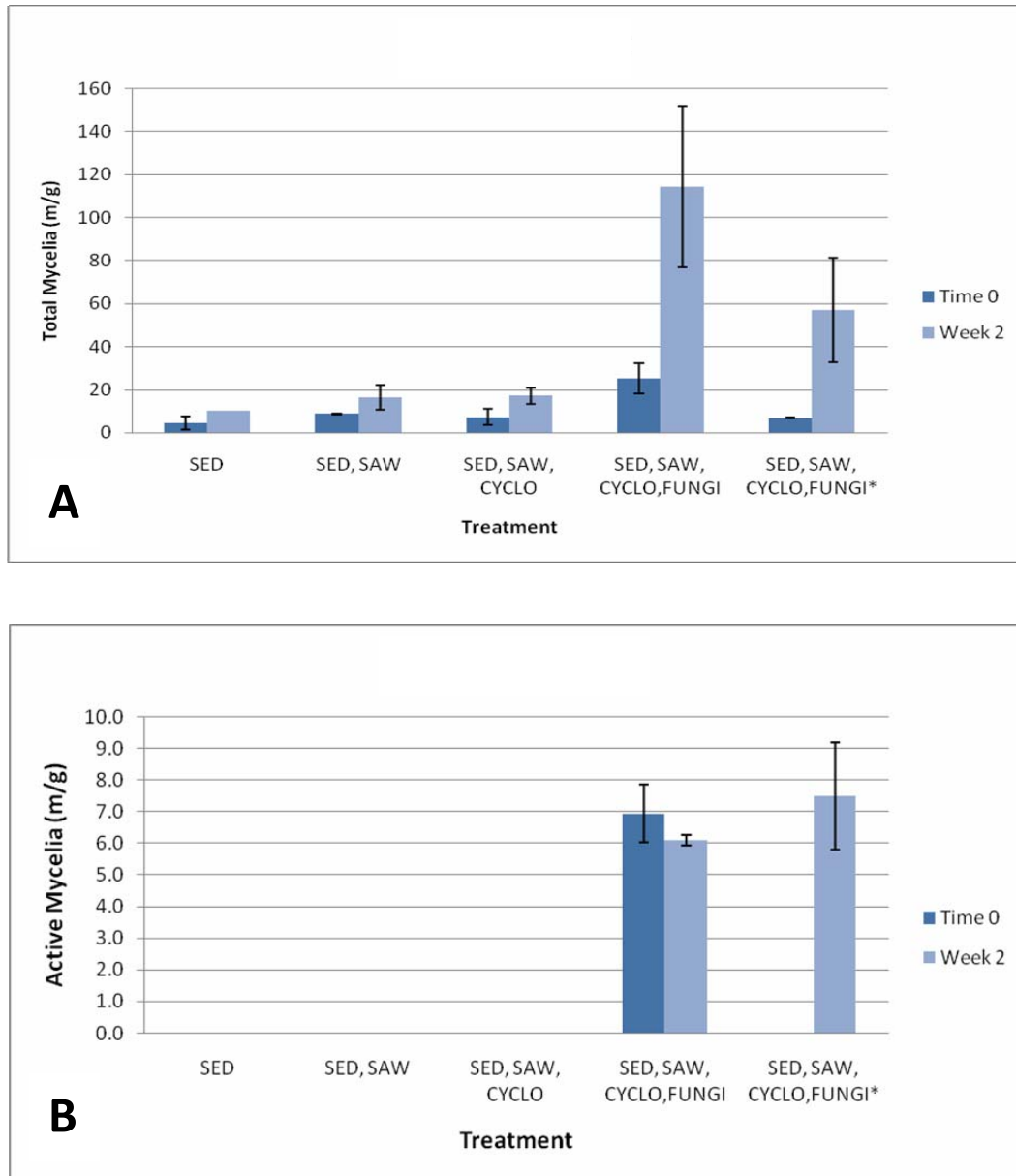


Figure 12. Cyclodextrin/fungi incubation data for total fungal biomass (A) and active fungal biomass (B) at time 0 (black), week 1 (dark grey) and week 2 (light grey). Standard deviation is shown as error bars. (N=2)

Molecular data indicated that *P. ostreatus* was present in inoculated sediment after 2 weeks. Analyses were performed on treatments containing fungi, nitrogen and sawdust. RFLP

analysis showed that colonies isolated after 2 weeks, that were similar in appearance to *P.ostreatus* displayed the same band patterns as found for *P. ostreatus* from pure culture (**figure 13A**). Samples isolated that did not appear to be *P.ostreatus*, had very dissimilar band patterns (**figure 13B**).

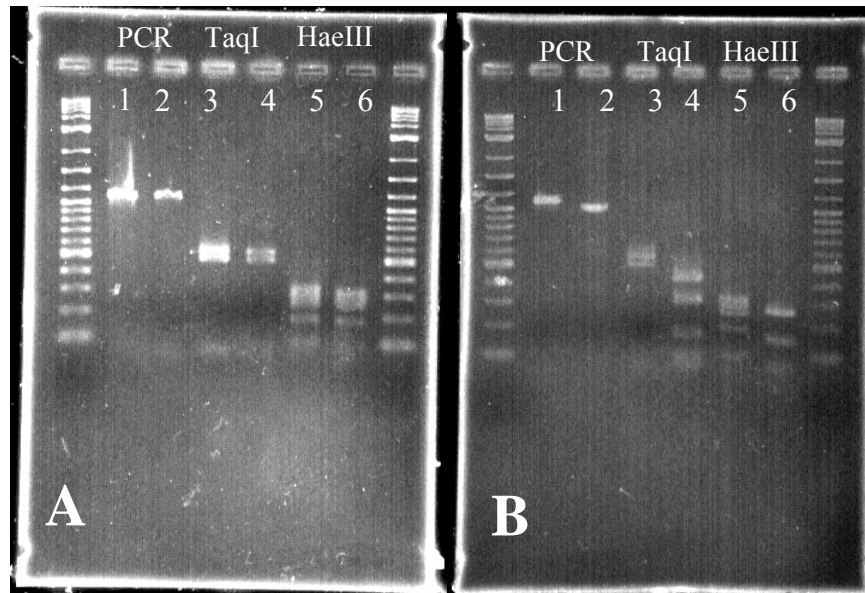


Figure 13. RFLP analysis of PCR amplified ITS DNA from fungi isolated from sediment amended with fungi, nitrogen and sawdust treatment after 2 weeks. The restriction enzymes used were *TaqI* and *HaeIII*. In A, PCR amplified DNA from pure culture *P.ostreatus* (lanes 1, 3 and 5) was compared to that isolated from colonies similar in appearance to *P. ostreatus* (lanes 2, 4 and 6). In B, PCR amplified DNA from pure culture *P. ostreatus* (lanes 1,3 and 5) was compared to that isolated from treatments that did not appear to be *P. ostreatus* (lanes 2,4 and 6). A 0.1-10.0Kb ladder was used in all gels.

The moisture content of the sediment during these incubations remained at approximately 45% moisture (**figure 14A and 14B**). The higher variability in moisture content for some samples was likely due to the presence of rocky debris. Some treatments appeared to be much wetter or dryer, however all values were relatively stable of the moisture. This suggests that a more accurate way of measuring the available moisture is required for further analysis.

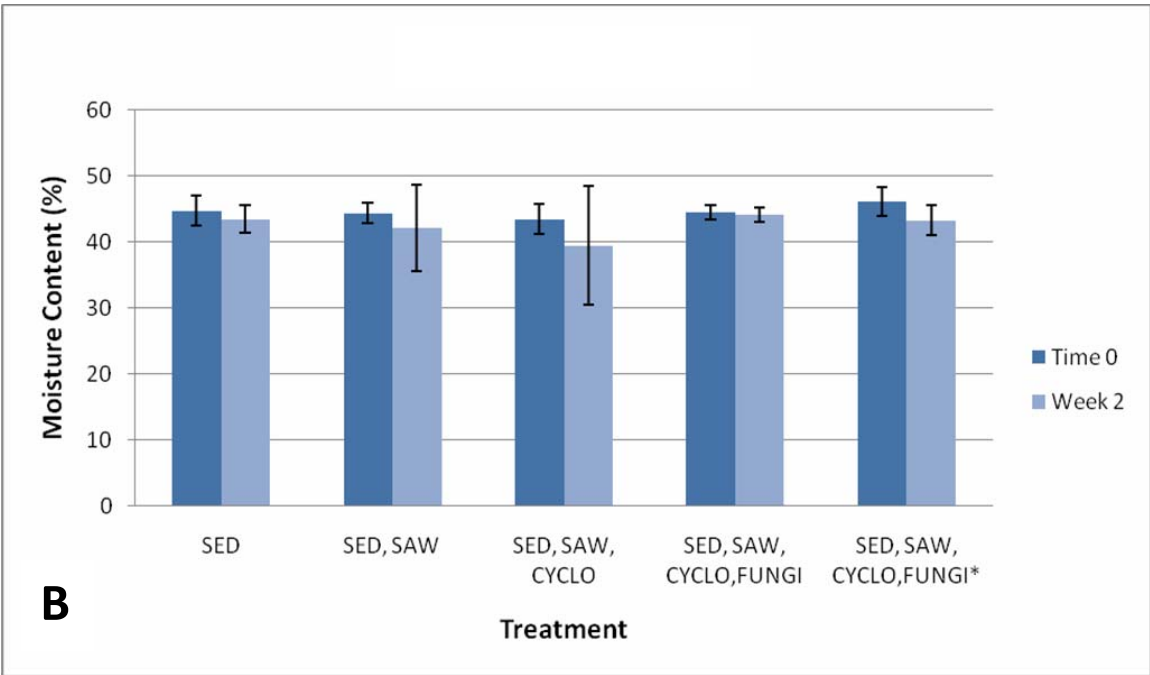
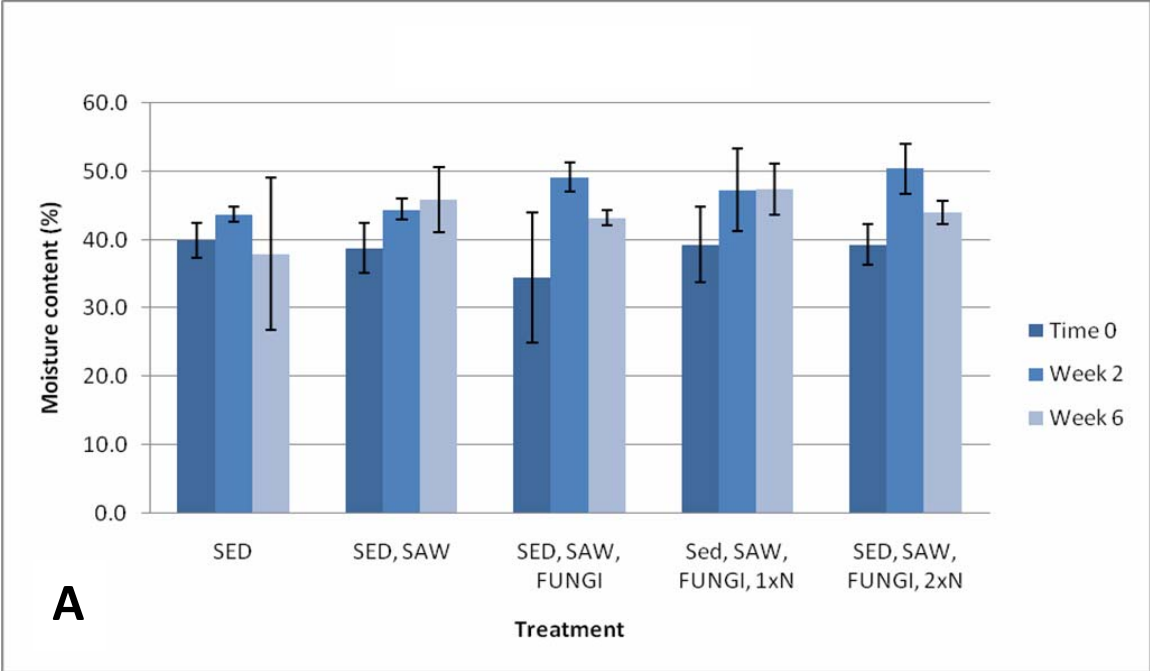


Figure 14. Sediment moisture content. Nitrogen/fungi incubation (A) and cyclodextrin/fungi incubation (B). Standard deviations are shown as error bars.

Table 3. Number of benzene rings within the chemical structure of detected PAHs

PAH	Number of Benzene Rings	PAH	Number of Benzene Rings
Napthalene	2	Benzo(a)anthracene	4
Acenaphthylene	2	Chrysene	4
Acenaphthene	2	Benzo(b)fluoranthene	5
Fluorene	2	Benzo(k)fluoranthene	5
Phenanthrene	3	Benzo(a)pyrene	5
Anthracene	3	Dibenzo(a,h)anthracene	5
Fluoranthene	3	Indeno(1,2,3-cd)pyrene	6
Pyrene	4	Benzo(ghi)perylene	6

To characterize PAHs and their degradation, PAHs were grouped based on their number of benzene rings (**table 3**). Starting total PAHs concentrations were approximately 140-160 ppm in the nitrogen/fungi incubation (**figure 15**). The greatest decreases in PAHs occurred between time 0 and week 2. Due to variability, high standard deviations, no treatment effects were detected in the degradation of total PAHs (**figure 15**), 2-ring PAHs (**figure 16**), and 3-Ring PAHs (**figure 17**). Sediment only controls appeared to have the same rate of degradation as other treatments. At week 6, PAH concentrations either remained the same as week 2, except for possibly 5-ring PAHs. This was most likely due to the presence of only low levels of metabolically active fungi (**figure 11B**) and the innate heterogeneity of the sediment.

The nitrogen amendments appeared to have had no significant impact upon the degradation of PAHs. However, nitrogen amendments did increase the fungal biomass to its highest levels (**figure 11**). The relatively high fungal biomass in this treatment included uncharacterized fungi associated with the sediment and sawdust. Uncharacterized fungi may not have been capable of degrading PAHs and likely outcompeted *P. ostreatus*. Sediment amended

with high levels of nitrogen did showed abundant spore forming fungi that appeared to be *Aspergillus*.

Sediment inoculated with *P.ostreatus* did appear to show increased degradation of 5-ring PAHs. Values of 5-ring PAHs decreased from 35.96 ± 5.61 ppm to 13.69 ± 2.00 ppm after 2 weeks in 5 ring PAHs (**figure 19**). The variability was too high to show a significant effect in fungal degradation of 6-ring PAHs (**figure 20**). Previous studies have shown that high molecular weight PAHs degrade much more rapidly in the presence of white rot fungi (**Johnsen et al. 2005 and Baldrian et al 2000**). They are better able to degrade higher molecular PAHs because their extracellular enzymes can degrade PAHs that are more tightly adsorbed to sediment and thus less bioavailable to other microbes.

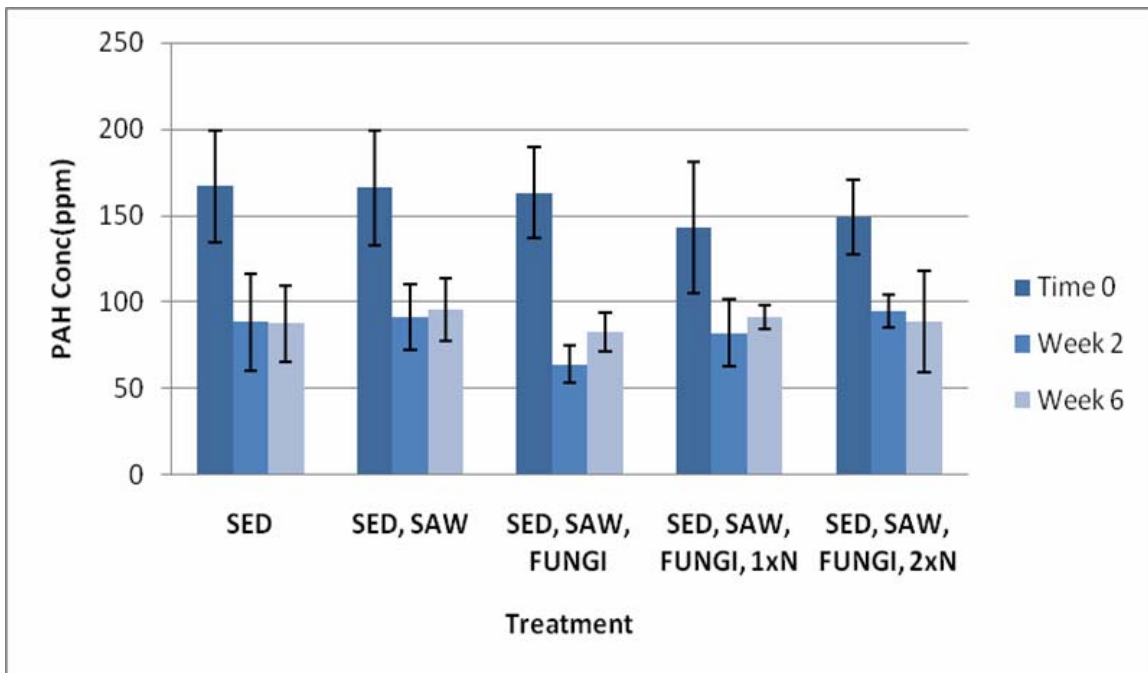


Figure 15. Changes in the concentration of **total PAHs** in the nitrogen/fungi incubation. Standard deviations are shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen, Fungi=*P. ostreatus*

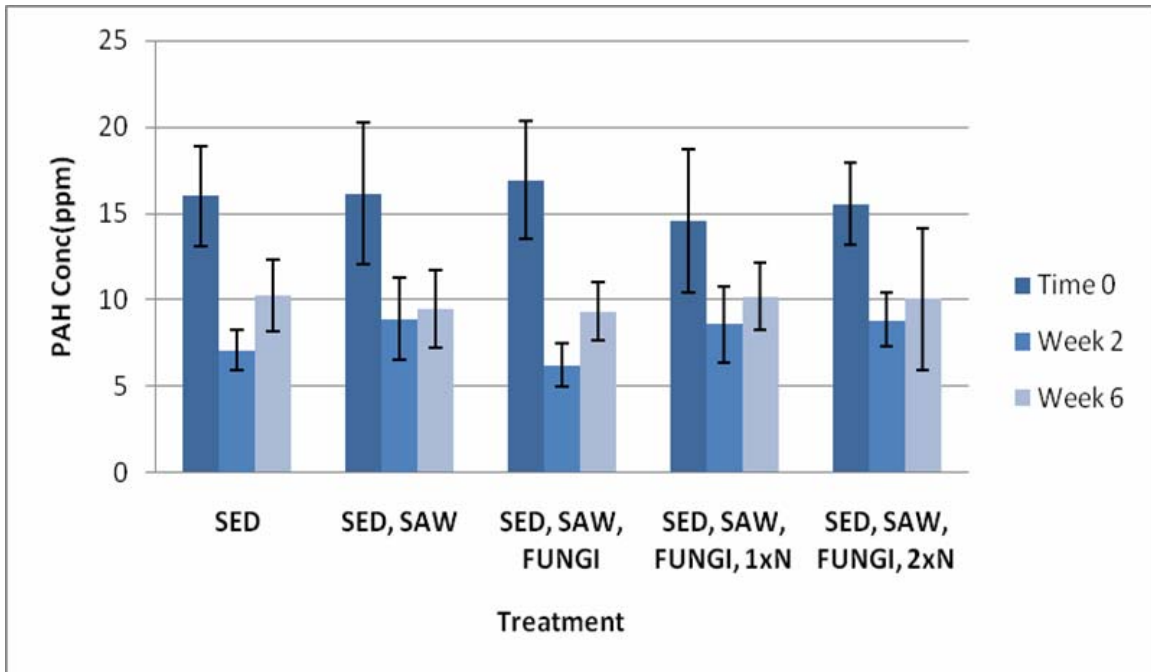


Figure 16. Changes in the concentration of **2-ring PAHs** in the nitrogen/fungi incubation. Standard deviations are shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen, Fungi=*P. ostreatus*

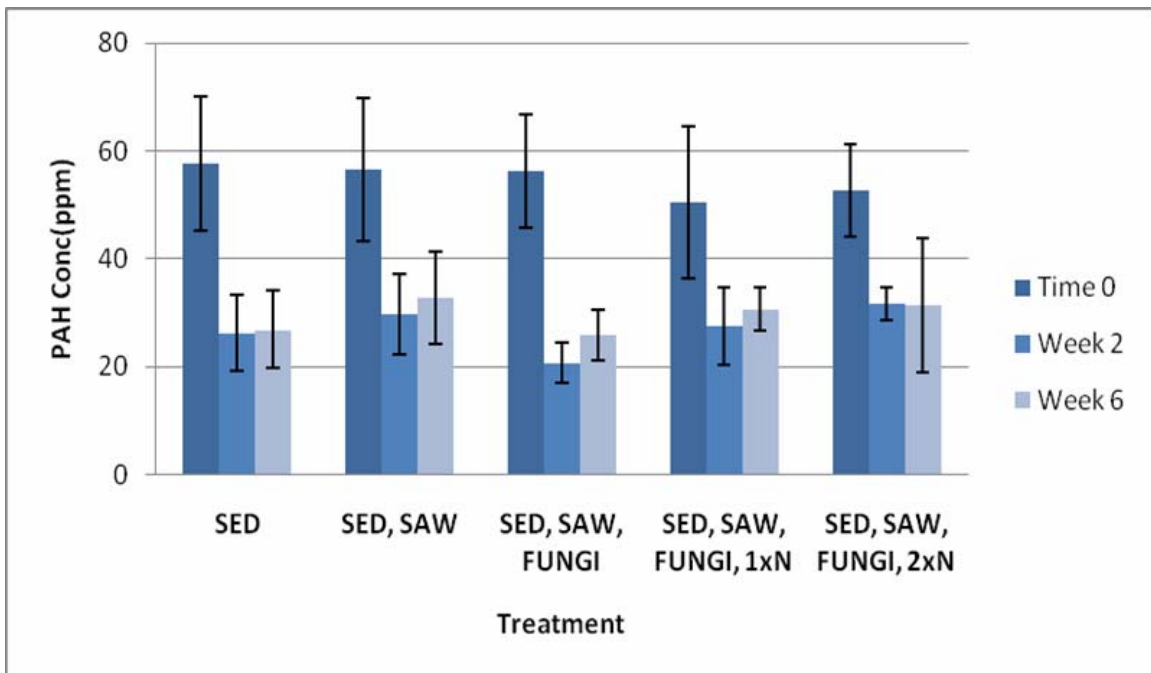


Figure 17. Changes in the concentration of **3-ring PAHs** in the nitrogen/fungi incubation. Standard deviations are shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen, Fungi=*P. ostreatus*

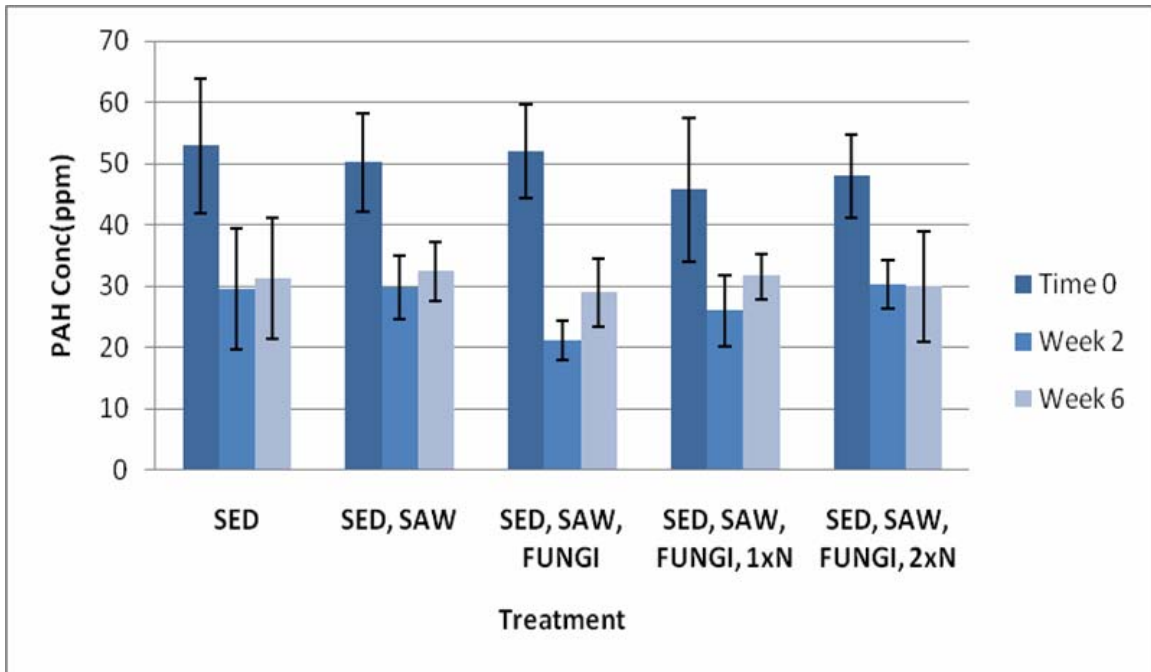


Figure 18. Changes in the concentration of **4-ring PAHs** in the nitrogen/fungi incubation. Standard deviations are shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen, Fungi=*P. ostreatus*

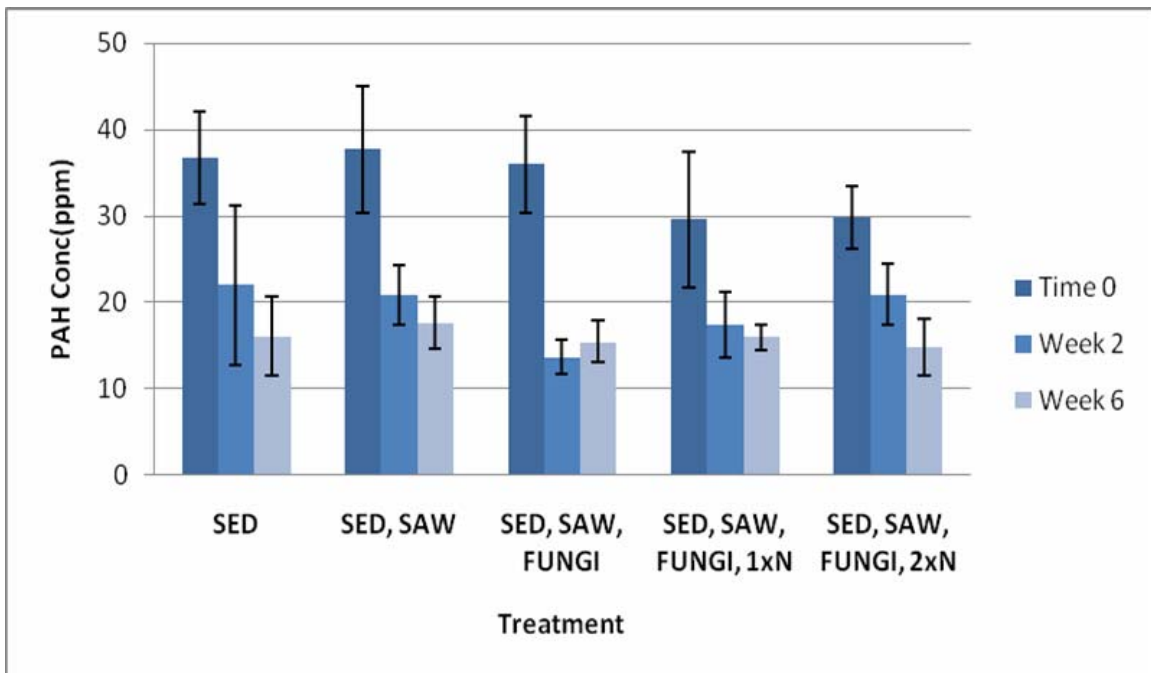


Figure 19. Changes in the concentration of **5-ring PAHs** in the nitrogen/fungi incubation. Standard deviations are shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen, Fungi=*P. ostreatus*

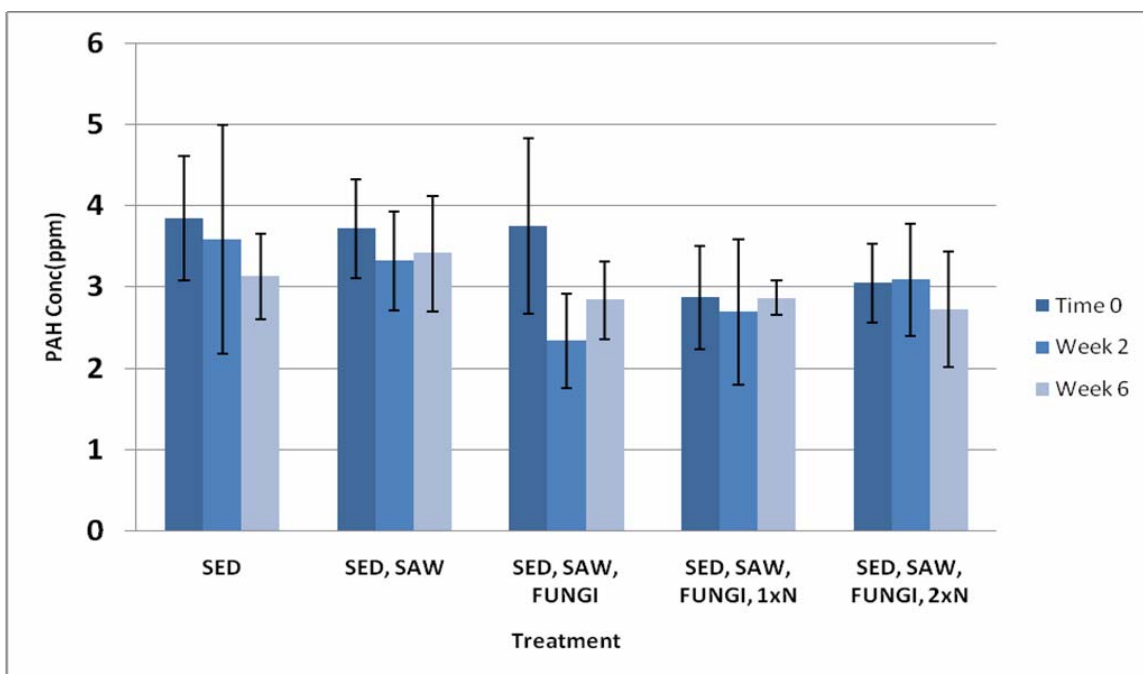


Figure 20. Changes in the concentration of **6-ring PAHs** in the nitrogen/fungi incubation. Standard deviations are shown as error bars. SED = Sediment, SAW= Sawdust, N=Nitrogen, Fungi=*P. ostreatus*

In the second larger scale cyclodextrin/fungi incubation, the total amount of PAHs found in all treatments at time 0 was between 63 and 90 ppm (**figure 21**). These values were 40-60% lower than found in the first incubation and were likely due to heterogeneity in the sediment collected. There was high degree of variability seen in the PAH concentrations, indicating that a further increase in replicates is needed. A possible treatment effect did occur in regards to the addition of sawdust. As can be seen in **figures 21-26** all sediment amended with sawdust showed lower PAH concentrations at week 2 than the sediment only control. A previous experiment performed in the lab on smaller scale showed similar results (data not shown).

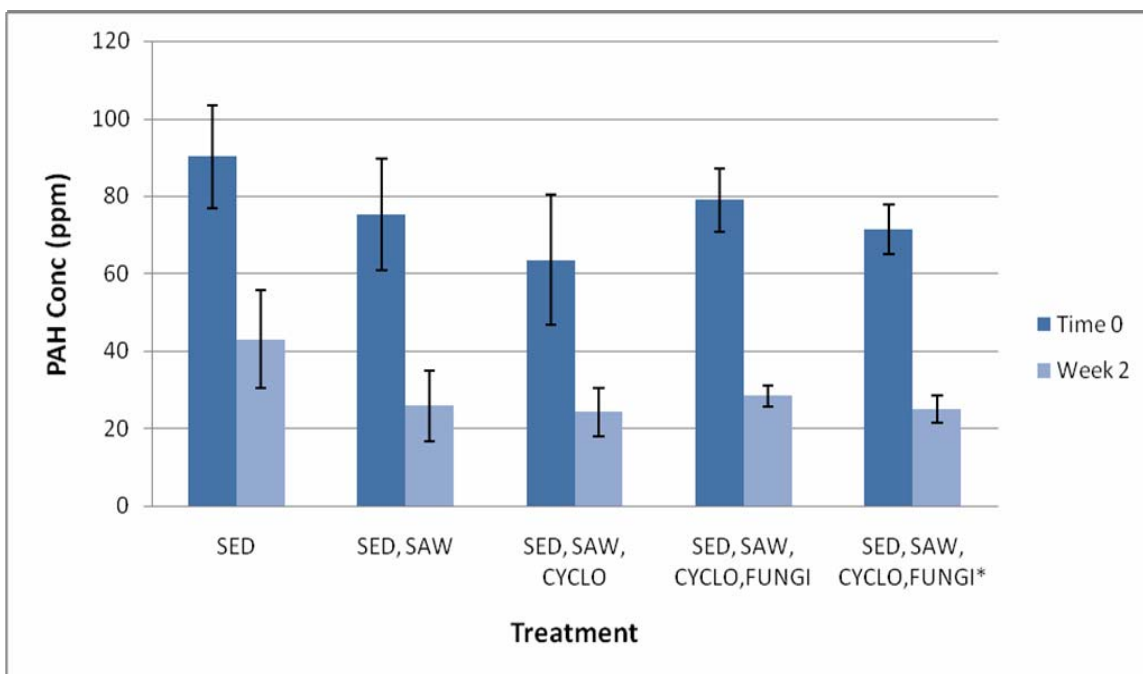


Figure 21. Changes in the concentration of **total PAHs** in the cyclodextrin/fungi incubation. Standard deviations are shown as error bars. SED=Sediment, SAW=Sawdust, CYCLO=Cyclodextrin, Fungi = *P. ostreatus*, Fungi*=Fungi added at 2 weeks

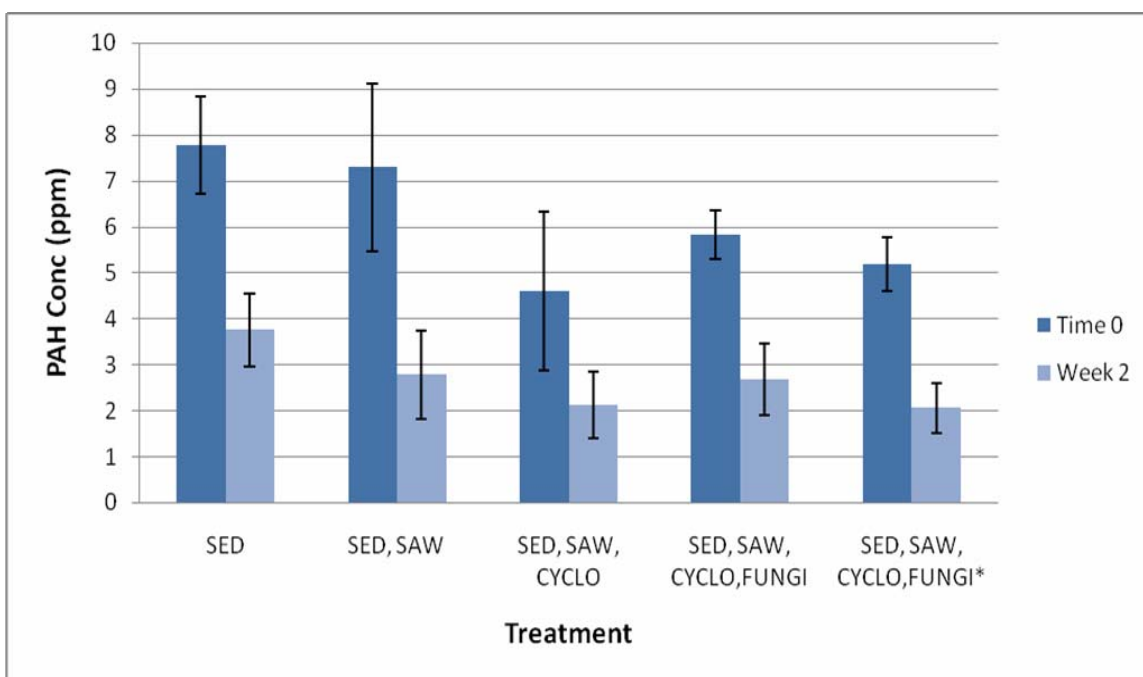


Figure 22. Changes in the concentration of **2-ring PAHs** in the cyclodextrin/fungi incubation. Standard deviations are shown as error bars. SED=Sediment, SAW=Sawdust, CYCLO=Cyclodextrin, Fungi = *P. ostreatus*, Fungi*=Fungi added at 2 weeks

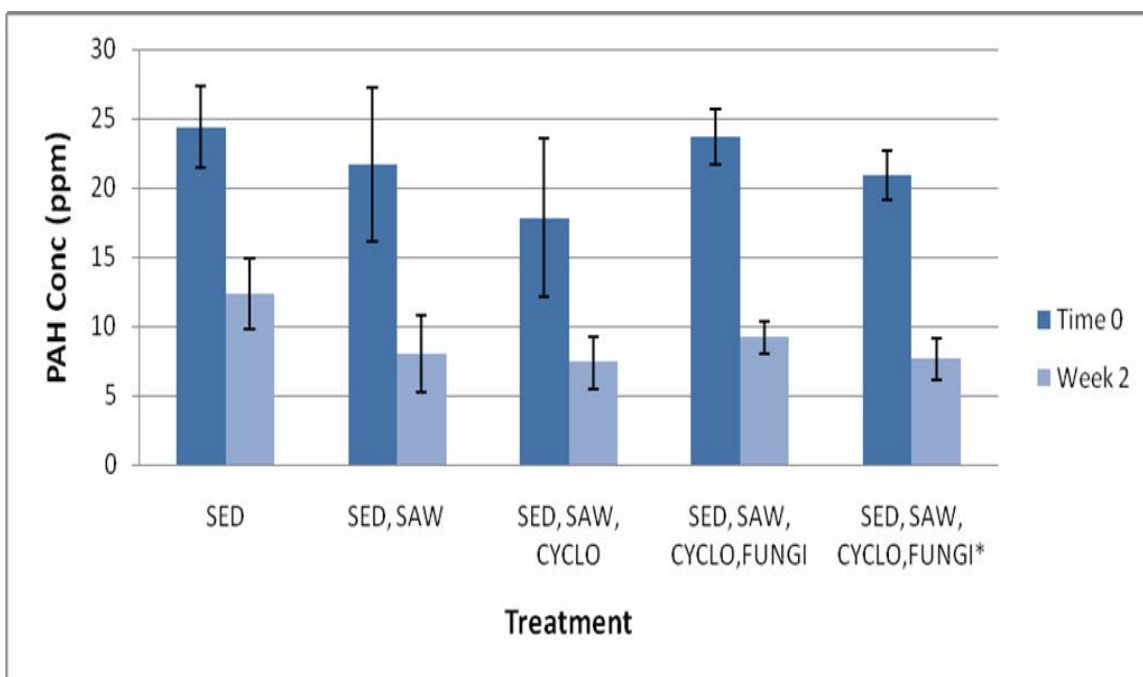


Figure 23 Changes in the concentration of **3-ring PAHs** in the cyclodextrin/fungi incubation. Standard deviations are shown as error bars. SED=Sediment, SAW=Sawdust, CYCLO=Cyclodextrin, Fungi = *P. ostreatus*, Fungi*=Fungi added at 2 weeks

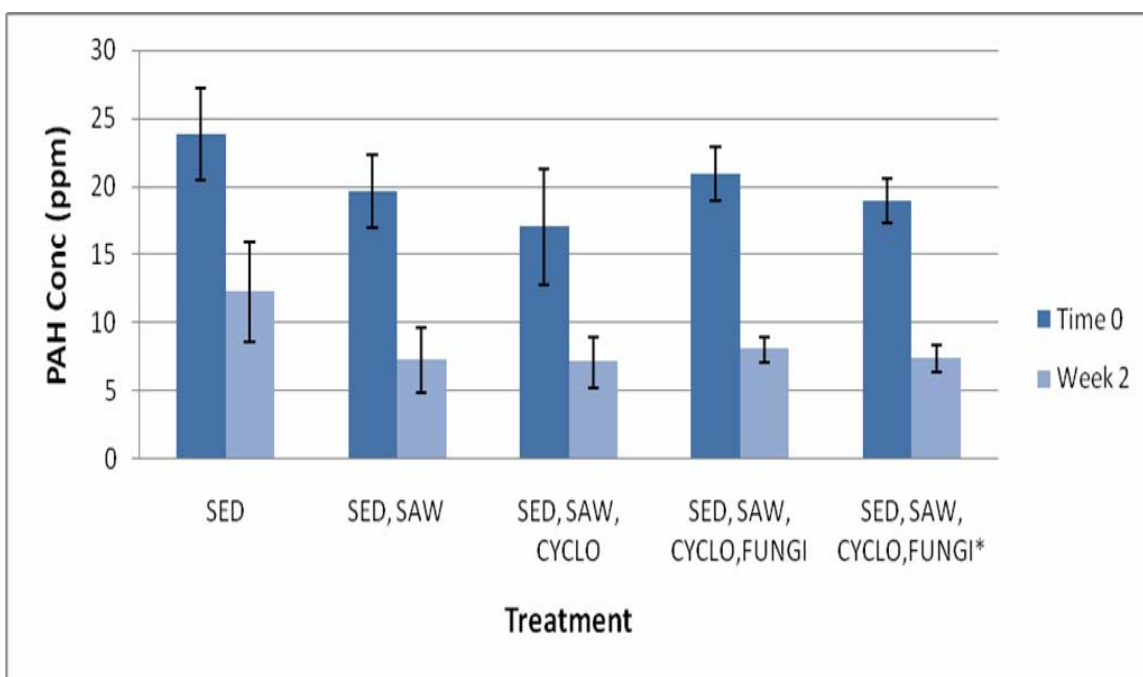


Figure 24. Changes in the concentration of **4-ring PAHs** in the cyclodextrin/fungi incubation. Standard deviations are shown as error bars. SED=Sediment, SAW=Sawdust, CYCLO=Cyclodextrin, Fungi = *P. ostreatus*, Fungi*=Fungi added at 2 weeks

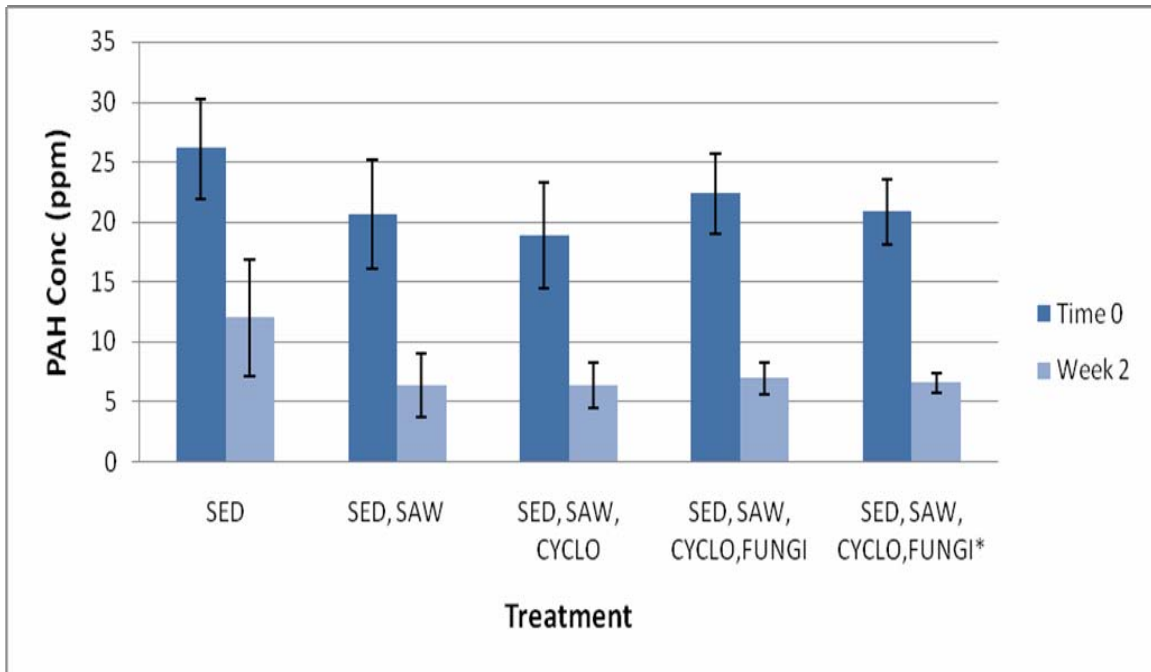


Figure 25. Changes in the concentration of **5-ring PAHs** in the cyclodextrin/fungi incubation. Standard deviations are shown as error bars. SED=Sediment, SAW=Sawdust, CYCLO=Cyclodextrin, Fungi = *P. ostreatus*, Fungi*=Fungi added at 2 weeks

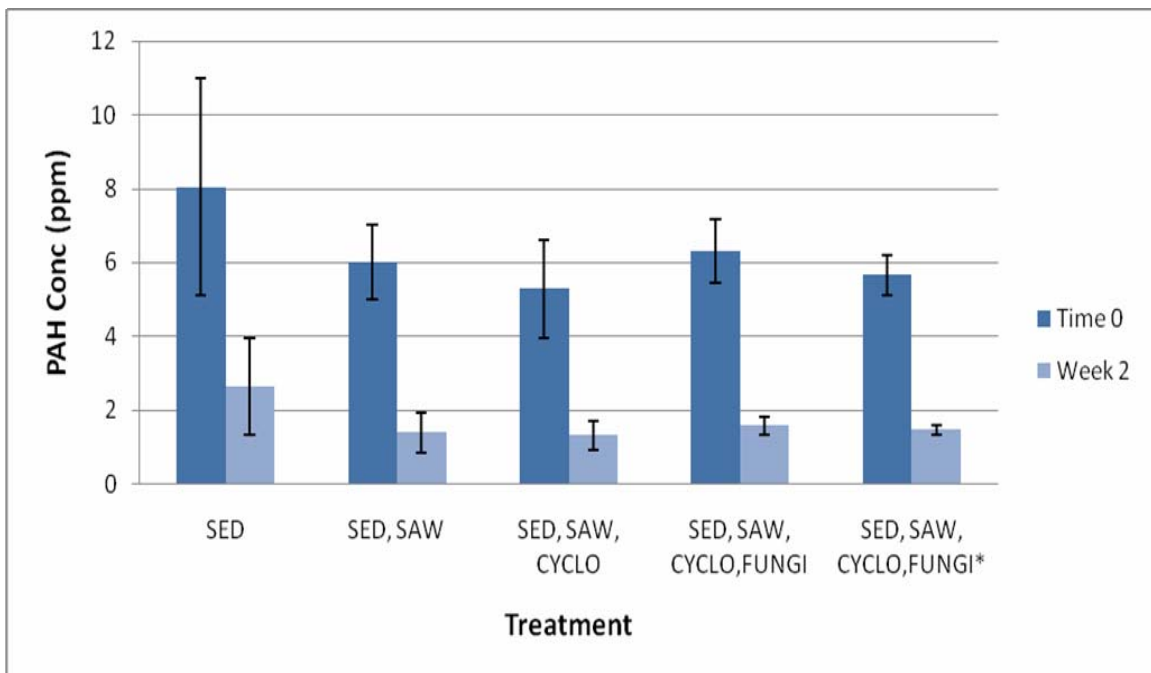


Figure 26. Changes in the concentration of **6-ring PAHs** in the cyclodextrin/fungi incubation. Standard deviations are shown as error bars. SED=Sediment, SAW=Sawdust, CYCLO=Cyclodextrin, Fungi = *P. ostreatus*, Fungi*=Fungi added at 2 weeks

It is important to note that sediment treated with and without fungi showed similar PAH degradation rates. With some exception, increased fungal biomass levels did not correlate with the greatest degradation rates, indicating that overall fungal PAH degradation was negligible. The disappearance of PAHs must have arisen from another factor. It is most likely due to a combination of aerobic degradation by native bacteria present within the sediment and volatilization. Many studies have shown that high levels of PAH degrading bacteria are prevalent within historically contaminated sediment. One such study successfully utilized indigenous bacteria from historically PAH contaminated sediment to degrade phenanthrene, a common PAH (**Tam et al. 2002**). Many species of bacteria are capable of breaking down PAHs and utilizing them as a carbon source, some of the more common species of PAH degraders include *Pseudomonas*, *Rhodococcus* and *Beijerinckia* (**Johnsen et al. 2005**). To increase bioavailability of some recalcitrant compounds, such as PAHs many bacteria can secrete biosurfactants which will increase their bioavailability and uptake (**Johnsen et al. 2005**).

Degradation of PAHs via bacterial mechanism can occur either aerobically or anaerobically. Studies have shown that aerobic degradation of PAHs is much more efficient, when compared to anaerobic degradation, by several orders of magnitude (**Rockne and Strand 1998**). Studies have shown that the addition of oxygen can be an effective treatment in the bioremediation of PAHs. In one such study it was found that exposure to oxygen was extremely effective in the degradation of PAHs contained within historically contaminated river sediment (**Lei et al. 2005**). During their study they were able to degrade significant levels of 2-ring, 3-ring, 4-ring and 5-ring PAHs in the presence of sufficient oxygen.

One of the most common methods used by bacteria for the aerobic degradation of PAHs is the use of dioxygenase enzyme mechanism (**Bamforth and Singleton 2005**). A simplified mechanism for the degradation of PAHs is that dioxygenase enzymes utilize oxygen and hydroxylize PAHs (**Bamforth and Singleton 2005**). After hydroxylation, the PAHs are then dehydrogenated by dehydrogenase enzymes. After being dehydrogenated PAHs can then be completely mineralized by catechols (**Bamforth and Singleton 2005**). The main problem with dioxygenase enzymes, in PAH degradation, is that these enzymes are bound to the bacteria and cannot diffuse into the environment (**Johnsen et al. 2005**). This leads to problems in the degradation of higher weight PAHs, which are more recalcitrant and less bioavailable.

4. Conclusions

The data found from this indicate that more work needs to be done to optimize PAH degradation by *P. ostreatus* in historically contaminated Mahoning river sediment. Increasing the number of replicates, controlling moisture and oxygen levels more effectively are all recommended for further studies.

The data shows that simply mixing and exposing sediment to oxygen correlated with a consistent and significant reduction in the concentrations of PAHs present within the sediment. This may be in part due to the oxygen increasing the volatility of the PAHs contained within the sediment. Another possibility is that exposure to oxygen stimulated the aerobic degradation of PAHs by native bacteria found within the sediment. This information will be useful for future applications in the restoration of historically PAH contaminated sediment. Aeration and mixing the sediment would be a cost effective treatment.

5. Recommended Work

Several subjects are recommended for further analysis. Completing the experiment is the most pressing. Data shown in this project is at week 2 for the larger scale cyclodextrin/fungi experiment. Time points for analysis still need to be completed at week 4, week 6, week 8 and week 10.

Another such subject would be the identification of microbial populations in the sediment samples and treatments using clone libraries, denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (TRFLP) analysis. Identifying bacterium and fungal populations present at time 0 and over time would be informative. For example one bioremediation study demonstrated that dramatic shifts in the populations of bacteria occurred during the bioremediation of creosote contaminated soil, with PAH degraders becoming more prevalent in soil (**Vinas et al. 2005**). Once certain conditions are found to induce the growth of populations of known PAH degraders, these conditions could be used in further bioremediation treatments.

Finally another area to pursue would be the quantification and analysis of expression of enzymes produced by fungi introduced into the sediment. Quantitating lignolytic enzyme activity, levels of lignolytic enzymes and mRNA of lignolytic enzymes would certainly help in completely optimizing fungal bioremediation in this system.

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Appendix 1

Nitrogen/Fungi Incubation Set Up

Contaminated river sediment (1000 ml) is removed from storage and placed into DCM washed mixing bowls. Non sterile sawdust (800 ml), with all rocks and large pieces of wood removed, is added to the sediment. Nitrogen is added using a commercially available fungal nitrogen source at either 25 ml or 50ml to low or high nitrogen treatments respectively. After all materials had been added the treatments are homogenized by hand mixing. Treatments with fungi added are mixed last and fungi is added last. Approximately 5 g aliquots are taken from the mixing bowls and placed into DCM cleaned VOA vials (Restek# 26121). Fungal inoculated grain (3 grains) is added to the sediment for fungal treatments. Only grains lightly coated with fungi are added to the treatments, no solid chunks of fungi were added DI water moistened paper towel stripes are then placed into the vials to keep treatments moistened. All vial lids are secured and vials are then placed into an incubator at 25°C. Every 3 days the lids are removed momentarily from the vials to avoid them from becoming anaerobic. Separate vials for PAH analysis and biomass are created in triplicate for each time point.

Cyclodextrin/Fungi Large Scale Incubation Set Up

Contaminated Mahoning River sediment (500 ml), all large twigs, leaf debris and rocks have been removed, is placed into a DCM washed mixing bowl. Depending upon the treatment different materials are added to the sediment inside of the bowl. β -cyclodextrin(fisher scientific #AC22728-1000) (4 g) is added to the sediment and homogenized by hand. Non sterile sawdust (400 ml), with all rocks and large pieces of wood removed, is then placed onto the sediment and

homogenized by hand. Fungal inoculated grain is then added to the sediment mix and homogenized. All fungal grain is lightly coated with fungi and no solid fungi is added to the sediment mix. The sediment is then placed into a DCM washed 2 L glass bowl with autoclaved gravel placed at the bottom for control of moisture. Two twist ties are then placed on top of the bowl to allow for a small Petri dish to hang over the sediment. Moistened paper towels are placed into the Petri to keep the sediment moist. Plastic wrap is then placed over the top of the bowl. The treated bowl is then placed into an incubator at 25°C.

Appendix 2

Total Fungal Biomass

For total fungal mycelia a modified version of a fluorescent microscopy method is used (Stahl et al. 1996). Approximately 0.23 grams of CalcofluorTM (Sigma Aldrich Product # F3543-5G) is added to 1 L of Milli-Q water. The reagent is then mixed by gentle shaking and can then be stored at 4°C until use.

Approximately 1 g of sediment is removed from the treatment and added into 100 ml of sterile water, inside of a sterile Waring blender head. The water and sediment are then mixed at low speed for 1 minute. The sample (1 ml) is then removed using a Luer Lock syringe (Fisher Scientific Product # AC22728-1000) and is passed through a 0.22 µm black mesh polycarbonate filter (Fisher Scientific Product # GTBP02500). CalcofluorTM solution (1 ml) is allowed to sit on the filter for 30 seconds and then removed. The filter is then placed onto a glass microscope slide and allowed to dry. Two drops of immersion oil are placed onto the dry filter and a glass cover slip is placed on top. Ten random fields of view are then taken using Ax70 fluorescent microscope at 100x magnification using cube D (432 nm). All mycelia that are found on the slides are measured and recorded using the Microsuite Special Edition program. The amount of fungal mycelia found on the slides is then converted into meters of mycelia per gram of sediment (m/g) with the following equation.

$$M * 100 / .1924 = \text{mycelia per gram of sediment}$$

M = The total length of mycelia found on ten images, 225 is the dilution of the sediment and 0.1924 is the amount of the filter observed with the ten fields of view. 0.1924 is calculated

by dividing the area of the 10 fields of view (58502 μm^2) by the total area of the filter (490625 μm^2).

Active Fungal Biomass

For the analysis of metabolically active fungal a fluorescent microscopy method is used (Stahl et al. 1996). Two grams of fluorescein diacetate (FDA) (Sigma Aldrich Product # F7378-10G) is hand mixed into 1 L of acetone. The solution is then stored at -20°C until use.

Milli-Q water (200ml) is placed into a sterile Waring blender head. Sediment (1 g) is added to the 200 ml of water. The water and sediment is mixed at low speed for 1 minute. FDA solution (25 ml) is added to the 200 ml sediment water solution. The solution is then allowed to sit for 3 minutes, with occasional mixing at low speed. The solution is then mixed again at low speed for 1 minute. Solution (2 ml) is removed from the blender head and passed through a 0.22 μm black mesh polycarbonate filter. The polycarbonate filter is then removed, placed onto a microscope slide and allowed to dry. Two drops of immersion oil are placed onto the dry filter and a glass cover slip is placed on top. Ten random fields of view are then analyzed using the Ax70 fluorescent microscope at 200x magnification, cube B (524 nm) is used for analysis. All mycelia that are found on the slides are measured and recorded using the Microsuite Special Edition program. The amount of fungal mycelia found on the slides is then converted into meters of mycelia per gram of sediment (m/g) with the following equation.

$$\mathbf{M * 112.5 / .11924 = \text{mycelia per gram of sediment}}$$

M = The total length of mycelia found on ten images, 112.5 is the dilution of the sediment and 0.11924 is the amount of the filter observed with the ten fields of view. 0.11924 is

calculated by dividing the area of the 10 fields of view (58502 μm^2) by the total area of the filter (490625 μm^2).

Appendix 3

PAH Extraction and Analysis

Approximately 5 grams of sample are weighed out in DCM cleaned 60 ml VOA (Volatile Organic Analysis) vials (**Restek product#26121**). The mass of sample is recorded for later calculations. Approximately 15 g of sodium sulfate (**Fisher Scientific Product# S421-1**) is then thoroughly mixed into the sediment sample using a DCM cleaned stainless steel spatula. DCM (20 ml) (**Fisher Scientific Product # D151-4**) is then added into the sample. B/N surrogate mix (10 ul), 2-fluorobiphenyl and p-terepheryl-d14, at a concentration of 1000 ug/ml (**Restek Product #31024**), is added into the sample. The sample is once again mixed using a DCM cleaned stainless steel spatula.

Samples are then placed onto a ultrasonic bath (**VWR product #98000-332**) and sonicated 3 times for 8 minutes each. After each sonication the sample is decanted off into DCM cleaned glass funnels lined with filter paper (**Fisher Scientific Product #1001-090**) and containing sodium sulfate (approximately 2 g). The samples are collected into DCM cleaned 150 ml beakers. Before each additional sonication 15 ml of DCM are added back into the samples. After all three sonications the remaining sodium sulfate contained within the VOA vials is poured onto the glass funnels lined with filter paper and sodium sulfate. An additional 2 ml of DCM is then pipetted onto the sodium sulfate in the funnels and allowed to drain. The volumes of samples contained within the 150 ml beakers are then recorded. The samples are then stored in DCM rinsed VOA vials at -20°C

Aliquots of the samples (1ml) are then placed into 2ml screw cap vials (**Sigma Aldrich Product # 27023**) with a screw cap containing a silicone septa (**Sigma Aldrich Product# 27262**). Internal standard (1ul), containing: 1,4-dichlorobenzene-d4, naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12 and perylene-d12, at a concentration of 2000ug/ml (**Restek Product# 31206**) is then added to each sample. The samples are then injected into a gas chromatograph mass spectrometer (Agilent 6890 GC System) with an auto sampler. The concentrations of PAHs found were converted to parts per billion (ppb) using the following equation:

$$(D*X)/W=C$$

D is the volume of DCM containing PAHs extracted from the sample (ml). X is the concentration of the sample given by the GCMS (ug/ml). W is the weight of the sample before extracting (kg). C is the concentration of PAHs in the sample (ug/kg or ppb).