Bioaugmentation using Pleurotus ostreatus to Remediate Polycyclic Aromatic Hydrocarbons (PAH) Contaminated River Sediment

By

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Abstract

The purpose of the study was to determine if polyaromatic hydrocarbon degradation in historically contaminated river sediment could be done when treated with the white-rot fungus *Pleurotus ostreatus. P. ostreatus,* grown on barley, was added to sediment with various amendments and controls, and incubated in triplicate at 25 ^{o}C for 42 days. Treatments included sawdust, shredded newspaper, a nitrogen source, and cyclodextrin. The most effective treatment included the addition of white-rot fungi, sawdust, nitrogen, and cyclodextrin. This treatment showed greater than 50% degradation of 9 our of the 11 PAHs with 95% degradation for benzo(b&k)fluoranthene. Fungal biomass (total mycelia and metabolically active mycelia) increased in all treatments with added fungi. The greatest increase in fungal biomass appeared in the same treatment with the greatest extent of PAH degradation (from 82 $\pm$ 10 m g sediment^{-1} at time 0 to 374 $\pm$ 18 m g sediment^{-1} at 42 days). These data shown that *P. ostreatus* is capable of colonizing highly contaminated Mahoning River sediment and degrading the PAHs present. With better optimization of amendments, this approach shows potential for remediating historically contaminated river sediment.

1. Introduction

1.1 Mahoning River

The Mahoning River is located in eastern Ohio and western Pennsylvania. The length of the river, stretches approximately 108 miles, and is associated with a watershed area of nearly 1,000 square miles (Figure 1). The Mahoning River is a part of the Ohio River watershed and connects to the Shenango River. The river itself travels through 5 Ohio counties and 1 county in western Pennsylvania. Approximately, 97 miles of the river are located within eastern Ohio.

Before industrialization, it was relatively undisturbed and the Mahoning River watershed served many important functions, both ecologically and economically. Industrialization, prior to 1970, in terms of runoff and dumping, has left both the river bottom and banks highly contaminated with PAHs and other hazardous materials. Today, there is approximately a 32 mile stretch of river bank and river bottom sediments that are highly polluted and requires attention in order to correct the contamination problem (US Army Corps of Engineers, Pittsburgh District, April 2004). The Mahoning River is one of the 5 most contaminated rivers in the United States. The Ohio Environment Protection Agency has placed a sediment contact ban on sediment of this region due to the specificity of the PAH content.The river supports life for over 70 different fish species, and some river areas themselves have a dense human population.

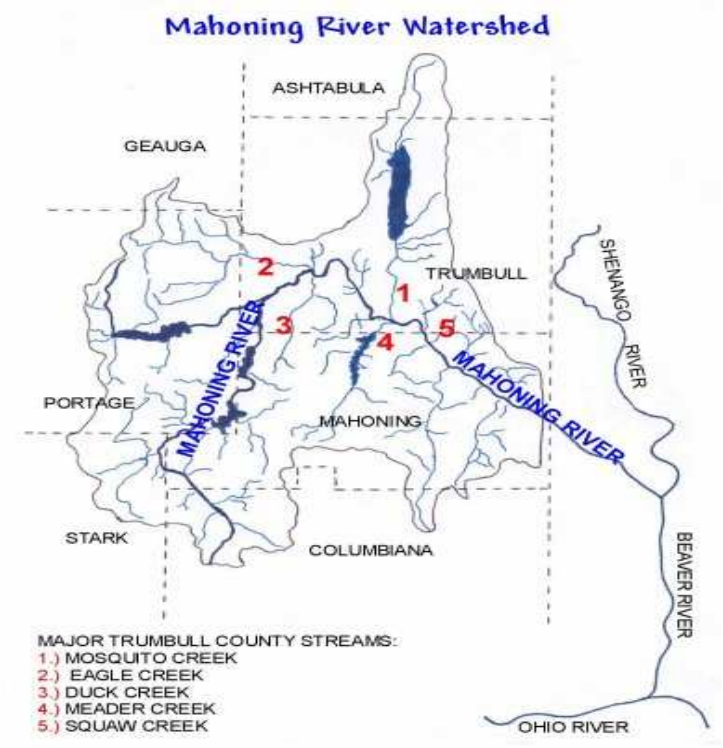

Fig.1 Picture of the Mahoning River Watershed
Courtesy of: www.swcd.co.trumbull.oh.us/.../mahoningmap.jpg

Contaminants currently found in the river sediment include PAHs, petroleum hydrocarbons, pockets of PCBs, mercury, and various metals (US Army Corps of Engineers, Pittsburgh District, 2006). Bioremediation of the PAHs is a possible solution for the clean-up of contaminated river banks. Remediation using white rot fungi, specifically *Pleurotus ostreatus*, poses no ecological concern. With the use of a waste cellulose substrate to act as a carbon source, it can be a cost effective solution.

Both the river bed and river banks are contaminated with various PAHs. Core samples from both river bank and bottom (Figure 2), show that the river sediment contamination usually occurs at a depth of 3 to 4 feet below the surface of the bank. This is ecologically important because the sediment located at this site is historically contaminated river sediment which has occurred pre-1970. This highly PAH

contaminated river sediment, is then capable of creating disturbances naturally to the watersheds animal and plant populations.

Sediment samples were collected from the river bank and bottom at a sample site in Lowellville, Ohio. The sediment located within sample site are the result of deposition of PAH contaminated sediment from industrial wastes due to slow moving areas of water. Since dumping of the industrial wastes has ceased, new uncontaminated sediment has been deposited over the previously hazardous sediments encapsulating the material within the river bank and bottom (Figure 4). The collection took place in this location due to the nature of contamination and accessibility of the contaminated sediment. Sediment was taken from the river bank via the water for ease of sampling in terms of amount of sediment needed for the incubations (Figure 3). Sediment composition in this location consist of clays and silts along the river banks. These sediment characteristics help to contribute to poor drainage and high susceptibility to PAH build-up. PAH build-up is generally located in areas of the river where free standing water is located, letting the PAHs accumulate in the river bank and bottom sediment.

Fig. 2 – Sample core of PAH contaminated sediment from Lowellville, Ohio river bank

Fig. 3 – Taking a contaminated sediment core from Lowellville, Ohio river bank via the water

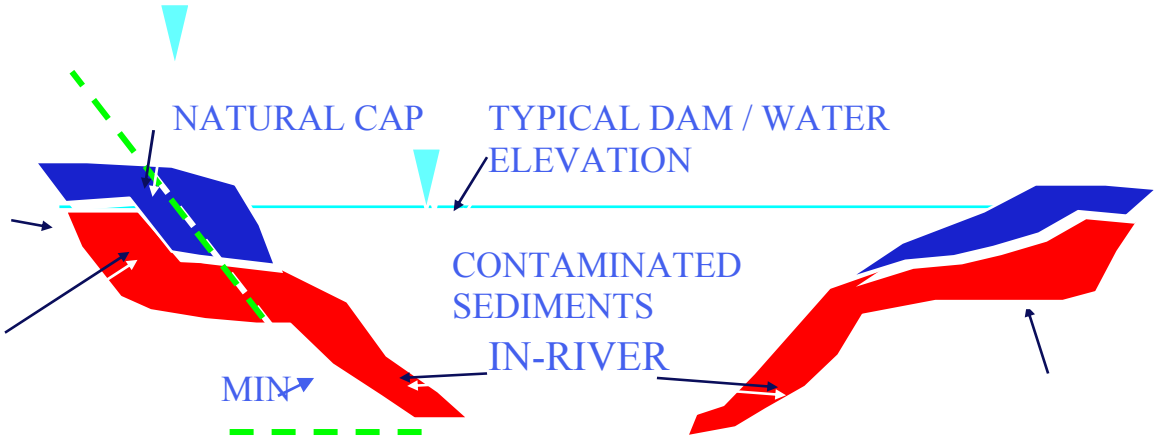

Fig. 4 – Cross section of Mahoning River and contaminated sediment deposition

1.2 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons that occur in the environment are usually the result of the incomplete combustion of carbon containing materials. The contaminated substances enter a water system through discharges from industrial plants. PAHs are insoluble by nature, leaving them to settle and deposit themselves to solid particles (sediment) in slow flowing areas of water. Due to their low water solubility and ability to adhere to soil particles, natural remediation becomes a relatively slow and inefficient process (Meulenberg et al., 1997). PAHs are known carcinogens and human contact can be harmful. PAH structure consists of multiple fused ring structures that make microbial degradation very difficult. Degradation in many cases requires the cleaving of the aromatic rings. PAHs can occur at many different molecular weights, with the higher weight PAHs being more difficult to degrade than the PAHs of low molecular weight. Common high molecular weight PAHs found in the system includes Pyrene, Flouranthene, and Chrysene (Figure 5). Common low molecular weight PAHs identified in the system includes Napthalene, Fluorene, Anthracene, and Phenanthrene (Figure 6). At the Lowellville location, 14 PAHs were detected.

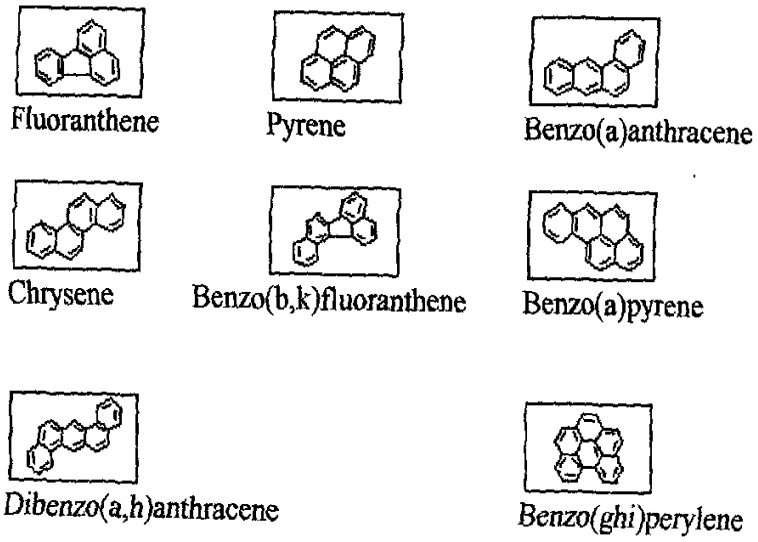

Fig. 5 – High molecular weight PAHs associated with the Mahoning River sediment

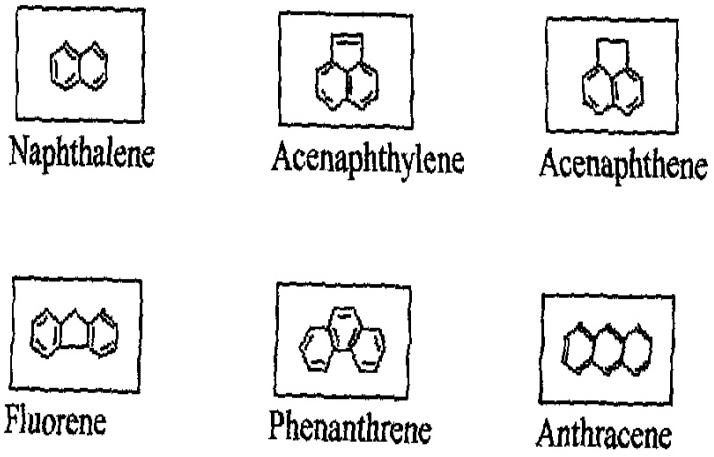

Fig. 6 – Low molecular weight PAHs associated with Mahoning River sediment

1.3 P. ostreatus and other White-rot Fungi

There are numerous white-rot fungi species capable of wood decay. Two of the most well studied for bioremediation are *Phanerochate chrysosporium* and *Pleurotus ostreatus*. This group of fungi utilizes extra-cellular ligninases for the initial degradation of wood. The extra-cellular enzymes which are produced are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase. What has also been discovered is that when wood is added to an incubation treatment, the enzyme patterns will change and *P. ostreatus* will secrete laccase in greater concentrations than other white-rot fungi (Schutzendubel et al., 1999). These enzymes play an important role in the cometabolic degradation of the PAHs. Both *Phanerochate chrysosporium* and *Pleurotus ostreatus* utilize a similar array of extra-cellular enzymes in a degradation model (Bezalel et al., 1997). The importance of these extra-cellular enzymes is that they are free radicals capable of cleaving lignin and other ring structures, including PAHs. White-rot fungi will partially oxidize the PAHs, converting the pollutants into more water soluble compounds with increased bioavailability. The newly created metabolites are then more easily degraded by indigenous microbes as by the white-rot fungi. The mineralization of these compounds in the soil is a result of the initial particle oxidization created by the white-rot fungi free radicals. In sludge and soil incubations, PAH metabolites were more readily mineralized by the natural mixed bacterial cultures once the PAHs have become partially oxidized. The results of these experiments suggest that white-rot fungi in association with indigenous bacteria will create an effective PAH degradative system (Meulenberg et al., 1997).

The importance of our study is discovering if *Pleurotus ostreatus* is capable of playing a major role in the degradation of PAHs in a wet, unique, historically contaminated, river sediment. Prior work has been conducted showing the effectiveness of *Pleurotus ostreatus* as a PAH degradative fungi (Baldrian et al., 2000). One study has shown that after only 3 days of incubation, *P. ostreatus* was capable of degrading fluorene and anthracene by 43% and 60% in liquid culture (Schützendübel et al., 1999). A study looking at creosote contaminated soil performed an incubation over a 70 day period using *P. ostreatus*. After 70 days, the two and three-ringed hydrocarbons were eradicated, leaving only small concentrations of four and five-ringed hydrocarbons (Atagana et al., 2006).

Another important reason for utilizing *Pleurotus ostreatus* is because it should be accepted by the public. *Pleurotus ostreatus* is an edible species of mushroom grown commercially for human consumption. Being that it is commercially available for human consumption; the general public should accept the introduction of this specie into the Mahoning River watershed for bioremediation purposes.

1.4 Goals

This study focused on the use of *Pleurotus ostreatus* in wet river bank/bottom sediment, with the use of easily available, economically friendly, and ecologically safe cellulose substrates and other amendments (Figure 11). The idea is to use a substrate that is an already readily produced human by-product (ex. newspapers and sawdust), and naturally reuse it for bioremediation and restoration of the Mahoning River watershed.

Some studies have shown that the introduction of a white-rot fungus to a PAH contaminated soil, will also promote PAH degradation by indigenous microbes. *P.*

ostreatus has the capability of oxidizing or partially oxidizing all of the PAH compounds found in contaminated sediment. The partially oxidized structures are generally the PAH molecules of low molecular weight. The partially oxidized PAHs are then more readily available for degradation from indigenous bacteria in the system (Meulenberg et al., 1997). Introduction of *P. ostreatus* into a natural non-amended soil, degradation of high molecular weight four and five-ring PAHs will be more successfully degraded in this cooperative manner. Due to the unspecific nature of enzymes created by white-rot fungi, the natural micro-flora found in the sediment will be able to focus on degrading the more readily available low molecular weight PAHs. The synergistic PAH degrading system that the *P. ostreatus* and the indigenous microbes create, should mineralize all the PAHs within the sediment with greater efficiency (Wiesche et al., 2003).

1.5 Hypothesis

The introduction of *P. ostreatus* into contaminated Mahoning River sediment will increase the efficiency of PAH degradation through the partial oxidation due to the extra-cellular ligninases being created. To test this hypothesis, two cellulose substrates were added to act as the carbon source: sawdust and shredded newspapers. In addition, amendments tested in the study included the introduction of a fungal nitrogen source and cyclodextrin. To evaluate white-rot fungi colonization, fungal biomass was measured by two staining procedures [see methods]. A direct correlation between total fungal biomass and metabolically active fungal biomass was expected. The treatment showing the highest concentration of total biomass is expected to show the highest metabolically active biomass as well.

1.6 Fungal Biomass/Hyphae Length

Two microscope techniques were used to monitor hyphae growth and metabolic activity of *P. ostreatus* colonization in the river sediment. The two techniques involve the Calcufluor M2R White stain and Fluorescein Diacetate stain respectively (Stahl and Parkin, 1996). Slides were prepared at every sampling point, in triplicate, throughout the incubation period. Total hyphae length was measured by using epifluorescence microscopy.

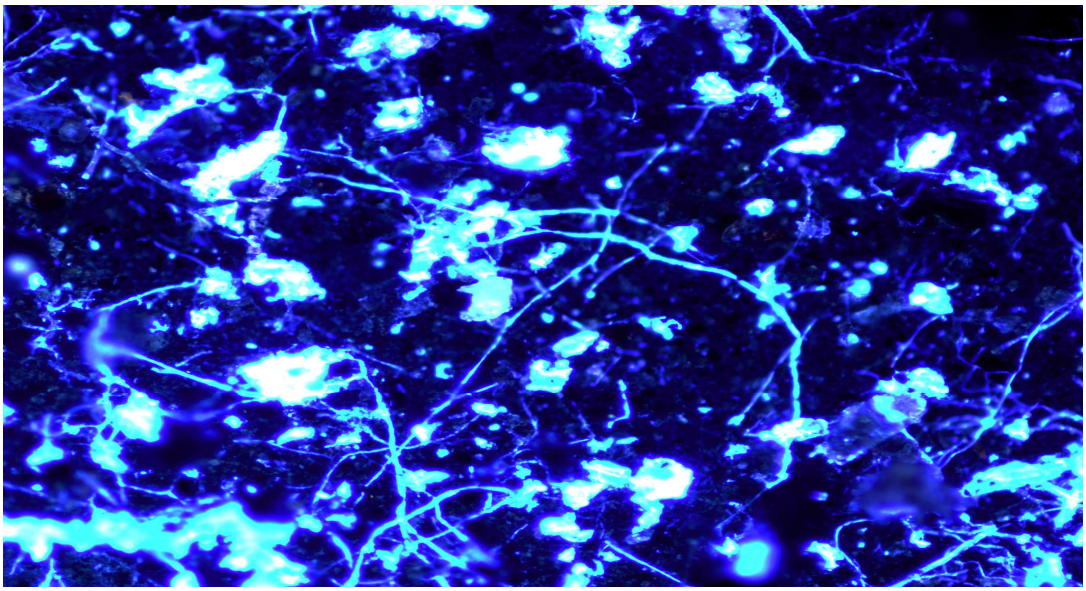

Fig. 7 – Calcufluor M2R White Stain example showing total fungal biomass at 21 days

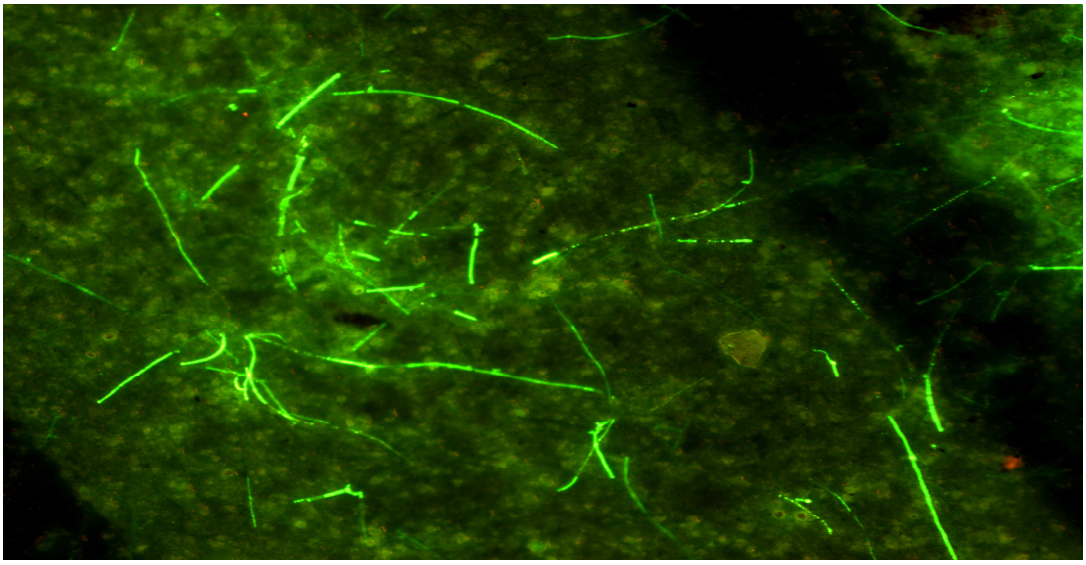

Fig. 8 – Fluorescein Diacetate stain example showing metabolically active fungal biomass at 21 days

1.7 Experimental Design

Contaminated Mahoning River sediment was inoculated with *Pleurotus ostreatus* and amendments (Figure 11). The sediment being used for the incubation was river bank/bottom sediment that was collected from the Lowellville Ohio site. The sediment at this site was collected from the water level of the Mahoning River from within the inner bank. To obtain the sediment, a plastic tube was pressed into the river sediment and a core was removed (Figures 3 and 4). The sediment was then refrigerated in a sealed plastic bucket until use. Once the parameters of the incubation were determined, the experiment was carried out by placing 1 L of sediment in glass incubation bowls with the proper amendments. Parameters being measured in the experiment included changes in PAH concentrations, changes in fungal biomass (both total and metabolically active), and changes in pH. The incubations for each treatment with the appropriate amendment was performed in triplicate over a 42 day incubation period at 25^{o}C.

2. Methods

2.1 Fungal Growth

P. ostreatus stock culture was obtained from ATCC (58053). The white-rot culture was then grown on white-rot selective potato dextrose agar (PDA) plates (Dietrich and Lamar,1990). The inoculated selective plates were placed in incubators at 25^{o}C for 72 – 120 hours. Cores of the plates were then taken and placed into sterile 250 mL Erlenmeyer flasks containing 125 mL of Potato Dextrose broth (Sigma). Broths were placed on a shaker at 250 RPM for 48 – 72 hours at room temperature.

After growth of inocula in 125 mL of Potato Dextrose broth, 500 mL of grain was placed into a spawn bag (Fungi Perfecti LLC) with 150 mL of MilliQ H_2O. The spawn bag containing the grain and water mixture was sealed using an impulse sealer and autoclaved at 121^{o}C. Once autoclaved, the spawn bag is then inoculated with 125 mL of the Potato Dextrose broth containing the *P. ostreatus* inocula. Spawn bags were then placed in a 25^{o}C incubator for 72 – 120 hours.

2.2 Sediment Incubation Set-up

1 L of PAH contaminated Mahoning River sediment was placed into a 1.9 L glass incubation bowls (in triplicate) per treatment. 60% by volume of sawdust and shredded newspaper were added to each glass bowl. 10% by volume of inoculated grain was then added to each bowl. Contents of the bowl were mixed to create a homogenous mixture. A glass petri dish filled with sterile water was suspended inside of the glass bowl to keep the moisture content constant. Incubation bowls were covered with plastic wrap, aluminum foil, and placed at 25^{o}C in an incubator (Figure 10). Amendments were added to the bowls at 21 days. Half of all incubation bowls were mixed to monitor the

effect of tilling on the system at 42 days. All samples containing sawdust had 1/3 of the set-up mixed with a nitrogen source to monitor the effect of nitrogen and tilling at 42 days. Growth of *P. ostreatus* in sediment after 21 days is shown in Figure 9.

Fig. 9 – P. ostreatus growth in sawdust + fungi treatment at 21 days in glass incubation bowl

Fig. 10 – Incubation run no. 1 containing all treatments in the 25^{o}C incubator

2.3 Treatments

There were 18 total treatments for the incubation with one sediment only control. The 18 treatments were conducted in a total of 8 glass incubation bowls, down in triplicate for a total of 24 bowls. At time 0, the treatments in the 8 bowls consisted of: control, control + cyclodextrin, sawdust only, shredded newspaper only, sawdust + cyclodextrin, sawdust + *P. ostreatus* inocula, newspaper + *P. ostreatus* inocula, and sawdust + *P. ostreatus* inocula + cyclodextrin. Sawdust and newspaper was added 60% by volume while *P. ostreatus* was grown on autoclaved barley grain and added 10% by volume and mixed.

At 21 days, half of all the incubation bowls to see the affect tilling would have as a treatment at 42 days. To the samples containing sawdust, half of the mix sediment was mixed with a commercial nitrogen source as a final treatment amendment. The fungal nitrogen source was added in10% by volume to mixed portion of sediment. The amendments gave a final total of 19 treatments to be sampled at day 42 of the incubation.

Treatment	Day Treatment Applied
Control	Day 0
Control Mixed	Day 21
Control + Cyclodextrin	Day 0
Control + Cyclodextrin Mixed	Day 21
Paper Only	Day 0
Paper Only Mixed	Day 21
Sawdust Only	Day 0
Sawdust Only Mixed	Day 21
Sawdust + Nitrogen	Day 21
Sawdust + Cyclodextrin	Day 0
Sawdust + Cyclodextrin Mixed	Day 21
Paper + Fungi	Day 0
Paper + Fungi Mixed	Day 21
Sawdust + Fungi	Day 0
Sawdust + Fungi Mixed	Day 21
Sawdust + Fungi + Nitrogen	Day 21
Sawdust + Cyclodextrin + Fungi	Day 0
Sawdust + Cyclodextrin + Fungi Mixed	Day 21
Sawdust + Cyclodextrin + Fungi + Nitrogen	Day 21

Fig. 11 – Treatment table and day the treatment was applied during the incubation

2.4 PAH – Extraction by Sonication Method

The method used was a modified version of the EPA Sonication Extraction Method (S. Pabba, 2008). A 10 g sediment core was extracted from the glass incubation bowl (in triplicate). Autoclaved sand was added to fill in any holes created from sampling. The sediment core was placed into a 125 mL Erlenmeyer flask. 50 µL of surrogate (Restek) was added to each sample to evaluate the extraction efficiency. Based upon the recovery of the surrogate from the extraction, an extraction coefficient could be obtained to evaluate the concentration of PAHs present. 10 g of sodium sulfate (Fisher Scientific) was added to each flask. 50 mL of DCM (methylene chloride) was added to the flask and the mixture was stirred using a sterile glass stirring rod. Flasks were covered with aluminum foil and the edges are sealed using parafilm wax. Flasks were placed inside the sonicator for 20 minutes.

After sonication, the liquid component to the flask was filtered onto sodium sulfate to dry the sample and collected in a 250 mL round bottom flask (Buchi). 40 mL of DCM was added back to the sediment remaining in the flask. The 125 mL Erlenmeyer flask was sonicated again for 20 minutes. The liquid component was collected in a corresponding 250 mL round bottom flask once more. A total of 3 sonications and DCM collection were performed.

After the third sonication, the round bottom flasks containing 130 mL of DCM were reduced to a 1 mL volume by using a Rotavapor [Büchi Rotavapor R-200]. The residual on the flask was rinsed with 5 mL of DCM and run through a clean prepared silica column containing a frit and 0.5 g of silica (Clarkson Chromatography Products Inc.) that had been conditioned for 2 hours at 103^{o}C. Samples from the silica column

were collected in clean conical tubes. These samples were ran through aminopropyl columns (J.T. Baker) and collected in clean conical tubes. The samples were dried under nitrogen to a volume of 1 mL and transferred to a 2 mL glass vial. 20 μL of internal standard (Restek) was to the vial added and samples were analyzed on a Hewlett Packard GCMS.

2.5 Quantification of Total & Metabolically Active Fungal Hyphae by Microscopy

Fungal biomass was performed using two specific dyes depending on the feature being monitored. To evaluate fungal mycelium length and number, calcofluor M2R white stain (Sigma) and the membrane filter method (Stahl et al., 1994) was used. The fluorescence in the calcofluor M2R white stain is ideal for studying basic mycelium features. Fluorescein diacetate (Stahl and Parkin, 1996) was used to measure metabolically active mycelium under epifluorescent microscopy.

For total fungal hyphae analysis, 1g of moist sediment was blended with 100 ml of nanopure water in a Waring blender at the highest speed for 1 minute. 1 ml of the mixture was then filtered through a 25 mm diameter 0.4 μm filter membrane (Millipore). The fungal material that was remaining on the membrane was then stained with 1 ml of calcuflour M2R stock solution for 15 to 20 seconds. The stain was removed with a vacuum and washed with nanopure filtered water. The filter was placed on a microscope slide and allowed to air dry. The filter was mounted to the slide by adding a couple drops of immersion oil, and covered with a cover slip. For evaluation of mycelia count and length, 20 random fields of view were observed on an Olympus epifluorescent microscope, for each slide, and pictures were taken using MicroSuite Special Edition software. Mycelia length values were adjusted accordingly to the area of a field of view.

For the metabolically active fungi, 1 g of moist sediment was blended with 200 ml of nanopure filtered water in a Waring blender at the highest speed setting for 1 minute. 20 ml of FDA stock solution was added to the blender mixture and allowed to stain for 3 minutes with occasional mixing. The final contents of the mixture were then mixed again for a few seconds to resuspend the sample. 1 ml of the mixture was passed through a 25 mm diameter 0.4 μm filter membrane (Millipore). As in the calculfuor white staining for the evaluation of mycelia count and length, 20 random fields of view were observed for each slide and pictures were taken. Mycelia length values were adjusted accordingly to the area of a field of view.

2.6 pH

Changes in pH were measured my using standard methods. 5 grams of sediment was blended with 50 ml of sterile water. After 30 minutes, pH measurements were taken using a calibrated pH meter (Oakton Model 35614-80). The measurements were performed in triplicate.

3 Results and Discussion

3.1 Degradation of PAHs

PAH degradation occurred in 9 out of the 11 compounds after 21 days of incubation (Figure 12). The two aromatic structures that did not show degradation were benzo(a)anthracene and chrysene. The addition of both *P. ostreatus* and either sawdust or shredded newspaper played an integral role in the initial degradation of the PAHs. PAH degradation in many of these treatments was most extensive in the 1st 21 days of the incubation.

The addition nitrogen, cyclodextrin, or both appeared to have a positive effect on the degradation of PAHs. Nitrogen or cyclodextrin being added to both cellulose substrates showed enhanced degradation after 42 days of incubation in comparison to the controls and samples without them. Nitrogen and cyclodextrin also had a positive effect on samples containing the *P. ostreatus* inocula. Increased degradation occurred in many of the treatments from the 21 to 42 day time period. PAH degradation was approximately 50% with the addition of sawdust, *P. ostreatus*, cyclodextrin, and the nitrogen source.

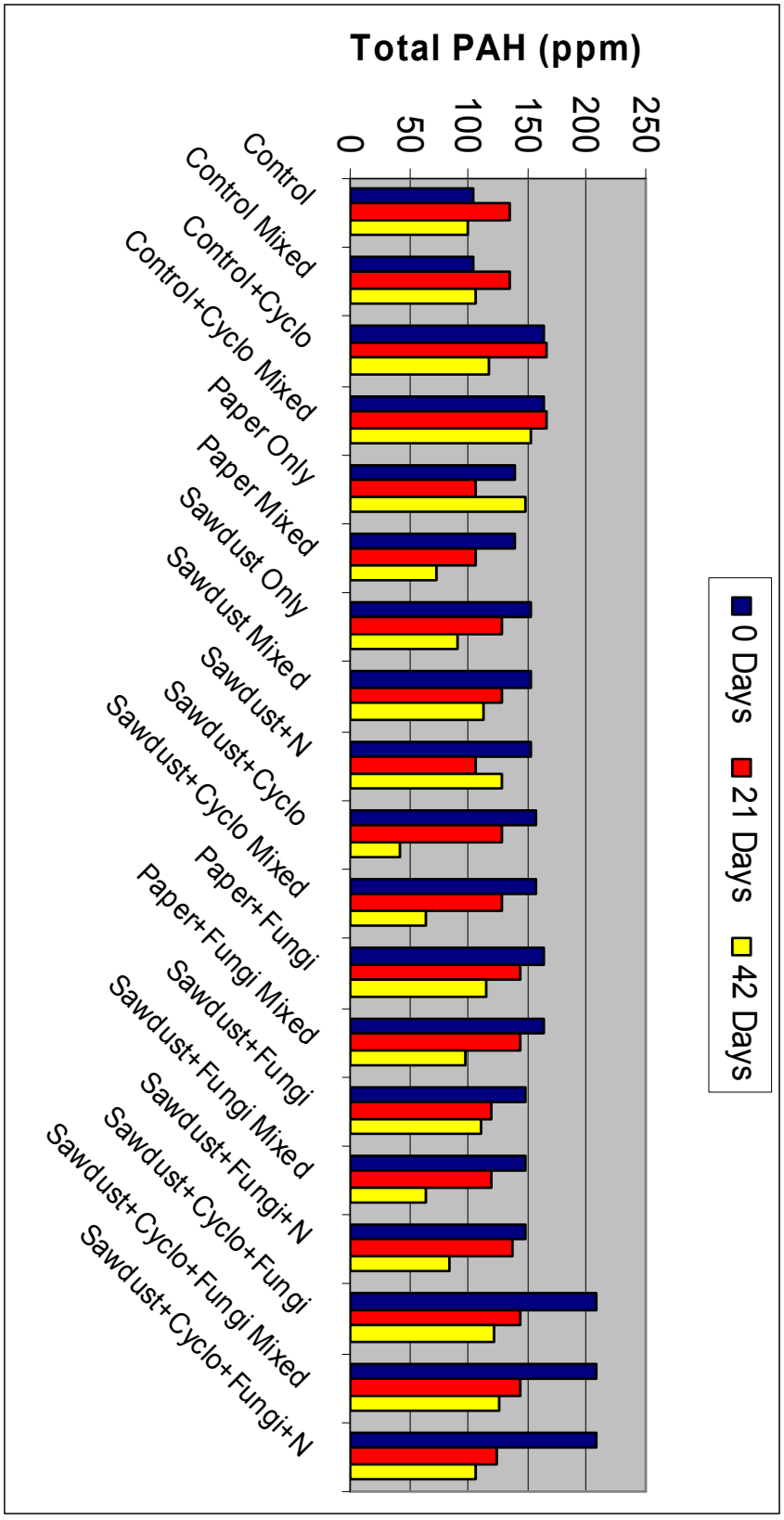

Fig. 12 – Changes in total PAHs associated with the Mahoning River sediment for incubation 1, all 19 treatments.

3.2 Changes in Hyphae Length and Biomass

Fungal biomass was monitored to correlate to the PAH concentrations being measured. Since it was determined that the most effect treatment was through the addition of all of the possible parameters, then that treatment should also show the greatest fungal biomass at day 42 if the *P. ostreatus* is playing a major role in the degradation of the PAH compounds. A correlation was drawn between total fungal hyphae, total metabolically active hyphae, length of both types of hyphae present, and PAH degradation in the river sediment. The importance of the metabolic activity measurement, is that it measures mycelium growth taking place at that specific time of the incubation. If increased length of hyphae, in both total and metabolically active slides is observed, then we hypothesized that they should directly correlate to a decrease in PAH concentrations in the river sediment.

There was a visual difference in fungal growth at 21 and 42 days when compared to time 0. The treatments containing *P. ostreatus* inocula and sawdust showed growth throughout the incubation bowl. Treatments containing *P. ostreatus* and shredded newspaper showed surface colonization. Treatments containing sawdust without fungi added showed green fungal growth on the surface of the incubation bowls. All other treatments showed no visible fungal growth. Fungal biomass was measured for each treatment to support the visual evidence. Treatments containing newspaper showed interference and were not analyzed for both total and metabolically active hyphal lengths.

Fungal biomass based on total hyphae length increased in fungal biomass from 0 days to 42 days (Figure 13). Biomass data was expressed in meters per gram of sediment (m/g). The range of biomass at day 0 was 0 to 82 $\pm$ 10 m g sediment^{-1}. At 21 days the

biomass range was from 18 $\pm$ 7 to 308 $\pm$ 180 m g sediment^{-1}. At day 42 the biomass range was from 5 $\pm$ 5 to 374 $\pm$ 18 m g sediment^{-1}. In general fungal biomass increased in all treatments, but some showed greater biomass at 21 days than at 42 days. This could be due to an initial consumption of the substrate, or the overall toxic environment of the sediment. Treatments that contained the nitrogen source showed a constant increase in total fungal biomass. Treatments containing *P. ostreatus* inocula showed the greatest overall hyphae length which correlated to the greatest fungal biomass value. Total fungal biomass data supported to the visual observations throughout the incubation.

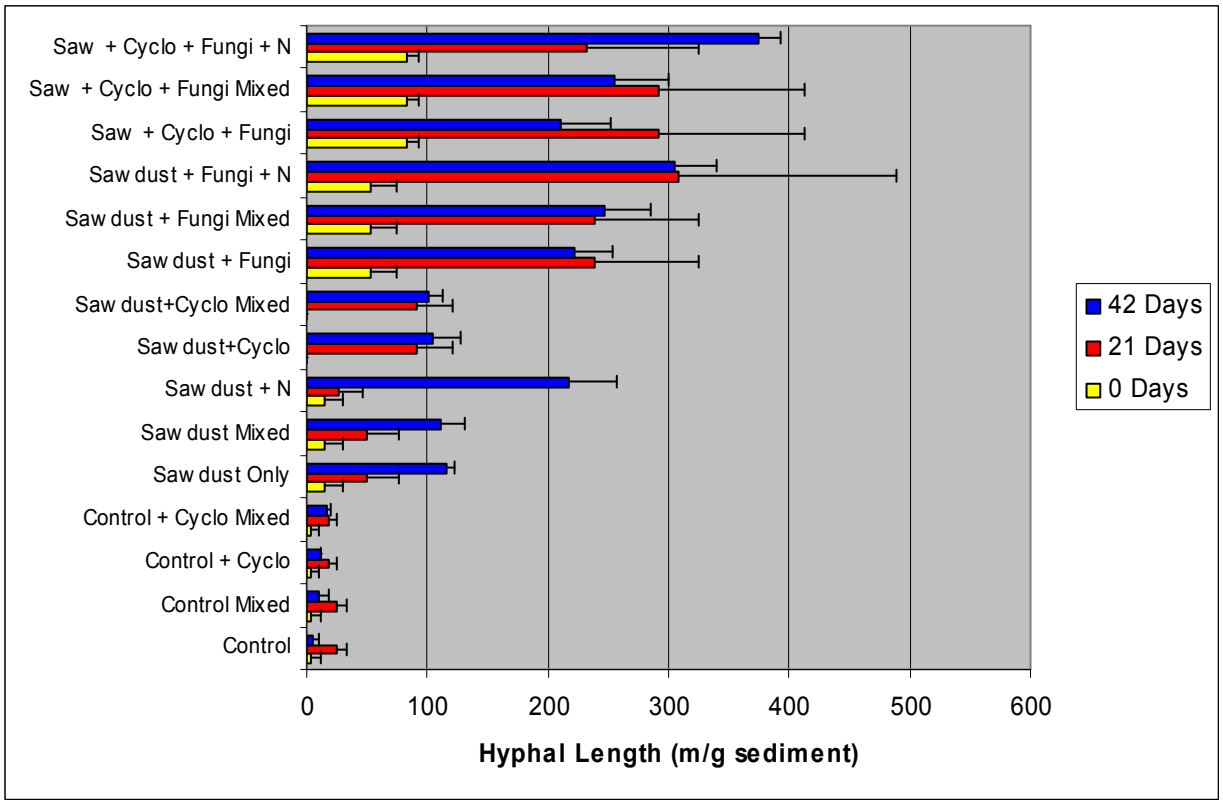

Fig. 13 – Total Hyphal Length in Mahoning River Sediment treated with fungi and other amendments as described in Figure 11.

To correlate the total hyphae length data with potential growth during the incubation, metabolically active hyphae length was measured (Figure 14). These data showed a general trend over the 42 day incubation. Total hyphal length was always greater than metabolically active hyphal length. Values were lower in treatments without fungi, sawdust, or newspaper. As expected, treatments containing the *P. ostreatus* inocula have the greatest active biomass at 42 days. The nitrogen source also appeared to have a positive effect on the enzymatic activity after 42 days of incubation. At 21 days, the treatment containing only sawdust and *P. ostreatus* had the greatest active fungal biomass (160 m g sediment^{-1}). The range at time 0 was 0 to 26 $\pm$ 1 m g sediment^{-1}. At 21 days the range was from 0 to 160 $\pm$ 64 m g sediment^{-1}. At 42 days, the range was from 4 $\pm$ 3 to 117 $\pm$ 32 m g sediment^{-1}. The treatment containing sawdust, surfactant, fungal inocula, and the nitrogen source showed the greatest biomass for both total and active hyphae length.

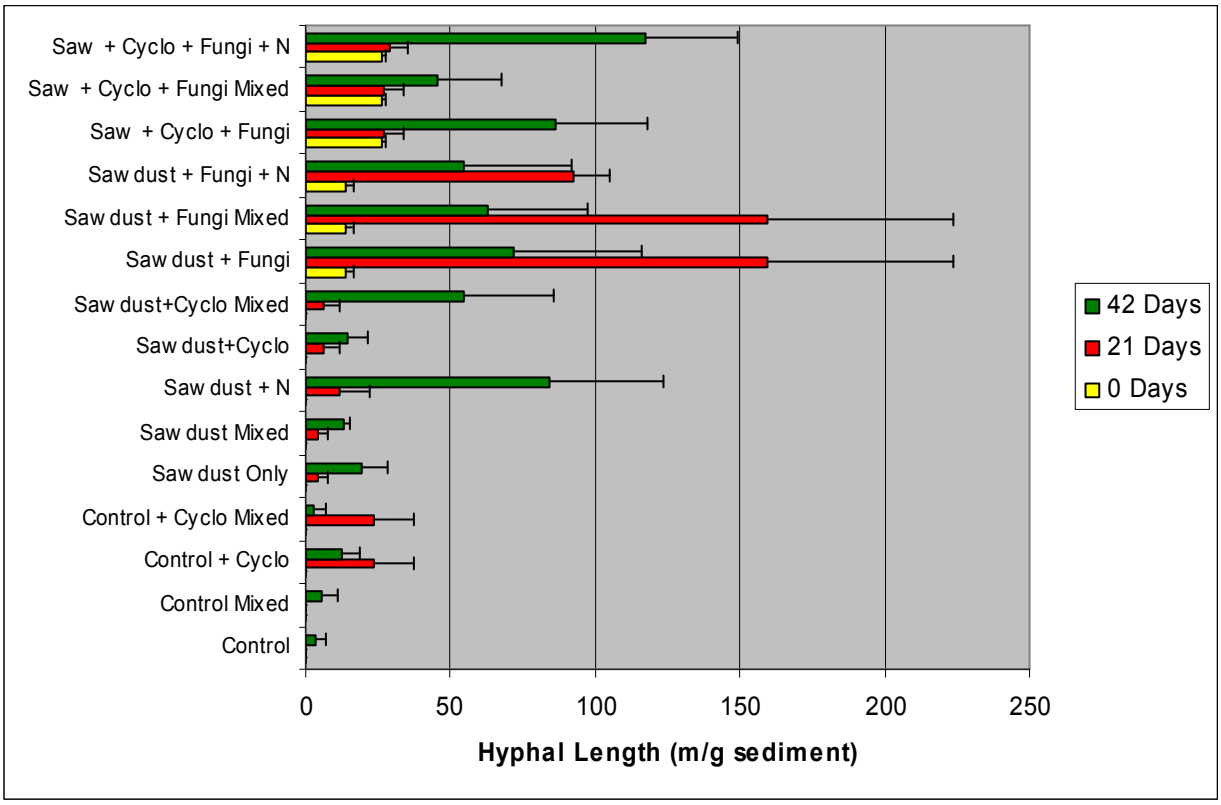

Fig. 14 – Metabolically Active Hyphal Length in Mahoning River Sediment treated with fungi and other amendments as described in Figure 11.

3.3 Changes in pH

To evaluate if pH had any effect on *P. ostreatus* growth or biomass, pH was measured in each treatment (Figure 15). The pH range for time 0 was 7.3 $\pm$ 0.4 to 7.6 $\pm$ 0.1. At 42 days the pH range was 7.9 $\pm$ 0.1 to 8.3 $\pm$ 0.2. Treatments with the highest fungal biomass also showed the greatest increase in pH from 21 days to 42 days.

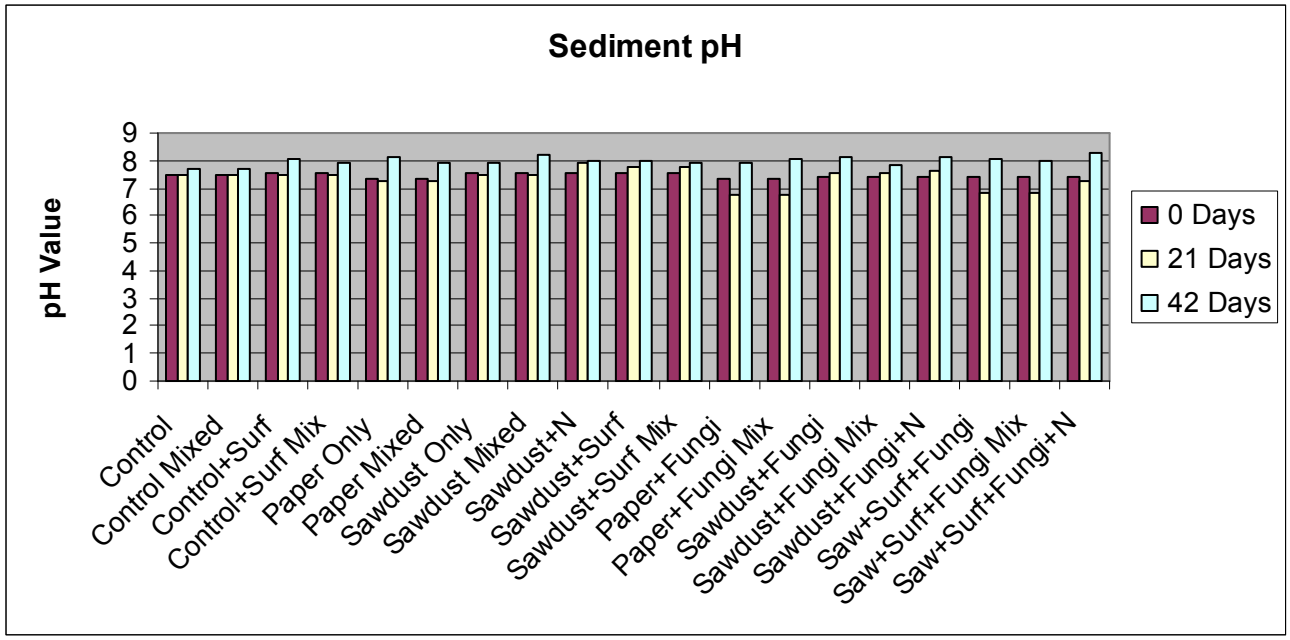

Figure 15 – Sediment pH Data (42 Day Incubation)

3.4 Discussion

The stock culture of *P. ostreatus* that was used in this study, was cultured on white-rot selective media to ensure the introduction of a pure strain into the sediment at the start of the incubation. The white rot selective media used for our study was made using potato dextrose agar which is a slight modification from the derived protocol which was using 2% malt agar. In the malt agar selective media, what was found is that 15 ppm of benomyl and 550 ppm of streptomycin sulfate had the greatest isolation effect when used on nonsterile forest soils for *Phanaerochaete chyrsosporium*. Benomyl at 1 ppm is capable of inhibiting the growth of non-wood decaying fungi (Dietrich and Lamar 1990). The fungal culture on selective media was then added to autoclaved barley. The white-rot colonized barley was used for all incubations which were derived from the pure stock culture. A past study has shown the effectiveness of culturing *P. ostreatus* on barley grain (Atagana et al., 2006). The addition of fungal inoculated barley to creosote

contaminated sediment, promoted better fungal growth and PAH degradation while we showed similar results. The absence of barley, directly related to a decrease in fungal colonization of the soil. The incubation had a final C:N:P ratio of 25:5:1, with barley acting as the nitrogen catalyst for fungal colonization Culturing techniques of the *P. ostreatus* at 25^{o}C took on average 72 to 96 hours.

The sediment used for the incubations, had the consistency of an oily sludge with the initial moisture content of 31%. Due to the high moisture content of the sediment, 60% by volume of the substrate (sawdust or newspaper) was added to ensure that *P. ostreatus* could aggressively colonize the sediment. There were many visual differences between the treatments during the incubation. All samples containing P. ostreatus and the sawdust substrate showed colonization of the white-rot fungi throughout the entire sediment. When these samples were mixed, fungal colonization decreased but began to rebound by day 42 of the incubation. Newspaper and *P. ostreatus* incubations had white-rot colonization only in the top third of the bowl, leaving only the heavy sludge behind. Mixing of the shredded newspaper ceased fungal colonization in that region for the rest of the incubation. The treatments containing no fungi, but a cellulose substrate, had a layer of green fungi covering the sediment by day 21. Mixing had no effect on this species ability to colonize. The controls for the experiment showed no visual fungal colonization. Addition of the nitrogen source had a positive effect on all fungal colonization, and was not specific to *P. ostreatus*. The nitrogen source promoted growth of both species of fungi that were visually evident.

PAH degradation was observed in all but two of the 11 PAHs being measured during the incubation (S. Pabba, 2008). The two that showed no apparent degradation

were benzo(a)anthracene and chrysene which are of high molecular weight. These two compounds showed a maximum concentration at 21 days. Other studies have shown that PAH degradation by *P. ostreatus* is due to the partial oxidation by the extra-cellular ligninases. Once PAHs become oxidized, their water solubility increases, facilitating a mineralization by the indigenous microbes as well as by white-rot fungus (Meulenberg et al., 1997). When *P. ostreatus* is introduced into a non-sterile PAH contaminated sediment, the white-rot fungi is capable of degrading the 5-ring PAHs while, the microflora can focus on the more accessible 3 and 4-ring structures. The cooperative system will increase the efficiency of the mineralization of the PAHs due the non-specific nature of the *P. ostreatus* (Wiesche et al., 2003). Some level of degradation was observed in these 9 PAHs as long as the minimal cellulose substrate was added to the system.

Cyclodextrin appeared to enhance degradation with or without fungi added. The commercial nitrogen source also showed a positive effect on the degradation of these 9 PAHs.

The treatment that had the greatest affect on the degradation of polycyclic aromatic hydrocarbons was the addition of sawdust, cyclodextrin, *P. ostreatus*, and the nitrogen source. The average degradation for this treatment in the 9 PAHs was over 50% with benzo(b&k)fluoranthene showing the greatest degradation (15 ppm to less than 1 ppm at 42 days).

For all samples being observed for total hyphae length, there was an increase in fungal biomass in all treatments in comparison to time 0 data. At day 42, the samples inoculated with *P. ostreatus,* sawdust, and the nitrogen source showed the highest fungal

biomass which correlates to the visual evidence. The treatment of sawdust, cyclodextrin, *P. ostreatus*, and the nitrogen source had the greatest total biomass at 42 days which correlates to the degradation of PAHs and that treatment being the most effective. The final biomass for this treatment was 374 m g sediment^{-1} with the next closest being 256 m g sediment^{-1}. Metabolically active fungal biomass showed the same trends in comparison with total biomass for every treatment. Again, the treatment that was most effective for degradation had the greatest metabolically active biomass. This treatment had a biomass of 117 m g sediment^{-1} with the next closest biomass being 87 m g sediment^{-1}.

There was a positive correlation in this study between all of the parameters being measured to see the effect of PAH degradation. *P. ostreatus* has shown its capability of degrading polycyclic aromatic hydrocarbons in a moist contaminated river sediment, though the incubation was not fully optimized. Sediment moisture should be looked at more carefully as well as the addition of the nitrogen source for better optimization of the system. Through better optimization, the *P. ostreatus* will have a greater chance of success showing results that can exceed the ones obtained in this initial experiment.

To find out the true potential of *P. ostreatus*, other factors that can change in the future include the introduction of the nitrogen source during the beginning of the incubation to see if that treatment promotes greater degradation throughout the entire incubation. Another factor will be looked at is the total organic nitrogen content should be measured in the sediment during the incubation to see the effect nitrogen is on the colonization of the white-rot fungi. Finally, since the work is being conducted with sediment that is not sterile and contains all of the native micro-flora, DNA analysis can be run on the sediment to determine what other species can be playing a cooperative role

in the degradation of the PAHs, either other fungi or native bacteria. Samples that did contain the green fungi, which was assumed to be an aspergillus species, did show a positive affect in the degradation of PAHs as well as an increase in fungal biomass.

Appendix I – Fungal Cultures and Media

White-Rot Selective Media – White-rot selective media (Dietrich, Lamar, 1990) was prepared by first adding 19.5 g of potato dextrose agar (PDA) to 500 ml of Milli-Q water in a 1,000 ml Erlenmeyer flask. The mixture was then autoclaved at 121^{o}C to put the PDA into solution. Benomyl solution (15 ppm) and streptomycin solution (550 ppm) are added from stock solutions. The stock solution of benomyl consists of 1,000 mg of benomyl per liter of acetone. The stock solution of streptomycin consists of 4,000 mg of streptomycin per liter of sterile water. Media was then poured in deep Petri dishes made for fungal cultures.

Fungal Culturing – White-rot mycelia was scrapped from inoculated sediment or prior inoculated media using a sterile swab. The swab was then streaked onto the white-rot selective media. The Petri dishes containing the media were place upside down in a 25^{o}C incubator for 72 – 96 hours.

Potato Dextrose Broth – Potato dextrose broth was made by adding 12 g of potato dextrose broth to 500 ml of Milli-Q water in a 1,000 ml Erlenmeyer flask. The flask is then autoclaved at 121^{o}C to bring the media into solution.

Potato Dextrose Broth Culturing – 125 ml of potato dextrose broth is added to an autoclaved 250 ml Erlenmeyer flask. To the flask, a core from an inoculated white-rot selective media plate is added and place on a rotary shaker at 250 rpm at room temperature. The flasks remain on the shaker for 72 – 96 hours.

Grain Preparation – 500 ml of grain is placed into a new spawn bag. To the spawn bag, 150 ml of Milli-Q water is added and the bag is sealed on an impulse sealer set to no. 4 setting. The spawn bag is then autoclaved at 121^{o}C and then cooled to room temperature.

Grain Inoculation – To the autoclaved grain in the spawn bag, 125 ml of white-rot inoculated potato dextrose broth is added and the spawn bag is resealed on an impulse sealer on setting no. 4. The spawn bag is then placed in a 25^{o}C incubator with the filter facing upward for 72-120 hours and then kept refrigerated until use.

Incubation Set-up – 1,000 ml of sediment is added to a 1.9 L glass bowl. For treatments containing a fungal nutrient source, 600 ml of cellulose (sawdust or shredded newspaper) is added and the contents of the bowl are mixed. For the treatments containing *P. ostreatus*, 100 ml of inoculated grain is added to the bowl and the contents are mixed. Treatments are then sampled for time 0 data and remixed to create a homogenous mixture. The bowls are then incubated for 21 days at 25^{o}C in an incubator. At 21 days, half of the mixture in the bowl is mixed by hand to see the effect of tilling as a treatment. To incubations containing sawdust, half of the newly mixed sediment was also mixed with 25 ml of a nitrogen source to see the effects of nitrogen. Sampling is down accordingly at 21 days, and bowls are incubated for another 21 days at 25^{o}C. Sampling is performed once more at 42 days.

Appendix II – Staining and Microscopy

Calcufluor M2R White – 0.23 grams of calcufluor M2R white is added to 1 L of sterile water (Stahl , Parkin, Each, 1995). The solution is mixed by hand shaking, and stored in a refrigerator until use.

Total Hyphae Length Analysis – Method is a modified fluorescent microscope method for measuring fungal biomass (Stahl, Parkin, 1996). 100 ml of Milli-Q water is added to an autoclaved Waring blender body. To the water, 1 g of sediment is added and the mixture is mixed on low speed for 1 minute. 1 ml of sample is extracted using a luer-lok syringe and passed through a 0.22 μm black mesh polycarbonate filter. To the filter, 1 ml of calcufluor M2R white stain is added, from a stock solution, for 30 seconds, and then filtered. Mesh polycarbonate filter is then placed on a microscope slide until dry. Once dried, a glass cover slip is affixed using two drops of immersion oil. Slides are analyzed using cube B on the fluorescent microscope using the 10X lens. 20 random fields of view are observed, with pictures taken for each field. Lengths are each mycelia present on the 20 fields are measured using a micrometer. $_{\text{Lengths}}$ are recorded, and biomass is calculated using the literature equation and diameter of white-rot fungi.

$$B_f = \pi r^2 L e S_c$$

B_f is the fungal biomass, r is the hyphal diameter (literature value of 3 μm), L is the total hyphal length per 20 fields of view, e is the hyphal density (1.3 g cm^{-3}), and S_c is the solids content (0.3). End product of the equation will be expressed at meters per gram of sediment.

Fluorescein Diacetate (FDA) – 2 g of FDA was added to 1 L of acetone. Solution was mixed by hand shaking and stored at -20 ^{o}C until use.

Metabolically Active Hyphae Length Analysis - Method is a modified fluorescent microscope method for measuring fungal biomass (Stahl, Parkin, 1996). 200 ml of Milli-Q water is added to an autoclaved Waring blender body. To the water, 1 g of sediment is added and the contents are mixed on low speed for 1 minute. 25 ml of FDA stain is added from the stock solution and let sit for 3 minutes with occasional mixing on low speed. Contents are then mixed again for 1 minute on low speed to resuspend the mycelia. 2 ml of solution is extracted using a luer-lok syringe and passed through a 0.22 μm black mesh polycarbonate filter. The mesh polycarbonate filter is placed on a microscope slide until dry. Once dry a glass cover slip is affixed using two drops of immersion oil. Slides are analyzed using cube D on the fluorescent microscope using the 10X lens. Pictures for 20 random fields of view are taken and fungal biomass is measured using the same equation for total fungal biomass.

$$B_f = \pi r^2 L e S_c$$

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