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Identification, Enumeration, and Diversity Determinations for Fungi Enriched on Phthalates as Sole Carbon Source from Riverine Sediments Using Molecular Methods

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Capstone Approval Form

WE, THE UNDERSIGNED MEMBERS OF THE COMMITTEE,

HAVE APPROVED THIS CAPSTONE PROJECT

IDENTIFICATION, ENUMERATION AND DIVERSITY DETERMINATIONS FOR FUNGI ENRICHED ON PHTHALATES AS SOLE CARBON SOURCE FROM RIVERINE SEDIMENTS USING MOLECULAR METHODS

By

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Abstract

Phthalates are a common chemical compound used as plasticizers in various industries that have been linked to several detrimental effects on health. Due to their widespread use, they have become a common environmental pollutant of soil and water. The persistence and distribution of phthalate esters in the environment has given rise to many microorganisms that are able to incorporate phthalates into various metabolic pathways and degrade them into harmless substances, with fungi being among those that are capable of such degradation of industrial polycyclic aromatic hydrocarbons. In this study, the diversity of fungal communities in response to the presence of phthalates within soil will be assessed using metagenomic analysis and their capacity for phthalate degradation will be examined. This research has shown that all fungal isolates obtained from contaminated and uncontaminated soil were able to grow on media with phthalate as the sole carbon source. Vermillion River saw a similar level of Shannon diversity at the species level when compared to Big Marsh. A principal components analysis showed the carbon usage between sites did not differ greatly and a two-sample t-test showed no significance in diversity between sites. Several notable known degraders of phthalates and polycyclic aromatic hydrocarbons were observed at each site including *Sarocladium strictum*, *Irpex lacteus*, and *Fusarium culmorum*. Several species of fungi not known to be capable of degrading phthalates were identified within phthalate-laced enrichments. This research suggests that the presence of such fungi at both sites may mean they are naturally predisposed to be capable of degrading phthalates and other xenobiotic compounds. Finally, the metagenomics analysis revealed the presence of several species not observed in the Unites States before.

Keywords: Fungi, phthalates, microbiology, diversity, biodegradation, metagenomics, carbon usage, dibutyl phthalate, DNA

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Microbial Bioremediation of Phthalate Esters

Significance

Phthalate esters are chemicals commonly used in the plastic manufacturing industry as plasticizers (Department of Health and Human Services 1994). These chemicals impart flexibility and strength to plastic compounds. Phthalate esters can be found in common plastics such as PVC and in items such as latex adhesives, cements, artificial leather, wire coatings, children's toys, and cosmetics. Because they are not chemically bound to plastics, they can enter the environment by means of industrial waste water, the burning of plastics, or due to plastic leachate entering soil or water from landfills (Heudorf et al. 2007, Schwarzbauer et al. 2002). Phthalates can also adhere to dust particles and be deposited by wind and rainfall (Weschler et al. 2008; Thuren and Larsson 1990). Given the ease with which phthalates can travel and the abundance of plastic waste generated by humans, it is no surprise that phthalate esters can be found in nearly every environment. With our constantly growing demand for plastics and other synthetic compounds, phthalate production has increased drastically. This is worrisome due to health concerns linked with phthalate exposure and that phthalate contamination can affect microbial ecosystems and possibly lead to a drastic loss of diversity. If other organisms are dependent on the microbial community in any way, this may have profound consequences.

Current Research

In recent years, phthalates have been a topic of discussion for researchers looking to determine how safe phthalate exposure is to biological systems. Research in this field has ranged from testing laboratory animals for adverse effects after exposure to varying doses of phthalates to monitoring aquatic food webs of ecosystems for the bioconcentration of phthalates in organisms (Mackintosh et al. 2004).

Studies have shown that specific types of phthalate esters can have adverse effects on organisms when exposed to specific concentrations of varying phthalate esters. These studies suggest that phthalate esters such as di-butyl phthalate (DBP) and di-ethyl phthalate (DEP) may interfere with normal growth and development of humans during gestation. It has also been reported that di-2-ethyl hexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) can lead to certain autoimmune diseases such as asthma and eczema (Braun et al. 2013). Certain phthalate esters have also been found to be slightly estrogenic, which can affect growth and development of organisms by mimicking the estrogen hormone and altering the body's normal hormonal balance (Jobling et al. 1995).

As far as bacteria are concerned, some phthalate esters have been demonstrated to have weak mutagenic effects when tested on *Salmonella typhimurium* (Seed, J. L. 1982). There is also evidence that the presence of DEP in soil can result in lower diversity in bacterial communities so that phthalate degrading bacteria become the dominant species (Kapanen 2007). Cartwright et al. (2000) demonstrated that DEHP was shown to have no significant effect on microbial communities, even at high concentrations. They also note that the lipophilic nature of phthalate

esters can disrupt critical cell membrane functions and cause cell death. Due to the negative impacts of phthalate esters on both higher organisms and bacteria, researchers are trying to determine how ubiquitous phthalate degradation is within the microbial world and are exploring the idea of utilizing bacteria to clean up sites with high levels of contamination. Research in this field is carried out largely by academic institutions or government-funded research labs.

The main route of degradation for phthalate esters such as dibutyl phthalate are photodegradation, hydrolysis, and aerobic degradation by bacteria (Hauser 2005). In hydrolysis, phthalate esters are first broken down into a mono-ester and an alcohol by water. Next, another hydrolytic reaction forms phthalic acid and a second alcohol (Cartwright, Owen et al. 2000).

Breakdown of phthalate esters involves the cleaving of the ester linkage between the alkyl groups and aromatic rings. From this point, phthalic acid can either enter the aerobic or anaerobic pathway. In the aerobic pathway, phthalic acid is converted to 3,4 dihydroxy phthalate or its isomer 4,5 dihydroxy phthalate. These are further converted to protocatechuate, at which point are broken down into either 2-hyroxy-4-carboxy-muconic semialdehyde or B-carboxy-cis-cis-muconate. The final metabolites of the aerobic pathway are acetate, carbon dioxide, succinate, and pyruvate. In the anaerobic pathway, phthalic acid is converted to a phthalic acid-CoA complex and is further broken down into benzoyl-CoA, cyclohax-1-ene carboxyl-CoA, 2-hydroxycyclohexane carboxyl-CoA, and pimeloyl di-CoA. The final product of this pathway is acetate, H_2, and carbon dioxide (Staples et. al 1997).

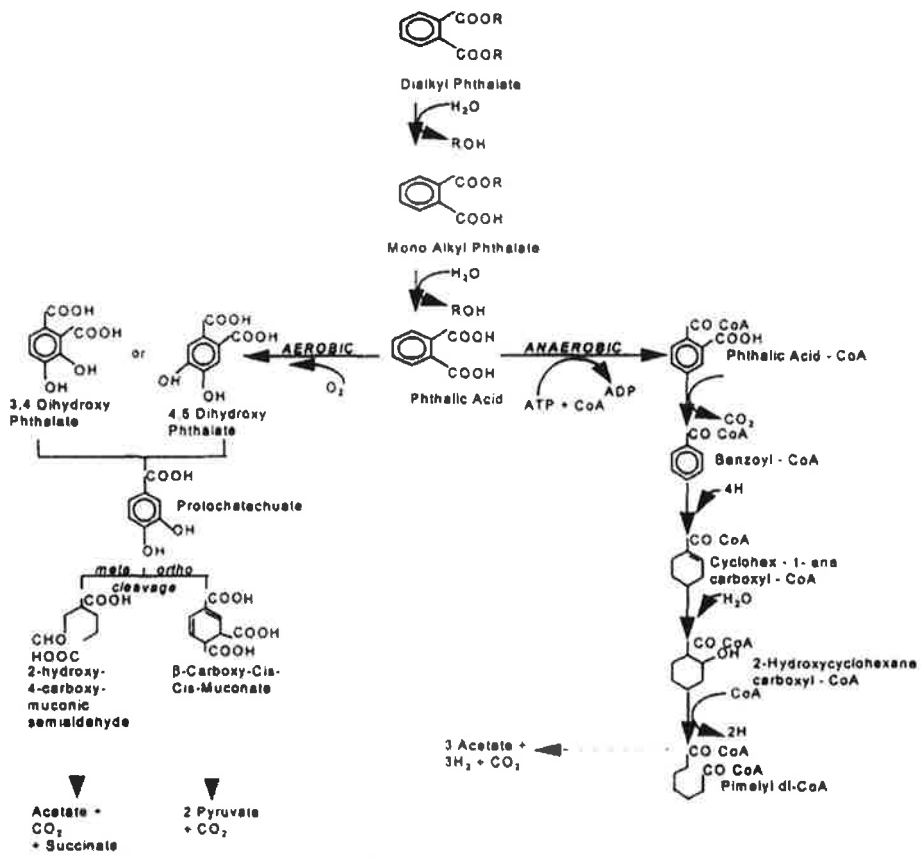

Figure 1. Phthalate degradation pathway (Source: Staples et al. 1997)

Photolysis of aqueous phthalate esters occur when UV light from the sun breaks the covalent bonds present in the molecule. Glendhill et al. (1980) estimated the half-life of BBP with respect to photodegradation of aqueous and atmospheric BBP to range from 144-200 days. For comparison, Howard (1991) lists the half-life of BBP under aqueous biodegradation by aerobic organisms as being 5-23 days. Studies have shown bacteria that are capable of degrading more than 90% of DBP in less than 48 hours (Chao et al. 2006). Aerobic biodegradation rates are

influenced by the length of the ester chain originating from the benzene dicarboxylic acid, with shorter chains being quickly mineralized and longer chains resisting degradation. This is thought to be due to steric hinderance of larger chains preventing hydrolytic enzymes from reacting with the molecule (Xia et al. 2004, Jianlong et al. 2000).

Both gram-negative and gram-positive bacteria are known to degrade phthalate esters (Engelhardt et al. 1976). Both types of bacteria are known to employ different pathways for breaking down phthalates. Gram-negative bacteria mostly employ the phthalate 4,5-dioxygenase pathway, while Gram-positive bacteria use the phthalate 3,4-dioxygenase pathway. In both pathways, phthalate esters are dihydroxylated to form phthalate dihydrodiol. Phthalate dihydrodiol is then metabolized into protocatechuate via enzymatic activity by either phthalate 3,4-dioxygenase or 4,5-phthalate dioxygenase (Pujar and Ribbons, 1985).

Bacteria can work in cooperation with each other to complete certain steps in the degradation pathway of phthalate (Gu, J. D. 2005). Some members of the genus *Nocardia*, *Arthrobacter*, and *Pseudomonas* are known to completely degrade phthalate by themselves using a phthalate esterase such as 3,4-dioxygenase (Engelhardt & Wallnöfer 1978). While many species of bacteria are themselves capable of degrading phthalate esters, bacteria have also been observed forming biofilms with yeasts and fungi to accomplish this (Oliver et al. 2007).

The number of microorganisms that have been documented as phthalate degraders has been well documented by another literature review composed by Liang et al. (2008). In it, the author compiled a comprehensive table detailing the species, where it was isolated from, what phthalate ester it was capable of degrading, and how fast it was able to degrade it. From this information,

we can gather that the total amount of species able to degrade phthalate is diverse, as are the locations they were isolated from. Deep sea sediment, soil, activated sludge, mangrove sediment, river water, and oil-field soil all contained phthalate-degrading bacteria. This data also show that most bacteria accelerate the degradation process drastically, typically within a matter of a day or two.

Like bacteria, fungi have also demonstrated the ability to degrade phthalates and other synthetic compounds. Fungi employ several enzymes to catalyze the degradation of phthalates. Fungi are able to oxidize some PAHs either directly or indirectly by means of either lignin peroxidase or manganese peroxidase. Lignin peroxidases are capable of oxidizing phthalates directly, while manganese peroxidase co-oxidizes PAHs indirectly by means of lipid-mediated peroxidation (Hammel, 1995).

Esterases are also thought to play a role in fungal degradation of phthalate. Fungal species have been isolated and observed to degrade phthalates by means of hydrolysis of the ester bonds within the molecule. Luo et al. (2012) noted that two isolates belonging to the genus *Fusarium* and *Trichosporon* were able to break down dimethyl phthalate (DMP) into monomethyl phthalate or phthalic acid. Esterases were found within fungal mycelium and also within the supernatant, with a higher concentration being found within the mycelium. Tests ran suggested that the esterase is highly substrate specific. Luo et. al (2015) further confirmed the esterase found in *Fusarium sp.* is specific for cleaving the two carboxylic ester bonds present in dimethyl phthalate esters. It is interesting to note that white-rot fungi, the fungi that subsist on the lignin within wood, seem to be proficient at degrading phthalates. Among the multitudes of fungi, a search through the scientific literature shows that the genus *Fusarium* is commonly seen to be capable of degrading phthalates and other PAHs.

Degradation rates of some species have also been studied. Using HPLC and GC/MS, *Polyporus brumalis* was able to break down dibutyl phthalate almost entirely within culture medium in 12 days. 50% of this concentration was found within the mycelium. The intermediate byproducts consisted of diethyl phthalate and monobutyl phthalate and the final degradation product was phthalic acid anhydride along with small quantities of other aromatic compounds. It is thought that *P. brumalis* uses both transesterification and de-esterification to intracellularly metabolize phthalates (Lee et al., 2007).

It has been suggested that fungi and bacteria work in tandem to break down aromatic hydrocarbons such as phthalates. Both fungi and bacteria have been shown to display synergistic degradation of crude oil. The addition of fungi to a bacterial culture promoted biofilm formation and increased the rate of crude oil degradation (Yuan et. al, 2018). Oil and PAH degradation were further studied with a mixed culture composed of both yeast and bacteria. After 48 days, 56% of total petroleum and 32% of PAHs were degraded. An increase of biomass and enzymatic activity were indicators of an accelerated level of degradation resulting from the synergistic breakdown of PAHs between the two species (Zhang et al. 2014). Other synthetic compounds have been seen to be degraded by bacteria and fungi consortiums. Purnomo et al. (2017) saw increased rates of degradation of DTT, an organic synthetic pesticide, when a culture of the white-rot fungus *Pleurotus ostreatus* was combined with *Psuedomonas aeruginosa*. By itself, *P. ostreatus* was able to degrade DTT by 19% over the course of 7 days. With the addition of *P. aeruginosa*, levels of DDT were decreased by 86% over 7 days.

Given the research that has already taken place demonstrating the synergistic degradation of hydrocarbons via bacteria and fungi and given that both bacteria and fungi are able to utilize phthalic acid in their metabolic pathways, it is not unreasonable to hypothesize that the two have

developed a synergistic and symbiotic degradation mechanism for phthalates. Biofilms where both bacteria and fungi are present could be able to efficiently degrade phthalates into harmless compounds. Further research would be helpful in elucidating these theorized symbiotic degradation pathways.

Methods for Isolating Phthalate Degraders

Isolating microorganisms from environments contaminated with various phthalates typically involves taking soil samples and enriching the bacteria or fungi in a mineral salts solution where phthalate is the only carbon source. This selects for phthalate degraders while eliminating other types of bacteria and fungi that subsist off other types of nutrients (Nomura et al. 1989). Yuan et al. (2000) carried out an experiment that followed the aforementioned protocol to observe the microbial degradation of phthalate by microbial communities in contaminated sediment collected from 5 sites along the Tanshui River. The aerobic degradation half-lives of DBP and DEHP were determined, and tests were run using several different treatments to determine if degradation by microbial activity could be sped up.

While Yuan et al. analyzed their soil samples for phthalate-degrading bacteria and the rates at which phthalate esters were broken down, they did not consider the effect that existing phthalate contamination might have on the diversity of microbial communities within a natural setting. Changes in community composition were observed in laboratory conditions where the soil samples were spiked with several types and several concentrations of phthalate esters. The

change in climates may cause significant differences in microbial growth and the degradation of phthalate esters due to differences in temperature and precipitation.

Molecular Methods

Several other methods for isolating phthalate degraders from the environment exist outside of traditional microbiological culturing techniques. These include molecular methods such as polymerase chain reaction fluorescence in situ hybridization. DNA probes can be created for segments of the bacterial or fungal chromosome that codes for phthalate degradation. The phthalate dioxygenase gene is often a target of such probes. Iwaki et al. (2012) developed a degenerate DNA primer to identify the presence of potential degraders in seawater off the coast of Japan. The primer was designed to bind with the partial gene for 4,5-dihydroxyphthalate decarboxylase. These primers were based off of the same gene in the terrestrial species *Psuedomonas putida* and *Burkholderia cepacia*. Phthalate degraders in marine environments had not been widely studied and it was not known whether or not phthalate degraders would be present in salt water. Using the primers mentioned above, the researchers were able to identify eleven phthalate-degrading bacterial strains. This seems to show that the genes involved in phthalate degradation are highly conserved between terrestrial and marine species.

Using the method described by Iwaki et al., it would also be possible to identify genes involved in phthalate degradation by using fluorescence in situ hybridization. This would involve using a degenerate primer and labeling it with a fluorescent tag. Once the primer anneals to its target DNA sequence, it can be immediately viewed under an epifluorescence microscope. This

method would eliminate the time consuming and often frustrating steps involved with polymerase chain reaction.

A simple technique for identifying phthalate degraders based on the initial step of phthalate degradation being present within cells. The technique identifies the presence or absence of phthalate 4,5-dioxygenase and 4,5-dihydro-4,5-dihydroxyphthalate dehydrogenase in the degradation pathways of bacteria. The procedure for determining if bacteria were actively breaking down phthalate involves culturing bacteria on minimal salts agar plates with disodium phthalate as the only carbon source. Once colonies grew they were sprayed with a mist of freshly diazotized p-nitroaniline (Bray & Thorpe, 1954). If bacterial colonies turned red after being sprayed, they were considered as testing positive for the initial stages of phthalate breakdown (Nomura et al. 1989).

Applications of Phthalate Degraders

The Lake Calumet area in Cook County, Illinois is well known for its contamination of polycyclic aromatic hydrocarbons and heavy metals. The contamination stems from a long history of industry in the area. Industry began occupying the area in the 1860's due to the location's close proximity to water and railways. The site quickly became host to iron and steel manufacturers. Since many current environmental regulations did not exist at the time, industrial waste was not disposed of correctly and often ended up within the soil and water. Some of these pollutants can still be found in the area (Ross et al. 1998). Sites such as these would be prime candidates for microbial bioremediation. Due to the potentially toxic conditions where exposure

to contaminants can be damaging to the health of humans, bacteria could easily begin to break down phthalates and other harmful contaminants. This would be accomplished quickly and would be exceedingly cost effective. Microbial bioremediation would eliminate the need for costly water pumps and filtration devices needed to remove phthalates and other similar chemicals from water supplies.

Future Research

Further research still needs to be completed in order to be able to fully understand and utilize microbes for bioremediation purposes. There are still many unanswered questions as to what role fungi might play in the breakdown of phthalates. Several studies have already identified species capable of phthalate degradation, but this group is most likely underrepresented in scientific literature as most research so far has revolved around bacteria. It may be that fungi are better candidates for bioremediation, as they can tolerate many environmental conditions, such as high pH, that some bacteria could simply not handle.

Finally, it has been documented that the phthalate dioxygenase gene is present both in the bacterial chromosome and within plasmids, depending on the species (Eaton, R. W. 2001). Obtaining this specific DNA sequence would be beneficial for a bacterial cell, as it opens up a new carbon source for it to exploit. It would make sense that bacteria would be actively sharing this plasmid within their communities as this trait would increase their fitness. Plasmid transfer of the phthalate degrading trait has been demonstrated in laboratory conditions with *Pseudomonas putida* and *E. coli*, but it has not been observed in a natural setting (Nomura et al. 1990). Furthermore, the extent to which this takes place in nature is not known. While it is

theorized that bacteria would share this trait, the opposite could be true. If the trait is present in such low copy numbers within the cell it may be that the likelihood of that plasmid ever leaving the cell and finding its way into another bacteria is close to zero.

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Chapter 2: Introduction

Phthalates are chemicals commonly used in the plastics manufacturing industry as plasticizers (U.S. Department of Health and Human Services 1994). Phthalate esters can be found in common plastics such as PVC, and in items such as latex adhesives, cements, artificial leather, wire coatings, children's toys, and cosmetics. Because they are not chemically bound to plastics, they can enter the environment by means of industrial waste water, the burning of plastics, or from plastic leachate entering soil or water from landfills (Heudorf et al. 2007). Phthalates can adhere to dust particles and be deposited by wind and rainfall (Weschler et al. 2008; Thuren, Anders, and Larsson 1990).

Phthalate esters have various routes of exposure in humans including inhalation, ingestion, and dermal contact (Hauser, Russ, and Calafat 2005). It has been suggested that phthalate esters are mutagenic, carcinogenic, and teratogenic. Tests conducted on animals have shown adverse health effects including teratogenic and mutagenic activity due to exposure to phthalates esters. Rats injected with DHEP or DMEP were shown to increase mortality in litters, reduce litter size, and produce offspring that often had skeletal deformations (Dillingham and Autian 1973). Also, bacterial models have shown that dimethyl phthalate, diethyl phthalate, and di-n-butyl phthalate cause mutations in *Salmonella typhimurium* (Seed 1982). Due to their usefulness and many applications in various industries, phthalates have become an increasingly abundant environmental pollutant. Phthalates commonly enter the environment by means of water runoff and sewage, where they enter water systems and become deposited in soil sediments (Fromme et al. 2002). While phthalates degrade slowly in the environment due to photodegradation and hydrolyzation, aerobic biodegradation by means of microorganisms is the leading cause of degradation (Gao and Wen 2016).

Like bacteria, fungi have also demonstrated the ability to degrade phthalates and other synthetic compounds. Fungi have demonstrated this ability in numerous studies where they have been observed breaking down several types of phthalates, with some species being more effective degraders than others (Ahuactzin-Pérez et al. 2014; Aguilar-Alvarado et al. 2015; Chai et al. 2008). Several enzymes appear to be responsible for the breakdown of phthalates. Among these are extracellular peroxidases that are typically involved in lignin breakdown (Hammel 1995; Hwang et al. 2012). Fungi are able to oxidize some PAHs either directly or indirectly by means of either lignin peroxidase or manganese peroxidase. Lignin peroxidases are capable of oxidizing phthalates directly, while manganese peroxidase co-oxidizes PAHs indirectly by means of lipid-mediated peroxidation (Hammel 1995).

Fungal diversity in response to the presence of polycyclic aromatic hydrocarbons (PAH) has been shown to decrease with an increase in PAHs (Kim et al. 2010). Furthermore, phthalate ester contamination can lessen the overall diversity of soil microbiomes (Wang et al. 2016). The overall effect of phthalates on fungal diversity remains a topic that requires more research. Furthermore, the genes coding for the enzymes in fungi responsible for phthalate breakdown need to be examined, as there is currently little work being done on the topic. Research into bacterial genes and enzymes is much more commonplace and many genes from multiple species have already been sequenced and analyzed (Jin et al. 2010; Chang and Gerben 1998; Wang et al. 2012). The same level of scrutiny is absent with fungal genes.

This research seeks to examine the diversity of fungi found at a site containing a significant amount of plastic litter and will attempt to identify phthalate degrading species of fungi from sediment samples. It is theorized that sediment samples obtained from areas with high levels of

manmade contamination will display a lower diversity than those samples obtained from a more pristine site.

Chapter 3: Materials and Methods

3.1 Sample Preparation and Collection

Sediment samples were obtained from the Lake Calumet area in Illinois (41.684334, −87.577017) and were collected in accordance with the methods described in Kolb et al. (2019). The Big Marsh (BM) area was chosen for this study due to high levels of polyaromatic hydrocarbons (PAHs) present resulting from pollution generated from surrounding industries since the early 1900's. Illinois Environmental Protection Agency detected various heavy metals and PAHs within the Lake Calumet area that exceeded the levels detected in off-site locations. Antimony, Arsenic, Cadmium, Vanadium, and Benzo(a)pyrene were all detected within environmental samples and are of concern due to the carcinogenicity of these pollutants. Kolb et al. led a study at this site that identified several species of bacteria that were able to survive on phthalate as the sole carbon source. This research warranted further investigation into if fungal species present within the area could also degrade phthalates and the impact these pollutants had on the overall diversity of fungi. A separate site at Vermillion River (VR) in Lasalle County, IL (41.292438, −89.030565) was chosen as a control site due to its relative isolation from major sources of pollution. Kolb et al. reported that only isolates obtained from the contaminated sediment samples of BM were capable of degrading phthalates. This finding suggests that bacterial species present at BM adapted to be able to degrade phthalates in response to their presence within the environment as no phthalate degrading bacteria were present in the non-contaminated sediments at VR. It can therefore be assumed that a similar effect might be seen in the populations of fungi at both sites.

Samples were randomly collected from shallow water ranging in depth from 0-5 cm into 50 ml sterile tubes. The distance between sample sites was between 500-1000 ft. The sediment samples were then brought back to the lab and stored at -20° C until the time of use. For this study, 10 samples were chosen from each site for analysis.

Enrichment for phthalate degraders followed the protocol outlined by Wu et al. (2010). Minimal salts medium (MSM) was used (5.8 g K_2HPO_4, 4.5 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.16 g $MgCl_2$, 0.02 g $CaCl_2$, .0024 g $Na_2MoO_4*H_2O$, .0018 g $FeCl_3$, and .0015 g $MnCl_2*2H_2O$/L) with a pH that has been adjusted to 7. MSM and was sterilized by autoclave for 20 mins at 121 °C. The sterile solution was mixed with 18g of agar per 1L of MSM for plating. Phthalate (DMP, DEP, DBP) was added to the MSM at 50mg of each phthalate per 1L. This yielded phthalate agar, where phthalates were the sole carbon source.

Enrichment cultures were prepared in 500mL Erlenmeyer flasks containing 200mL of MSM containing a mixture of DMP, DEP, and DBP (each at 66.66mg/L^{-1}, 200mg/L^{-1} total These were inoculated with 5g (wet weight) of sediment sample. The cultures were incubated for 10 days in the dark at 30° C on a rotary shaker operated at 130 rpm. After incubating for 10 days, 1% by volume of each enrichment was serially transferred to a higher concentration of mixed phthalates (200→300 → 400→500mgL^{-1}) with a 10d incubation following each transfer. In the final enrichment, DNA was extracted for sequencing of the 18S rRNA gene to identify the enriched members of the communities.

To isolate fungi, samples taken from BM and VR were serially diluted and plated in triplicate onto Sabouraud dextrose agar (pH 5.6) via sterile swabs. Plates were incubated for 7

days at 23°C. Plate counts were obtained from each sediment sample upon completion of the incubation. Isolates were transferred several times from the original culture to new SDA plates to ensure a pure colony was obtained. Once a pure colony was isolated, it was plated on phthalate agar to test for phthalate usage. Hyphal growth throughout the plate was deemed indicative of phthalate utilization as previous studies have shown a positive correlation between hyphal growth and phthalate degradation (Ahuactzin-Pérez et al. 2014, Suárez-Segundo et al. 2013).

3.2 Metabolic Activity and Identification

The metabolic activity of the soil microbiome was compared between BM and VR using Biolog Ecoplates (Biolog Inc.). A soil dilution 0.5g of soil suspended in 50 ml of sterile water was pipetted into each well via multichannel pipette and allowed to incubate for 7 days at 23°C. Plates were read at 500nm on a plate reader to determine carbon usage patterns of the microbiome. A principal components analysis was performed on the resulting data using RStudio. Identification of the isolates was attempted using Biolog FF fungal identification plates coupled with the Biolog Gen III database.

3.3 Metagenomics Analysis

DNA was extracted from sediments using the MO BIO Ultra Clean® Mega Soil DNA Kit and from enrichments using the MO BIO UltraClean® Microbial DNA Isolation Kit. DNA obtained from the sediment samples was sent to Argonne National Laboratory's Environmental Sample Preparation and Sequencing Facility (Lemont, IL) for metagenomics analysis. Samples were run using the Illumina MiSeq platform for 18S rRNA amplicon sequencing. Primers

targeting the internal transcribed spacer (ITS) region of the 18S rRNA gene of fungi were also used (Martin and Rygiewicz 2005). This served to aid in the identification of fungi as the ITS region shows more variability when compared to the 18S gene and can more easily differentiate between species of fungi.

The data collected was used to obtain the overall diversity of fungi present in the sediment samples, the distribution and proportions of each genera, and how BM and VR differ in species community composition. To determine the overall diversity of each site, a Shannon Diversity Index value was calculated for each sample site. Since only a small portion of DNA from each sample was able to be identified, this study only focuses on identifiable sequences and ignores all unidentifiable sequences (86.7%) present in the metagenomic analysis.

3.4 Statistical Analysis

A principal components analysis (PCA) was generated from the data obtained via the Biolog Ecoplates to determine if carbon usage patterns at VR and BM differ. A PCA was also performed at the species level of diversity comparing the species composition of both sites to see if any significant difference exists (see Appendix). This analysis was made under the assumption that each of the 10 samples from each site were independent of each other.

The PCA was generated using the "prcomp" function in RStudio which uses singular value decomposition to covariances/correlation between sample sites. For this analysis, only fungi that were identified down to the species level were used and the average abundance of each species were calculated from all ten replicates. A two-sample t-test was performed at the species level to

further determine if the communities present at BM and VR differed significantly. A two-sample t-test was also performed on the plate counts derived from SDA plates.

Chapter 4: Results and Discussion

4.1 Plate Counts

SDA plate counts from VR and BM differed both in number of colonies present and the number of different morphologies that appeared on the plates. BM had an average count of 2560 colonies per 1.0g of soil while VR had an average of 466 colonies per 1.0g of sample. BM displayed a higher level of visual diversity on a plate-for-plate basis with an average of 2.9 different colony morphologies per plate with values ranging from 1 to 6 types per plate. VR had a lower diversity, with an average of 1.9 different morphologies per plate. The two-sample t-test revealed a significant difference between the counts obtained from the sediment samples at both sites ($p = 0.008$).

All fungal isolates were found to be able to grow on phthalate agar containing DMP, DEP, DBP with the exception of one VR sample that showed no hyphal growth. Only one plate bore visible fruiting bodies, suggesting some inhibitory effect on reproduction resulting from phthalate exposure. Microscopic analysis of hyphae showed sexual reproduction in the form of zygospores on several plates (See fig. 1). Asexual reproduction was noted on one plate by the presence several *aspergillus* sporangia.

Identification of individual colonies was attempted using fungal identification microplates (Biolog). No identification was able to be made using this method. This could be due to the colonies not being pure cultures, or cultures not having a reference within the GEN III database to be compared to.

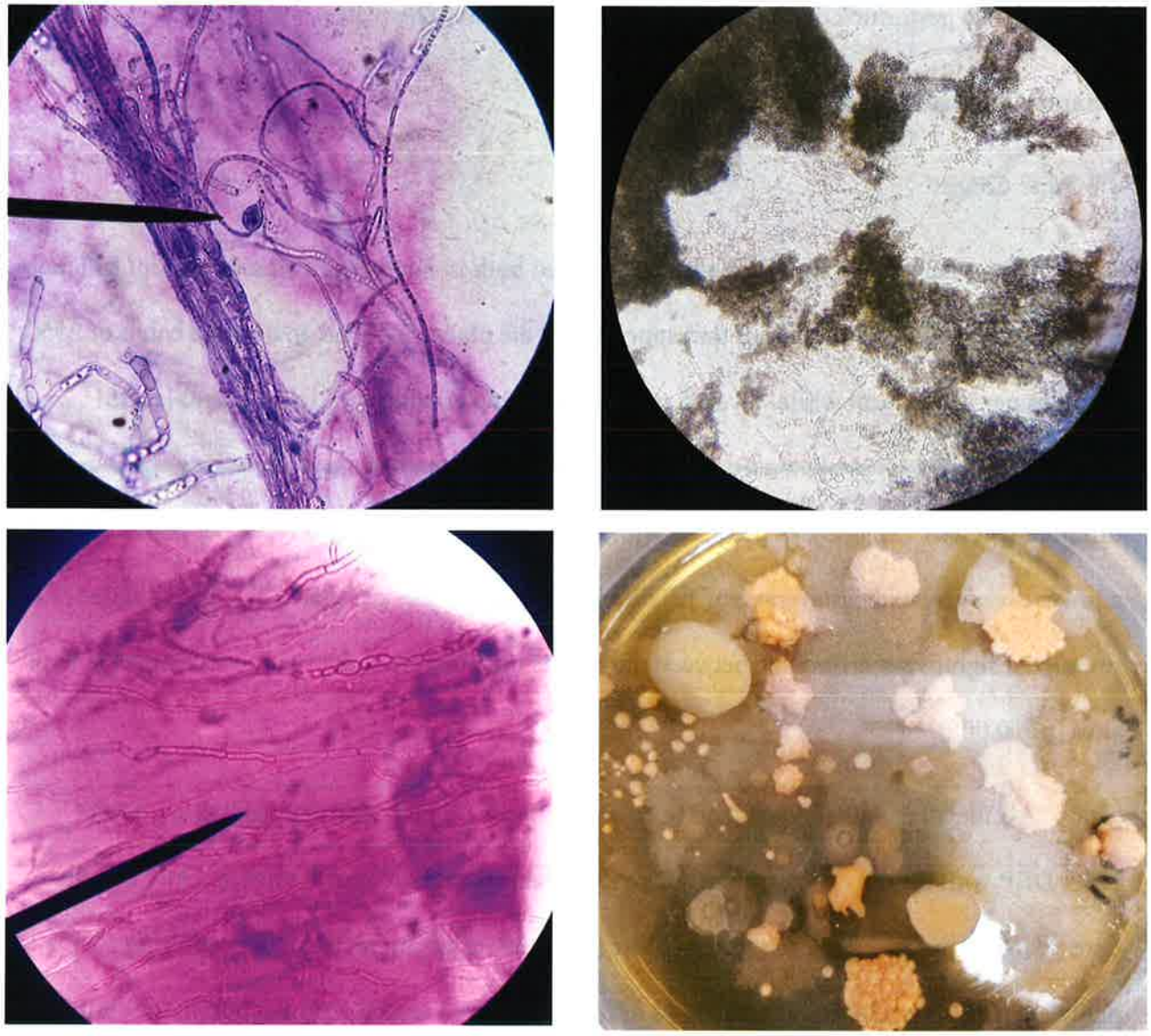

Figure 2. Microscopic images depicting hyphae from samples obtained at Big Marsh along with several different species of macroscopic fungi growing on the surface of a SDA plate.

4.2 Metagenomics analysis

At the phylum level, both VR and BM had Ascomycota and Basidiomycota as the prominent identifications. At the class level, VR had 9 classes identified and BM had 11 identified. At VR, the most prominent classes were Tremellomycetes (61%), Sordariomycetes

(15%), Dothideomycetes (8%), Agaricomycetes (8%), and Leotiomycetes (3%). Classes found at BM included Sordariomycetes (55%), Agaricomycetes (11%), Dothideomycetes (9%), Laboulbeniomycetes (8%).

At the order level, VR saw 27 orders identified and BM saw 26 identified. The most prominent orders found at VR were Cystofilobadiales (33%), Hypocreales (25%), Pleosporales (9%), Polyporales (7%), and Helotiales (6%). Orders at BM included Hypocreales (45%), Pyxidiophorales (8%), Filobadiales (7%), Agaricales (5%), and Pleosporales (4%).

At the family level of classification, VR had 50 families identified and BM had 32 families identified. The most prominent families at VR included *Mrakiaceae* (28%), *Hypocreales incertae setis* (22%), *Ganodermataceae* (6%), *Cystofilobasidiales incertae sedis* (5%), and *Didymellaceae* (4%). The most prominent families found at BM included *Hypoceales incertae sedis* (56%), *Pyxidiophoraceae* (10%), *Nectriaceae* (5%), and *Pezizaceae* (4%).

There were 33 genera identified at BM and 13 genera identified at VR (See fig.2 and fig.3, respectively). The most prominent genera observed at VR were *Tausonia* (39%), *Sarocladium* (30%), and an unidentified genus belonging to the order *Cystofilobasidiales* (8%), *Phoma* (6%), and *Neobulgaria* (4%). *Pyxidiophora* was the most abundant genera found at BM (95.67%), followed by *Acremonium* (0.16%), *Disculoides* (0.13%), *Pseudallescheria* (0.06%), and *Tomentella* (0.05%).

There were 85 different species identified at BM and 158 species that were identified at VR (see fig.4 and fig.5, respectively). The most abundant species present at BM was *Sarocladium strictum* (46.10%), followed by *Pyxidiophora microspora* (5.12%), *Naganishia*

globosa (3.17%), *Coprinellus verrucispermus* (1.90%), and *Fusarium culmorum* (0.95%). The most abundant species present at VR were *Sarocladium strictum* (25.73%), *Tausonia pullulans* (11.45%), *Preussia flanaganii* (1.77%), *Chalara pseudoaffinis* (1.39%), and *Mrakiella aquatica* (1.39%).

Table A.1 (see Appendix A) shows a distinct difference in species composition between BM and VR. Visually, VR (designated by the letter "C") seems to have a greater distribution of species that is consistent with all 10 samples derived from VR. BM9 and B10 have a similar pattern to what is shown in the VR samples, but all other samples show a pattern with discernably less diversity. Samples labeled SC and BC were obtained from the gut microbiome of two species of Asian Carp. While this study does not focus on these samples, it should be noted that they display a similar pattern to the phthalate isolate enrichments in that *Naganishia globosa* is the dominant species. The soil enrichments (designated by "ln") also show a similarity among themselves. Once again, *Naganishia globosa* is the predominant species.

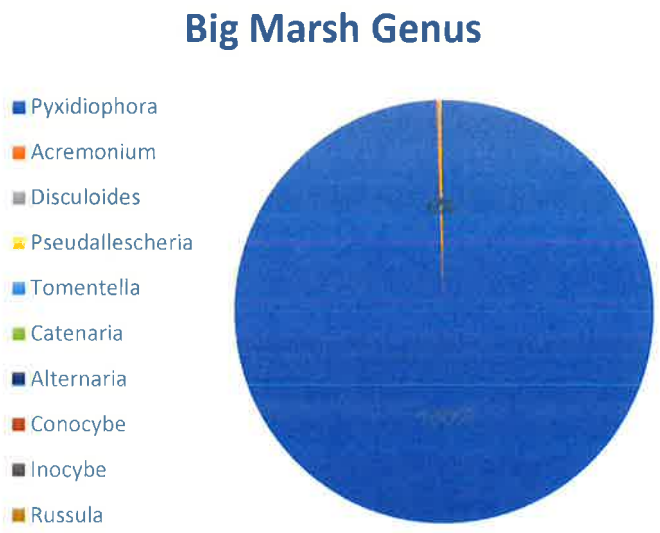

Figure 3. Distribution at the genus level of classification for BM showing the ten most-abundant genera. BM contained 33 genera total.

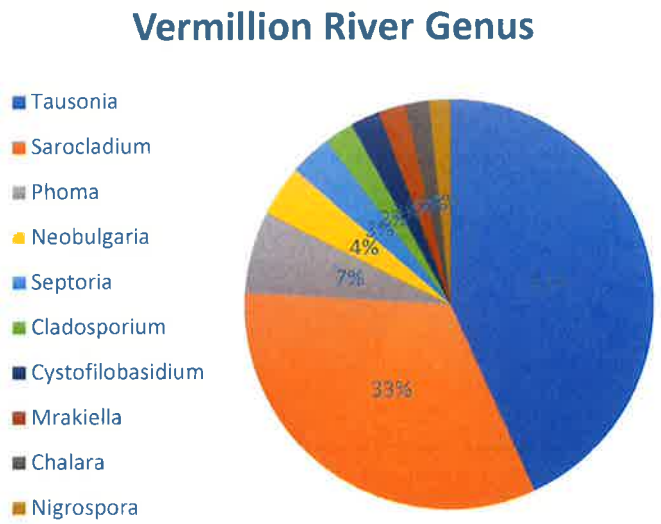

Figure 4. Distribution at the genus level of classification for VR showing the ten most-abundant genera. VR contained 13 genera total.

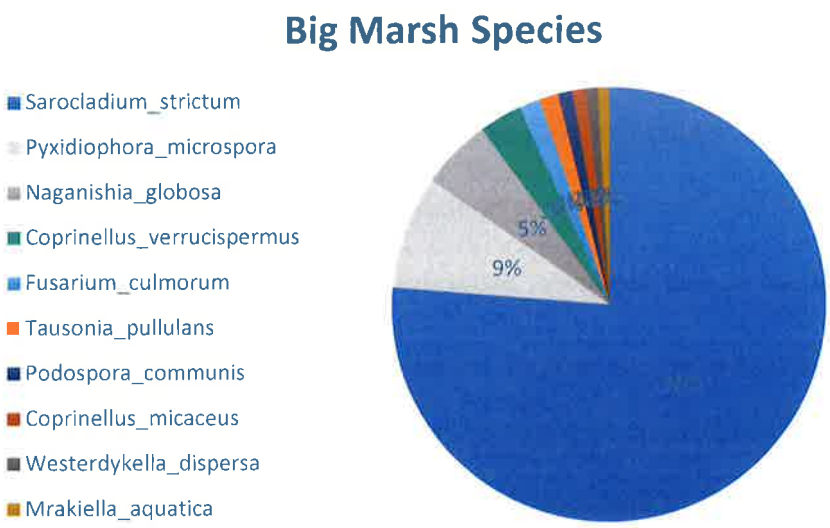

Figure 5. Distribution at the species level of classification for BM showing the ten most-abundant genera. BM contained 85 genera total.

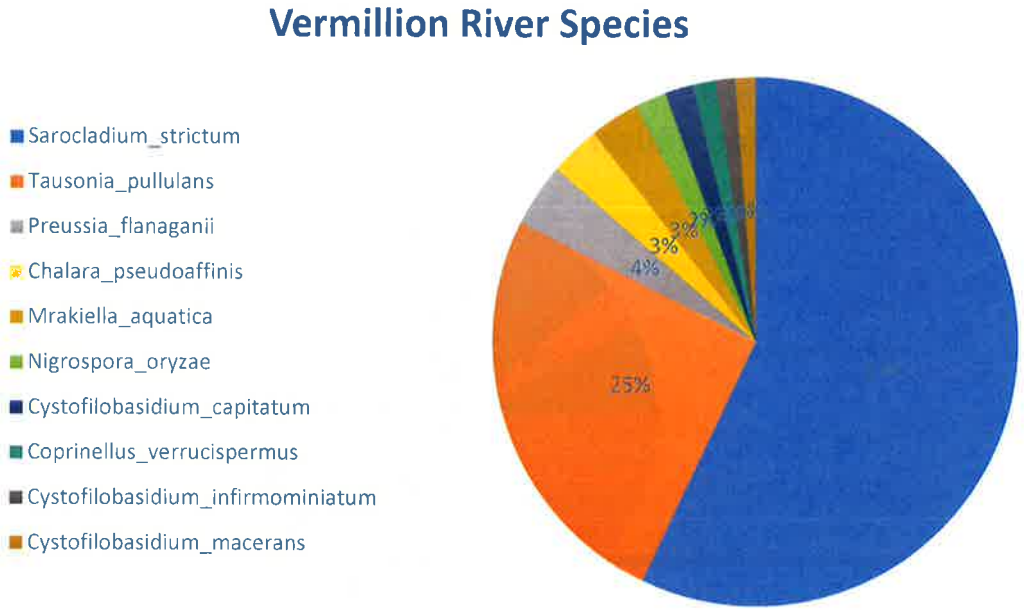

Figure 6. Distribution at the species level of classification for VR showing the ten most-abundant species. VR contained 158 genera total.

The phthalate enrichments of Kolb et al. containing bacteria isolates showed the presence of several species of fungi. The dimethyl phthalate enrichment contained *Mycosphaerella tassiana*, *Penicillium chrysogenum*, *Penicillium polonicum*, and two unidentified species of *Penicillium* (see fig.7). The diethyl phthalate enrichment contained *Cladosporium* sp., *Phoma sp.*, *Candida spencermartinsiae, Meyerozyma caribbica, Naganishia globosa,* and a member of *Dothideales* (see fig.8). The dibutyl phthalate enrichment contained *Coniosporium apollinis*, *Naganishia globosa*, *Fusarium* sp., and *Schwanniomyces sp.* (see fig.9). The species found within these enrichments are not known to be degraders of phthalates. These identifications may represent strains of these species that carry mutations that enable them to utilize phthalates in their metabolism. Another possible explanation may be that these fungi were obtaining nutrients from the bacteria present in these enrichments.

Kolb et al. identified several genera of bacteria within the soil-amended phthalate enrichment cultures. These included members of *Pseudomonas*, *Arthrobacter*, *Ochrobactrum*, *Stenotrophomonas*, *Chryseobacterium*, *Micrococcus*, *Pigmentiphaga*, *Wautersiella*, *Variovorax*, *Enterobacter*, *Novoshingobium*, *Comamonas*, and *Acinetobacter*. These samples showed *Achromobacter*, *Pseudomonas*, and *Ochrobactrum* were most prominent.

Several genera of bacteria isolated by Kolb et al. along with fungi identified by this study have also been found together in PAH enrichments from other research. *Pseudomonas*, *Rhodococcus*, and *Fusarium* were both observed in an enrichment of ethylene glycol dibenzoate, a plasticizer similar to phthalate (Jackson, Labeda, and Becker 1996). A different study isolated various species of fungi from sewage treatment biofilms that actively degraded DEP and di-2-ethylhexyl phthalate in enrichments (Oliver, May, and Williams 2007). This study identified hyphae present in media containing the aforementioned phthalates alongside bacteria such as *Sphingomonas* and *Agrobacterium* that were also present in the enrichments of Kolb.

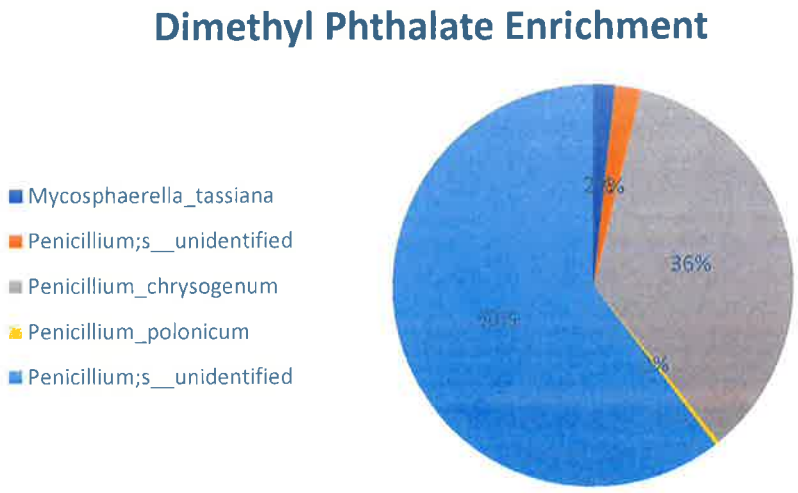

Figure 7. Distribution at the species level of classification for DMP.

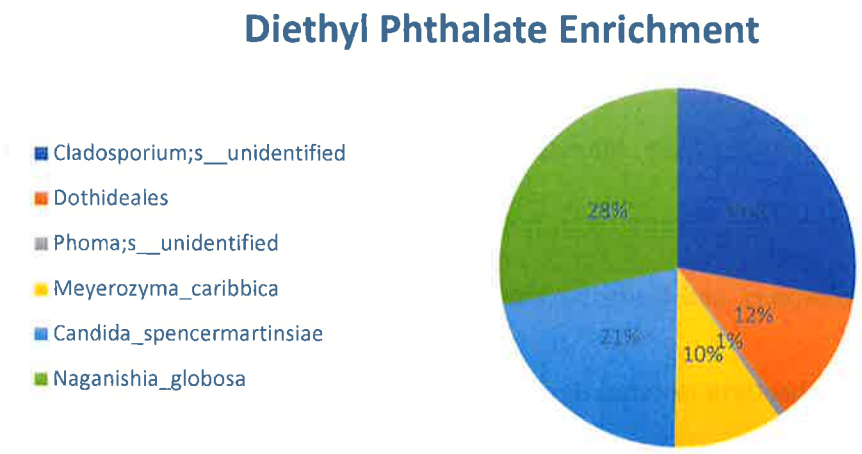

Figure 8. Distribution at the species level of classification for DEP.

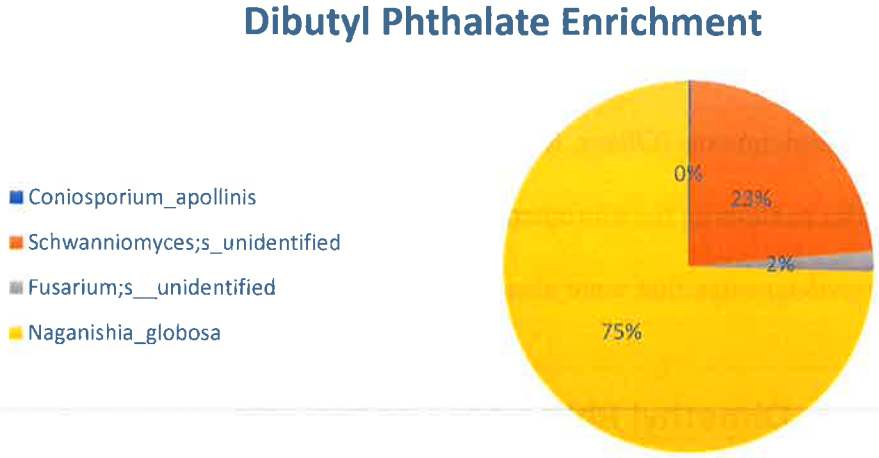

Figure 9. Distribution at the species level of classification for DBP.

4.3 Biolog EcoPlates

The PCA performed on the Biolog Ecoplate data shows most variation occurring on PC1 (see fig.10). No discernable grouping is present between BM and VR loadings, indicating that carbon usage between the sites does not significantly differ. Data obtained from the Biolog Ecoplates showed ubiquitous carbon usage for most carbon sources across both BM and VR. Nearly all samples from both sites showed a strong affinity for utilization of D-Galactonic Acid

γ-Lactone, L-Arginine, Pyruvic Acid Methyl Ester, D-Galacturonic Acid, L-Asparagine, Tween 40, Tween 80, D-Mannitol, 4-Hydroxy Benzoic Acid, L-Serine, D-Cellobiose, D-Xylose and N-Acetyl-D-Glucosamine. Samples from BM did not appear as efficient at utilizing D-Glucosaminic Acid, Itaconic Acid, Glycyl-L-Glutamic Acid, or Phenylethyl-amine when compared to VR.

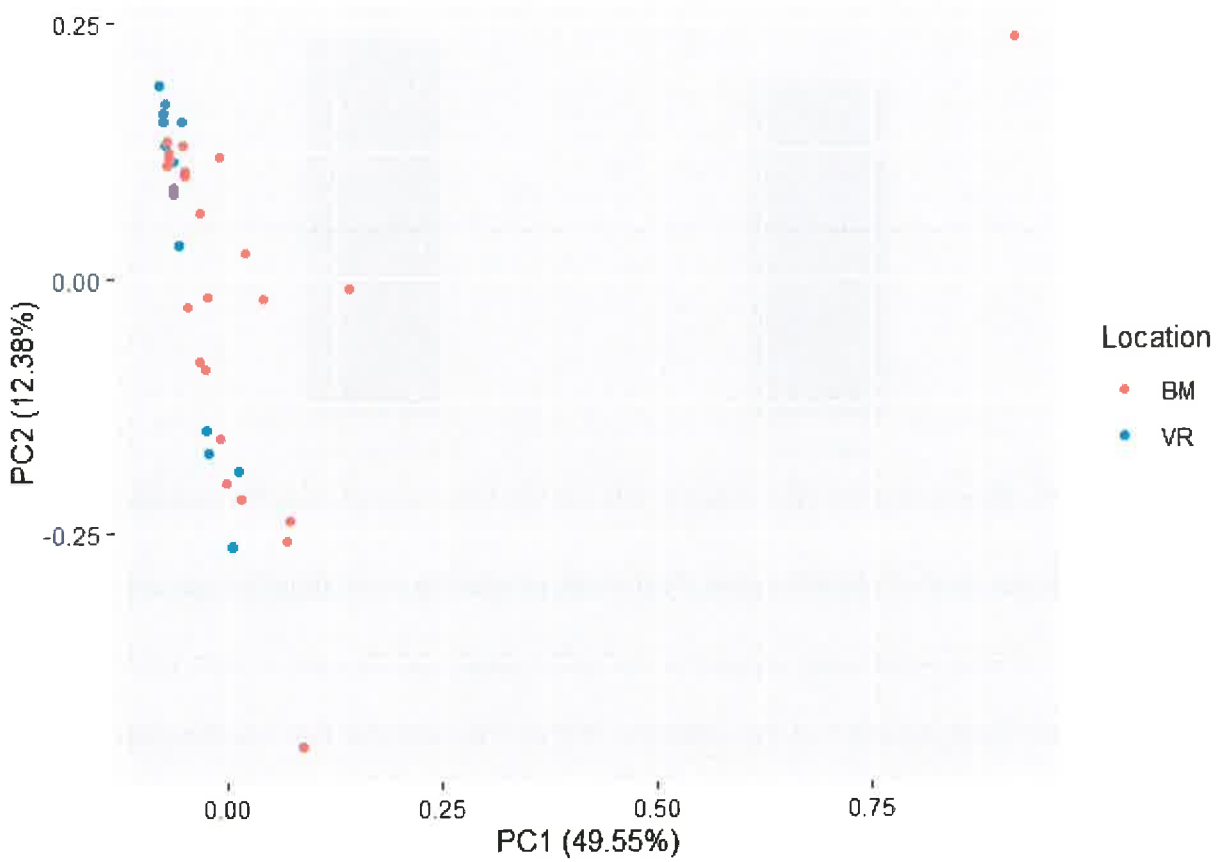

Figure 10. PCA of Biolog Ecoplate data showing carbon usage between BM and VR.

4.4 Community composition and diversity

The Shannon Index value for BM using the metagenomics data at the species level was calculated to be 0.06 and the value for VR was calculated to be 0.07 (see fig.11). Equitability for BM and VR were 0.014 and 0.013, respectively. Results show low species evenness at both sites and a slightly higher diversity for VR. Results of the two-sample t-test indicate that the

difference in species composition is not significant (p = 0.857). A Levene's test for homogeneity of variance was also performed to ascertain if the population variances were equal. Levene's test showed that the two variances between the sites were equal (p = 0.8116).

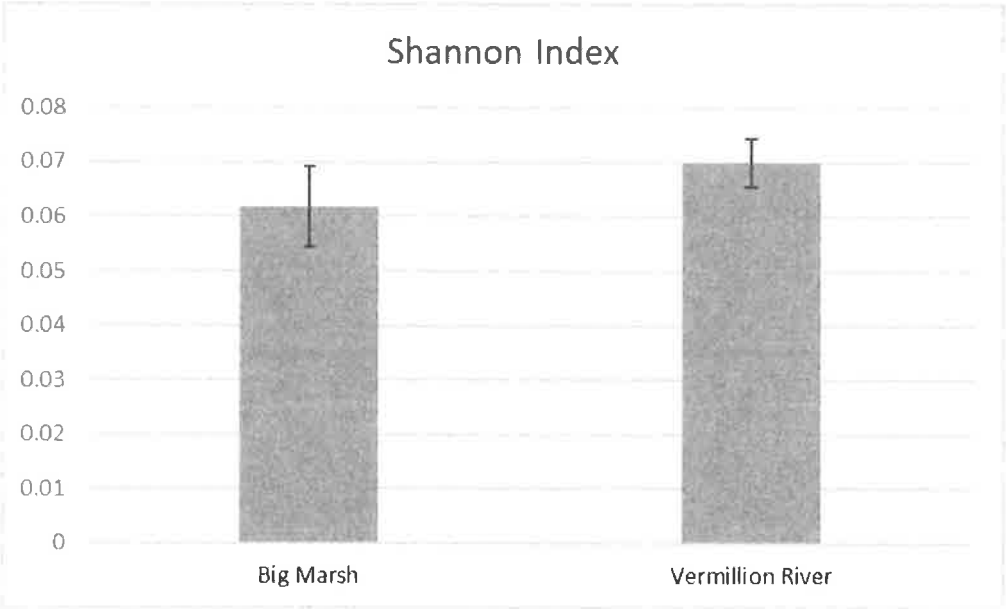

Figure 11. Shannon diversity index values for BM and VR. Error bars represent 95% confidence interval.

At the genus level of identification, *Pyxidiophora* was the most abundant genera within BM. This genus is most commonly associated with arthropods and they are not known to be degraders of man-made pollution in any capacity. Within VR, *Sarocladium* was the most dominant genera, followed by *Tausonia*. At the species level, VR contained almost twice as many species when compared to BM. 158 species were identified at VR and 85 species were identified at BM. *Sarocladium strictum* was the most prevalent fungi at both sites with a higher abundance found at BM (76%). Other fungi identified in significant amounts at BM include *Pyxidiophora microspora* (9%), *Naganishia globosa* (5%), *Coprinellus veruccispermus* (3%), and *Fusarium culmorum* (2%). The most abundant species found at VR included *Sarocladium strictum* (57%), *Tausonia pullulans* (25%), *Preussia flanaganii* (4%), *Chalara pseudoaffinis* (3%), *Mrakiella* aquatica (3%). 23 species were unique to BM while 94 species were unique to

VR. The species identified included yeasts, plant pathogens, soil fungi, wood rot fungi, and aquatic fungi.

Several notable degraders of phthalates and other pollutants were identified as being present at each site (see Tables A2 and A3 in Appendix). 25 species at BM and 28 species at VR have previously been documented as being able to either degrade either phthalates, PAHs, or heavy metals. *Sarocladium strictum* was responsible for most of the identifiable DNA sequences present at both sites. *Sarocladium* is a genus of phytopathogens that have shown to have some affinity for degrading PAHs. The genus consists of the so-called white rot fungi that breakdown wood and other detritus via ligninolytic enzymes. The ability to degrade PAHs has been amplified in experimental conditions when grown with another species of yeast (Kamyabi, Nouri, and Moghimi 2017). This genus has also been observed growing on tarballs present in marine environments (Shinde, Suneel, and Shenoy 2017).

Fusarium is a prolific phthalate degrader that has been observed participating in the breakdown of several different varieties of phthalates. Members of *Fusarium* have been seen to employ the enzyme cutinase to degrade phthalates. Cutinase derived from *F. oxysporum* was able to degrade 60% of a butyl benzyl phthalate solution within 7.5 hours under experimental conditions (Kim et al. 2002). A species of Fusarium isolated from mangrove sediment was also observed to degrade di-(2-ethylhexyl)-phthalate. This area also exhibited high levels of PAHs and heavy metal contamination and appears to be a similar environment to what is found at BM (Luo et al. 2009).

Fusarium culmorum was observed at BM and has been noted by several different papers to have the capability to break down phthalates such as dibutyl phthalate and 2-ethyl-hexyl phthalate (Ahuactzin-Pérez et al. 2016; Ahuactzin-Pérez et al. 2018). This species was also

present at VR but was not as abundant. *Fusarium solani* was present at BM and VR and has been shown to degrade PAHs as well (Wu, Luo, and Vrijmoed 2010).

Coprinellus micaceus was observed at both sites. It is a member of Basidiomycota and is commonly found around rotting wood. *C. miraceus* has been observed metabolizing 2,7-dichlorodibenzo-p-dioxin and is exceedingly capable of biosorption of lead from the environment (Albert et al. 2020; Suhara et al. 2011). *Pseudallescheria boydii* is a plant pathogen capable of infecting humans as well. It was able to remediate PAH contamination when in a consortium of other species of fungi (Greco et al. 2019). *Penicillium chrysogenum* is capable of degrading di-2-ethylhexyl phthalate and is capable of leaching a wide array of heavy metals from contaminated soils (Crow 2013; Deng et al. 2012). *Rhodotorula graminis* is an endophytic yeast found in riparian areas and can degrade toluene (Hesham, Alrumman, and ALQahtani 2018). *Stropharia coronilla* has been shown to break down benzo[a]pyrene in liquid culture (Pozdnyakova 2012). *Trametes versicolor* is known to degrade various phthalates and PAHs via an increased production of laccase when exposed to such chemicals. It is also capable of biosorption of heavy metals (Kim et al. 2008; Majcherczyk, Johannes, and Hüttermann 1998). *Meyerozyma caribbica* is also capable of the biosorption of heavy metals such as manganese (Amorim et al. 2018). *Trichoderma asperellum* belongs to the ascomycetes and is from a genus that is known for degrading PAHs. Species within this genus use an array of enzymes, such as laccases and dioxygenases, to accomplish this (Zafra and Cortés-Espinosa 2015). *Trichoderma longibrachiatum* was also present at both sites. *Nigrospora oryzae* is another species found at BM and VR that has been known to degrade PAHs. This plant pathogen was able to reduce concentrations of naphthalene and phenanthrene by up to 85% in experimental settings (Kannangara et al. 2016).

Irpex lacteus uses a manganese peroxidase to degrade PAHs and has been noted to degrade numerous compounds in various studies. This white rot fungi appears to be a good candidate for the bioremediation of contaminated soils because of this (Baborová et al. 2006). *Scytalidium lignicola* can break down PAHs and *Tomentella stuposa* has been observed growing in areas contaminated with heavy metals resulting from mining activity (Cerniglia and Sutherland 2001; Huang et al. 2014).

Those species capable of degradation that were unique to BM include *Periconia byssoides*, *Rhodotorula dairenensis*, *Coprinopsis atramentaria*, *Candida parapsilosis*, *Inocybe curvipes*, *Thielaviopsis basicola*, *Saccharomyces cerevisiae*, *Macrophomina phaseolina*, *Aspergillus flavus*, and *Stereum hirsutum*. While none of these species are known phthalate degraders, they have all been observed to at least be capable of PAH degradation or have some tolerance to heavy metals.

Cystofilobasidium capitatum, Wickerhamomyces anomalus, Abortiporusb iennis, Trichoderma atroviride, Westerdykella dispersa, Coprinellus disseminatus, Trichoderma virens, Alternaria alternata, Curvularia lunata, Trichoderma harzianum, Phlebia tremellosa, Mucor circinelloides, and *Coprinus cordisporus* were all found exclusively at VR and were noted in various scientific literature to be able to either degrade/show resistance to phthalates, PAHs, and heavy metals (see Table A.3 and accompanying references in Appendix).

While these fungi have been noted to be degraders of various xenobiotic pollutants, it should be noted that because they have been seen degrading these compounds in experimental settings or elsewhere in nature does not mean they are necessarily degrading pollutants at BM or VR. Likewise, just because a fungus has never been observed degrading pollutants does not exclude fungi within these samples from having that ability. While highly unlikely, it is possible

that some of the species present within BM have obtained genes from other degraders via horizontal gene transfer enabling them to degrade the pollution inherent in that area. Horizontal gene transfer has been associated more with prokaryotes, but a growing body of evidence shows that this mechanism is present in fungi as well (Fitzpatrick 2012). Occurrences of horizontal gene transfer have been recorded involving bacteria transferring genes for specific enzymes to fungi (Garcia-Vallve, Romeu, and Palau 2000). A far more likely explanation is that fungi naturally possess many of the tools they need to degrade many man-made compounds.

It was thought that BM would carry the bulk of degraders, but many degraders were also found to exist at VR. This may not be as odd of an occurrence as it seems when you consider the purpose of fungi within the ecosystem as saprotrophs. As previously mentioned, fungi employ a variety of enzymes to break down plant matter and other detritus. Enzymes such as lignin peroxidases, manganese peroxidases, versatile peroxidases, and laccases evolved to help fungi degrade incredibly sturdy molecules such as lignin. Lignin is composed of aromatic rings linked together primarily by ester bonds, although other linkages can occur between lignin monomers. This structure is very similar to phthalates and PAHs. This similarity may allow fungi to degrade some synthetic man-made compounds with ease.

These enzymes can attack various structures of the lignin molecule. For example, lignin peroxidase (LiP) is able to attack aromatic rings directly using H_2O_2. (Umezawa et al. 1986). LiP was observed cleaving aromatic components of a synthetic lignin via oxidation of the protein surface by a long-range electron transfer process. This process was elucidated by NMR and MS (Higuchi 2004). LiPs are similar to other peroxidases in that they are relatively nonspecific in the substrates that can react with. Laccases are another enzyme with the potential to be naturally compatible with degradation of phthalates and PAHs. This enzyme is found in many of the

white-rot fungi and has a substantial role in the depolymerization of lignin. Laccase breaks down lignin in several ways including ether cleavage and aromatic ring cleavage (Wong 2009). Laccase activity has been noted to increase in some species of fungi when introduced to phthalates, indicating that these enzymes are used by the fungi to metabolize phthalates (Yeo, Kim, and Choi 2008).

One final group of enzymes employed by fungi that could degrade phthalates into harmless phthalic acid are the esterases. In nature, these enzymes are used to break down plant polysaccharides by cleaving the cross linkages that occur between the polymers that constitute the cell walls of plants. Feruloyl esterase is an example of such an esterase produced by many species of fungi (Donaghy and McKay 1997). Regarding phthalate degradation, an esterase would cleave the ester bonds of phthalate and free the side chains from the aromatic ring structure. This would have significant implications for the molecule since the toxicity and hydrophobicity of phthalates depends on the length of the side chains. Such an esterase has been discovered when examining a soil microbiome metagenomic library (Wu et al. 2019). Another phthalate degrading esterase was found in a species belonging to the genus *Fusarium* and was specific to the hydrolysis of dimethyl terephthalate (Luo et al. 2012).

4.5 Identification of noteworthy species

Several species were identified in the metagenomics analysis that warranted mention in this research. These species are noteworthy because they have not previously been observed in North America previously. The first species, *Helicascus uniseptatus*, is a member of Ascomycota that was recently discovered for the first time living on submerged wood in Thailand (Luo et al. 2016). *Lyophyllum favrei* is a basidiomycote that inhabits beech forests. It is only known to inhabit central Europe and Russia (Dahlberg and Croneborg 2006). *Coprinellus radicellus* was

recently discovered in 2011 in Scandinavia and to date has not been observed in any other location (Házi et al. 2011). It should be noted that these identifications by the metagenomics analysis are not absolute, instead being the closest match possible from the DNA within the sample to the database used.

Chapter 5: Conclusions

Statistical analysis showed a significant difference in plate counts between BM and VR with BM having a significantly higher microbial load per unit of soil when compared to VR. Metagenomic data from BM and VR revealed a slightly higher species diversity at VR and nearly twice as many species identified. Shannon index values were low due to extremely low abundances of most species present at both sites. A two-sample t-test indicated that there was no significant difference in species composition between sites. *S. strictum* was the most abundant species at both sites but ultimately the species composition and proportions varied between the two sites. Phthalate enrichments showed high abundances of several species including *N. globosa* and several species of *Penicillium*.

The Ecoplate PCA showed no significant difference in carbon usage patterns between BM and VR. Each site contained species known to utilize phthalates, PAHs, and heavy metals. Also, fungi plated on phthalate agar from BM and VR both showed signs of hyphal growth. This could be explained by the arsenal of enzymes that fungi naturally employ to break down plant matter and the structural similarity that plant polysaccharides share with phthalates and PAHs. Several species not previously found in North America were identified in the metagenomics analysis.

Further research is needed to determine if the fungi present at both sites can truly degrade phthalates and PAHs. Isolates should be individually identified, and phthalate degradation measured by analytical techniques such as HPLC. Transcriptome analysis should be performed on cultures amended with phthalates and other PAHs to ascertain what genes are activated in the presence of such pollutants. This analysis could also be extended to include the addition of bacteria to fungal cultures to determine if a synergistic degradation occurs between the two.

A full genetic analysis would also provide information on the number of genes responsible for degradation and where these genes are located within the genome. Investigation into fungal plasmids to determine if traits such as phthalate degradation can be shared between individuals or among different species would be helpful, as this area of research is currently lacking. It is known that some species of fungi contain plasmids, however most of these are mitochondrial in nature and do not replicate inside the host cell, instead needing to be integrated into the host's genome in order to be expressed (Griffiths 1995). Such research could provide valuable information as to the mechanisms behind the degradation of xenobiotic substances and lead new and more efficient applications of fungi in bioremediation.

Appendix

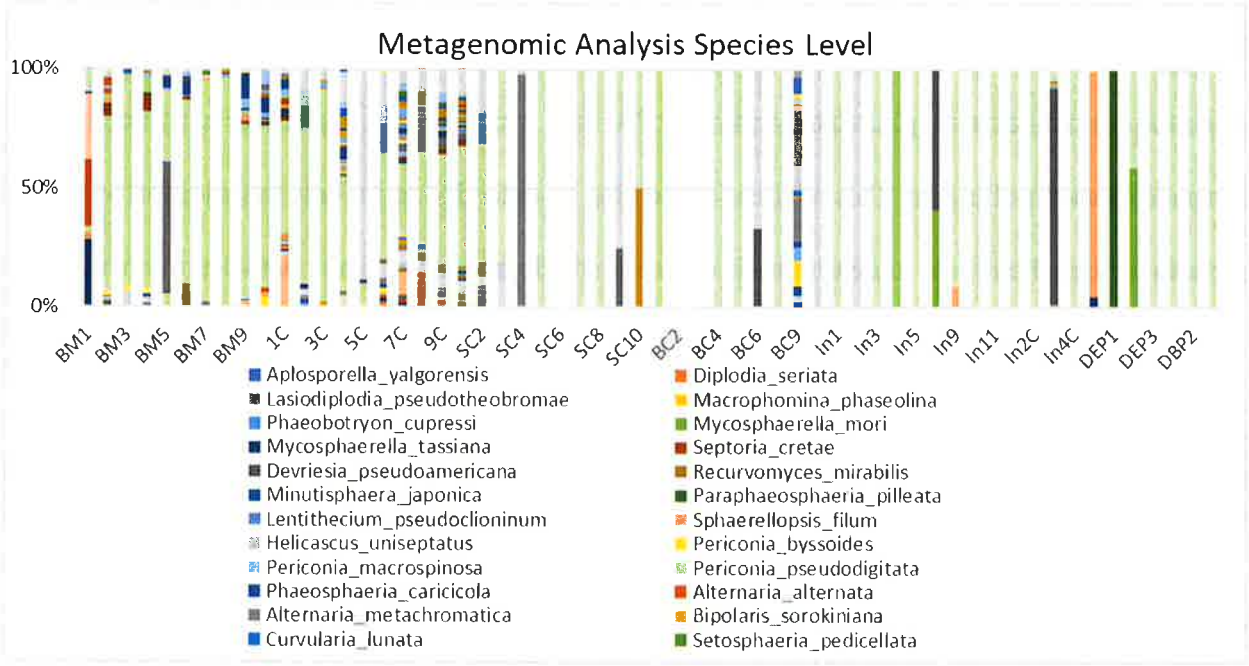

Table A.1. Metagenomic analysis at the species level for BM, VR, and phthalate enrichments. The vast majority of DNA that was sequenced was unidentified via ITS sequencing. 95.67% of extracted DNA from Big Marsh and 92.02% of extracted DNA from Vermillion River could not be identified. The following results ignore the unidentified sequences and are comprised of the sum of sequences that were identifiable.

Big Marsh					
Species	*Abundance*	*Phthalates*	*PAHs*	*Heavy Metals*	References
Sarocladium strictum	46.10%	N	Y	N	1
Fusarium culmorum	0.95%	Y	Y	N	2,3
Coprinellus micaceus	0.62%	N	Y	Y	4, 5
Pseudallescheria boydii	0.50%	N	Y	N	2
Penicillium chrysogenum	0.38%	Y	N	Y	6,7
Rhodotorula dairenensis*	0.18%	N	Y	N	8
Fusarium solani	0.13%	N	Y	Y	9,10
Coprinopsis atramentaria*	0.13%	N	N	Y	11
Rhodotorula graminis	0.13%	N	Y	Y	12,13
Stropharia coronilla	0.11%	N	Y	Y	14,15
Trametes versicolor	0.10%	Y	Y	Y	16,17,18
Candida parapsilosis*	0.06%	N	N	Y	19
Inocybe curvipes*	0.06%	N	N	Y	20
Meyerozyma caribbica	0.03%	N	N	Y	21
Saccharomyces cerevisiae*	0.02%	N	Y	Y	22,23

Trichoderma asperellum	0.02%	N	Y	Y	24,25
Trichoderma longibrachiatum	0.02%	N	Y	Y	26,27
Nigrospora oryzae	0.02%	N	Y	N	28
Macrophomina phaseolina*	0.01%	N	N	Y	29
Aspergillus flavus*	0.01%	N	Y	Y	30,31
Scytalidium lignicola	0.01%	N	Y	N	32
Irpex lacteus	0.01%	N	Y	Y	33
Stereum hirsutum*	0.01%	N	Y	Y	34,35
Tomentella stuposa	0.01%	N	N	Y	36

Table A.2. Species present at Big Marsh showing either degradation or resistance to phthalates, PAHs, or heavy metals as described in scientific literature. References found after Table A3.

*Denotes species unique to Big Marsh

Vermillion River

Species	*Abundance*	*Phthalates*	*PAHs*	*Heavy Metals*	*Reference*
Sarocladium strictum	25.73%	N	Y	N	1
Nigrospora oryzae	0.87%	N	Y	N	28
Cystofilobasidium capitatum*	0.77%	N	N	Y	37
Irpex lacteus	0.46%	N	Y	Y	33
Coprinellus micaceus	0.34%	N	Y	Y	4,5
Fusarium solani	0.32%	N	Y	Y	9,10
Trichoderma asperellum	0.30%	N	Y	Y	24,25
Fusarium culmorum	0.24%	Y	Y	N	2,3
Meyerozyma caribbica	0.20%	N	N	Y	21
Wickerhamomyces anomalus*	0.19%	N	N	Y	38
Abortiporus biennis*	0.18%	N	N	Y	39
Trichoderma atroviride*	0.16%	Y	Y	Y	40,41,42
Scytalidium lignicola	0.11%	N	Y	N	32
Penicillium chrysogenum	0.11%	Y	N	Y	6.7
Pseudallescheria boydii	0.11%	N	Y	N	2
Westerdykella dispersa*	0.10%	N	Y	Y	43,44
Coprinellus disseminatus*	0.09%	N	Y	N	4
Trametes versicolor	0.09%	Y	Y	Y	16,17,18
Trichoderma virens*	0.05%	N	Y	Y	45,46
Rhodotorula graminis	0.04%	N	Y	Y	12,13
Alternaria alternata*	0.03%	N	N	Y	47
Curvularia lunata*	0.03%	Y	Y	N	48.49
Trichoderma harzianum*	0.03%	Y	Y	Y	41,50,51
Phlebia tremellosa*	0.03%	Y	N	N	52
Tomentella stuposa	0.03%	N	N	Y	36

Trichoderma longibrachiatum	0.02%	N	Y	Y	26,27
Mucor circinelloides*	0.02%	N	Y	Y	53,54
Coprinus cordisporus*	0.01%	N	Y	N	55
Stropharia coronilla	0.01%	N	Y	Y	14,15

Table A.3. Species present at Vermillion River showing either degradation or resistance to phthalates, PAHs, or heavy metals as described in scientific literature. See below for references.

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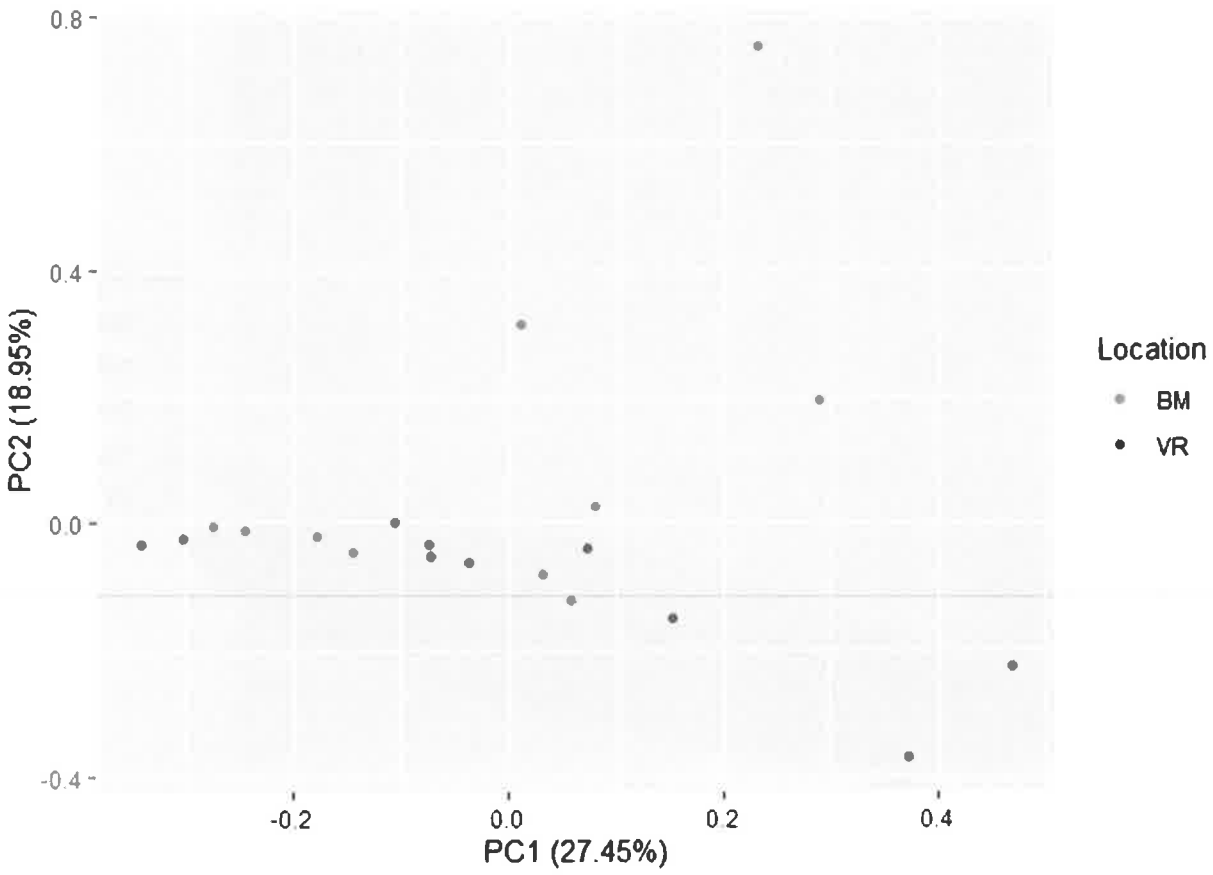

Figure 1A. PCA of diversity between Big Marsh and Vermillion River at the order level of classification.

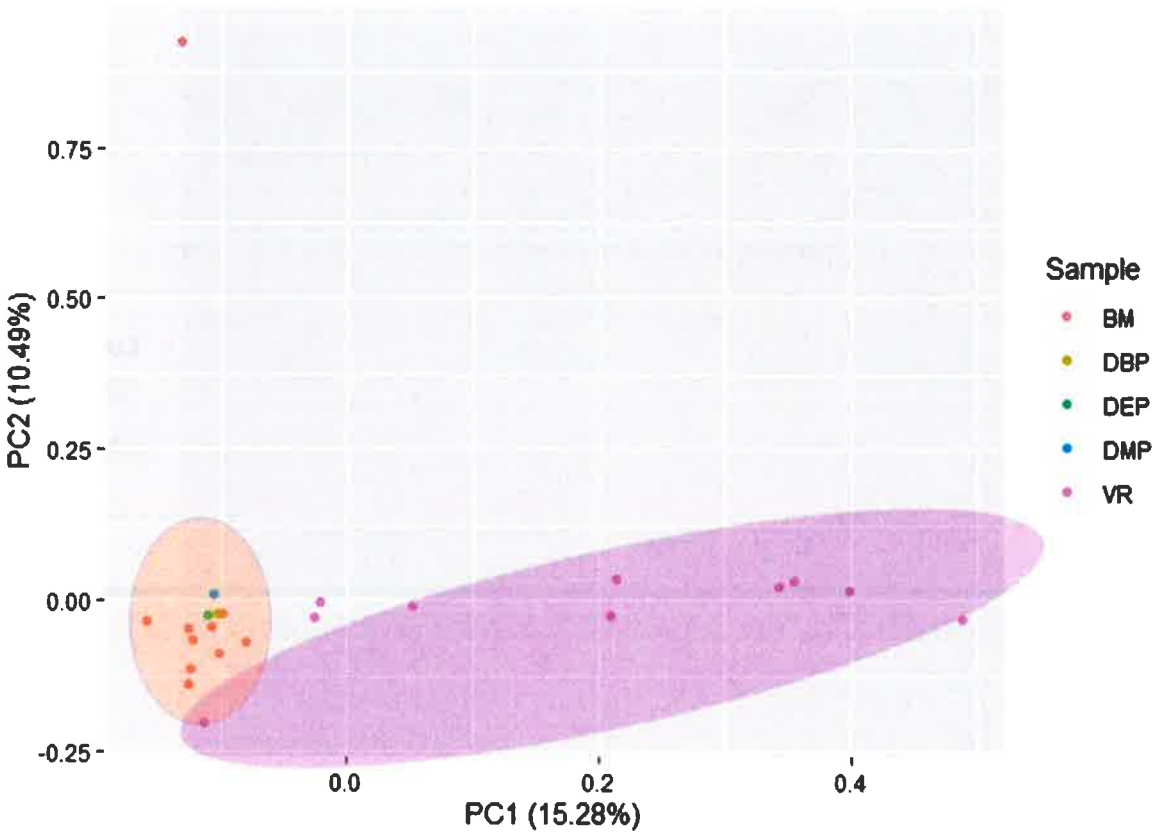

Figure 2A. PCA of species composition between BM, VR, and phthalate enrichments under the assumption that all 10 replicates from each site were independent samples. Clustering is present with VR samples and with BM samples. Phthalate enrichments are clustered together with BM samples.

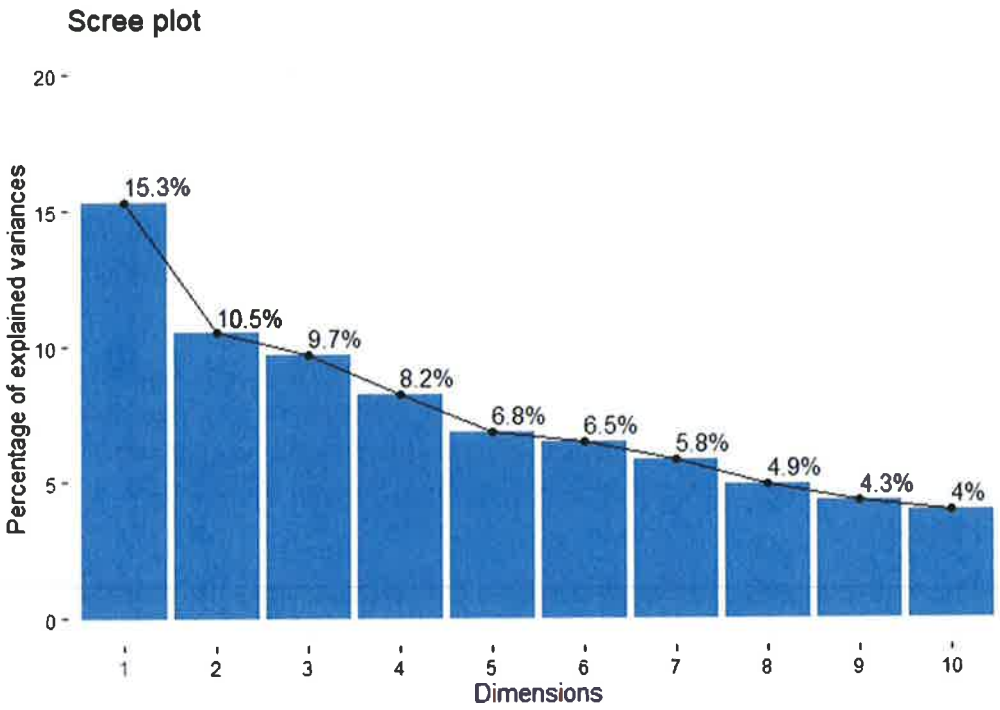

Figure 3A. Scree plot showing the variance for each loading present in Figure 2A.

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