DETERMINING THE POTENTIAL OF PAPER MILL BIOSOLIDS TO PRODUCE A COMPOST USING *EISENIA FETIDA* AND FUNGAL INOCULUM, AND ITS ABILITY TO REMEDIATE PETROLEUM HYDROCARBON CONTAMINATED SOIL

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Abstract

The pulp and paper industry produces large amounts of organic waste products in the form of paper mill sludge or biosolids (PMB). Current practices of incinerating PMB contribute to climate change and are wasteful and costly for the producer and the environment. PMB has the potential to be a useful product that could become a secondary revenue stream for paper mills. In this study, PMB and bark were composted to test the effect of fungal inoculum on the degradation of PMB, with and without *Eisenia fetida*, against controls with no additives. The composted product was then used to assess the potential of remediating soil contaminated with motor oil. PMB composted but did not reach maturity based on the C:N ratio. Based on the germination index (GI) 100 % of treatments with *E. fetida* (Ef+) reached maturity, while only 33.33 % of treatments without *E. fetida* (Ef-) reached the GI threshold. Compost nutrient levels were acceptable for land application, and contaminants were below thresholds set by the Canadian Council of Ministers of the Environment, indicating the PMB composted or in raw form is suitable for land application. The soil remediation was successful, with 10 % and 20 % compost addition being the most effective at removing oil. The potential of PMB as a soil amendment and for remediation is significant and further research is required to determine the best use for this valuable product.

General summary

The pulp and paper industry produces large amounts of organic waste products in the form of paper mill sludge or biosolids (PMB). Current practices of incinerating PMB contribute to climate change and are wasteful and costly for the producer and the environment. PMB has the potential to be a useful product, that could become a secondary revenue stream for paper mills. In this study, PMB and bark were composted to test the effect of four species of fungi on the degradation of PMB, with and without *Eisenia fetida*, a species of earthworm, against PMB controls with no additives. The composted PMB was then used to assess the potential for remediating soil contaminated with motor oil. PMB composted but did not reach maturity, based on the carbon to nitrogen ratio, and 100 % of treatments with *E. fetida* reached maturity based on a germination index (GI), while only 33.33 % of treatments without *E. fetida*, reached the GI threshold of 60 %. Four species of were tested, however, none appeared to affect the composting process. Nutrient levels in all treatments were acceptable, and contaminants were below Canadian Council of Ministers of the Environment thresholds, indicating that PMB, composted or raw, is suitable for land application. The soil remediation was successful with 10 % and 20 % compost addition being the most effective at removing oil. The potential of PMB as a soil amendment and for remediation is significant, and further research is required to determine the best use for this valuable product.

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List of Abbreviations

ADF – Acid detergent Fibre

ADL - Acid detergent Lignin

C:N – Carbon to Nitrogen ratio

CBPPL – Corner Brook Pulp and Paper Ltd

CCME – Canadian Council of Ministers of the Environment

EC – Electrical Conductivity

Ef- – Without *Eisenia fetida*

Ef+- With *Eisenia fetida*

EM – Effective Microorganisms

FAMES – Fatty Acid Methyl Esters

FC – Fungal Consortium (including *T. versicolor*, *G. applanatum*, *F. betulina*, *F. pinicola*)

GI – Germination Index

MSW – Municipal Solid Waste

NL – Newfoundland and Labrador Province, Canada

NLSPF – Newfoundland and Labrador Soil Plant and Feed Laboratory

OM – Organic Matter

PAHs – Polycyclic Aromatic Hydrocarbons

PLFA – Phospholipid Fatty Acid

PMB – Paper Mill Biosolids (Paper Mill Sludge)

SE – Standard Error

SS – Sewage Sludge

WAS – Waste Activated Sludge

1.0 Introduction and overview

Pulp and paper mills generate large amounts of solid organic waste in the form of sludge or biosolids. Waste management is a concern for the pulp and paper industry worldwide with waste in Canadian pulp and paper mills generating 1.7 million dry tones of paper mill biosolids (PMB) annually (Mccarthy et al., 2003). Similarly, Corner Brook Pulp and Paper Ltd (CBPPL) produces approximately 12 Mg of this organic waste each day, this is known as PMB or sludge. At CBPPL, PMB is primarily composed of wood material originally from black spruce (*Picea mariana),* balsam fir (*Abies balsamea),* and microbial waste from the wastewater treatment process. Incineration is a common practice for dealing with this waste product which can be costly, due to the addition of fuel, and is suspected to emit dioxins, furans, trace metals, and toxic organic compounds into the environment (Corbel et al., 2016). At CBPPL PMB is incinerated, however, due to the high-water content of the PMB (~70%), it often requires the addition of bunker fuel or waste oil, which produces CO_2 emissions and contributes to climate change. CBPPL uses mechanical separation techniques to pulp the wood and there is very little chemical or metal contamination in their waste products making it ideal for post-production use. CBPPL's PMB consists of sludge from primary and secondary clarifiers as well as small amounts of fly-ash scrubber water and on-site greywater. Due to the environmental impact of the large amounts of waste being incinerated the industry is under pressure to convert waste into a useful product that could also become a secondary revenue stream (Corbel et al., 2016). To reduce the amount of PMB being incinerated, and the difficulties associated with its high water content, composting and subsequent land application, has been suggested as a possible secondary use for this organic waste.

PMB has been composted with amendments in several studies around the world and found to produce a nutrient-rich compost suitable for land application (Dinel et al., 2004; Evanylo & Daniels, 1999; Faubert et al., 2016; Foley & Cooperband, 2002; Hackett et al., 1999; Hazarika & Khwairakpam, 2018; Thyagarajan et al., 2010). Due to the high lignin content in PMB (30.7%) the composting process can be slow and may require the addition of microorganisms to assist in the degradation process (Jurado et al., 2015). Lignin degradation is primarily carried out in nature by ligninolytic enzymes present in fungi and actinomycetes (Abdel-Hamid, Solbiati, & Cann, 2013; Cragg, et al., 2015; Datta, et al., 2017). White-rot fungi are the most effective microorganisms at lignin degradation in nature with the use of lignin-degrading enzymes, while brown rot fungi modify lignin to reach and degrade cellulose and hemicellulose. Both white and brown rot fungi could significantly increase the rate of decomposition in composting PMB either individually or as a consortium (Cragg, et al., 2015; Datta, et al., 2017). These wood loving fungi may require the addition of bark to acclimate to a compost environment as they grow on trees in nature. CBPPL has large piles of bark on site that can act as a secondary substrate for fungi during composting. Fungi can be added to compost in a liquid inoculum consisting of fungal mycelium grown in a nutrient broth. Along with these microorganisms, macro-organisms such as earthworms and arthropods are also responsible for wood and litter degradation in nature and have also been previously employed in composting studies (Negi & Suthar, 2018).

One of the most commonly used macro-organisms in composting is the earthworm *Eisenia fetida* which has been used to increase decomposition rates. *E. fetida* is a species of epigeic earthworm known for quickly producing nutrient-rich compost from organic waste material including PMB (Elvira et al., 1998). Numerous studies have found that PMB should be

mixed with other N-rich organic wastes such as animal manure and sludge to produce a quality substrate for *E. fetida* (Butt, 1993; Elvira et al., 1998; Gratelly et al., 1996). Due to the high N content of PMB a secondary substrate for *E. fetida* was not necessary, however, bark was added for the fungal inoculum which reduced the N content. Producing quality compost requires a carbon-nitrogen ratio (C:N) of 20-30 for microorganism metabolism, which performs the transformation from organic matter into stable compost, this level was achieved in the present study with the addition of urea N fertilizer.

Composting is a bio oxidative process that results in the mineralization and humification of organic matter, making nutrients more available to plants (Bernal et al., 1998). Compost addition to the soil can increase nutrient levels, raise the pH, and reduce bulk density to foster a healthier soil microbial community. This could be beneficial in Newfoundland and Labrador (NL) where rocky soils with low pH, and low nutrient content are widespread. The addition of locally sourced, nutrient-rich organic matter (OM) could increase the productivity of NL's agriculture sector while reducing waste from CBPPL and saving shipping costs of bringing OM to the island.

The increased use of petroleum products has led to large areas of contaminated soil worldwide; NL is no exception. The use of compost to remediate petroleum hydrocarbon contaminated soils has been successful in many studies however no studies have been done with composted PMB in NL soils (Bastida et al., 2016; Chen et al., 2015b; Taiwo et al., 2016; Wu et al., 2013). The addition of composted PMB to contaminated soil can increase OM and microbial activity allowing the faster breakdown of petroleum hydrocarbons and benefiting overall soil health. This is another potential use for the waste from CBPPL.

This study evaluated compost trials using PMB and bark with the addition of four species of fungal inoculum and the earthworm *E. fetida* to determine the most effective combination for efficient decomposition. Furthermore, the study aimed to determine the effectiveness of the composted PMB at remediating petroleum contamination from the soil in a lab setting. It was hypothesized that: 1) Adding fungal inoculum and/or *E. fetida* would increase the rate or degree of decomposition in PMB and bark obtained from CBPPL. 2) Composted PMB and bark would produce a good quality compost of both indoor and outdoor experiments. 3) Composted PMB would effectively remediate petroleum hydrocarbon contamination in soil under controlled conditions.

2.0 Literature review

2.1 Papermill biosolids characterization and management

PMB, also known as dewatered paper mill sludge, is a waste product of the pulp and paper industry. The production of pulp and paper requires a large amount of water and PMB is the residual organic material after the treatment of wastewater in pulp and paper mills (Faubert et al., 2016). Many studies have composted municipal solid waste (MSW) or sewage sludge (SS) for use as a soil amendment or fertilizer; however, MSW and SS can be highly variable in composition and contain more potential biohazards (Corbel et al., 2016). PMB is more consistent in its composition and has less variability and risk of biohazards than MSW and SS (Corbel et al., 2016). The PMB at CBPPL is a mixture of primary sludge, secondary sludge, small amounts of fly-ash scrubber water, and small amounts of greywater from the water used on site. Primary sludge is the residual solid material from the primary clarifier, the first step in the wastewater treatment process, and is high in fibrous and organic material. It is a mixture of

coarse and fine woody fibers obtained through sedimentation and floatation in the primary clarification process and often has a high C:N (Faubert et al., 2016). Secondary sludge is also called waste activated sludge (WAS) and consists mainly of microorganisms and their waste from the secondary clarifier in the wastewater treatment process. Microorganisms are used to reduce the biological oxygen demand (BOD) and chemical oxygen demand (COD), N and P are added to this process to maintain a suitable environment and provide a food source for the microorganisms, due to these additions secondary WAS often has a low C:N (Faubert et al., 2016). At CBPPL primary and secondary sludges are mixed with small amounts of fly-ash scrubber water and on-site greywater and dewatered on a shaker table, followed by a screw press to achieve a water content of approximately 60-70%. The final product is mostly organic matter with a high water-content referred to in this document as PMB. Some pulping processes such as Kraft and chemical separation often have the added issue of chemical contamination in their sludge. However, CBPPL uses thermo-mechanical separation for pulping and their sludge is therefore lacking any significant chemical contamination.

Management of PMB varies widely between different pulp and paper producers and between countries. Faubert et al. (2016) reported that PMB in Quebec is managed 29% by landfilling, 31% by land application, 35% by energy recovery, and 5% by other practices. CBPPL manages its PMB mainly by energy recovery and steam production with incineration, along with bark and other woody biomass, in the boiler on site. However, CBPPL has its own hydroelectric facility which provides clean energy for the mill and PMB has a high water content making it more difficult to burn than bark reducing energy output. The main concerns with land application as a PMB management strategy are often heavy metal contamination, odor, and transportation costs. The PMB at CBPPL has no observed heavy metal contamination, nor a

significant odor that would be a concern for land application making land application a feasible option for management.

2.2 Effects of PMB as a soil amendment on soil properties and plants

PMB has been shown to increase microbial biomass and activity when added to soil, as well as increasing N and available P (Abdi et al., 2018; Faubert et al., 2016; Foley & Cooperband, 2002; Gallardo et al., 2012). Foley & Cooperband (2002) found that total soil C and plant available water were increased by the amendment of soil with composted PMB and bark while the bulk density was decreased creating improved physical soil conditions. One study applied pulp fibre residue to a field crop and found the soil had improved C and N levels, increased plant-available P and potassium (K), and improved bulk density and water holding capacity (Fahmy et al., 2010). The authors further reported the increased yield in all rotations of pea, corn, and potato with the application of pulp fibre residue (Fahmy et al., 2010). While a similar study found that applying PMB over three years with recommended rates of N fertilizer showed a highly variable yield of *Zea mays* but an increased yield of *Glycine max* compared to recommended rates of N fertilizer alone, it was hypothesized that the PMB helped with N uptake (Price et al., 2009).

CBPPL performs clear cuts throughout the island of Newfoundland and low-cost forest restoration is in demand to increase tree turnover without affecting fibre quality. Clear cuts expose forest soils and reduce the OM and nutrient content in the soil, however, composted PMB has been shown to significantly increase wood growth in coniferous trees and could be used to restore forest soils (Ortega Rodriguez et al., 2018). Ortega Rodruiguez et al, (2018) found that applying composted PMB increased wood production in *Pinus taeda* in a managed forest setting.

A significantly increased biomass of *Lolium perenne* and increased availability of micro and macronutrients were observed when composted secondary sludge was added to the soil (Gallardo et al., 2012). An increase in OM, N, C, and microbial activity has been observed with the addition of both fresh and composted secondary sludge (Gallardo et al., 2010). This could not only protect the soil in the forest but also increase tree growth for a faster turnover which would benefit the environment, NL economy, and CBPPL.

2.3 Increasing compost degradation with microorganisms

Composting with microorganisms has been found to increase the rate of decomposition or improve the quality of the final product (Awasthi et al., 2014; Jurado et al., 2015; Negi & Suthar, 2018; Parveen & Padmaja, 2011; Thyagarajan et al., 2010; Voběrková et al., 2017). Microorganisms that are specialized for the type of OM that is being composted can be more effective than those microorganisms that are naturally occurring, especially when working with waste products that are not exposed to the native microbes in the environment. Thyagarajan et al. (2010) found that the addition of effective microorganisms (EM) to PMB, cow dung, and sawdust produced mature compost in 90 days while the control without EM did not reach stability in this time frame, showing the effectiveness of including targeted microbial inoculum in the composting process. Another study composted tomato plants and pine chips with an inoculum of 30 strains of microorganisms including bacteria, actinobacteria, and fungi that were isolated from compost sources (Jurado et al., 2015). The inoculated pile degraded the lignocellulosic fraction more efficiently than the control, and while both piles reached maturity the inoculated pile had better quality in a shorter time (Jurado et al., 2015). Composting is an activity that is dependent on microorganisms and the addition of specialized microorganisms for

the product is an effective way to increase the efficiency of composting (Thyagarajan et al., 2010).

2.4 Degrading lignin in compost with fungal additives

Composting industrial organic waste like PMB can improve local sustainability and reduce the environmental impact of industry. PMB is a wood product that in nature would be primarily degraded by fungi. Fungi are considered fundamental in the composting process along with bacteria and archaea (Wright et al., 2016). Different fungi are present at different stages of composting and isolating these from the substrate being used can help determine which fungi would be effective at increasing the rate of degradation (Wright et al., 2016). Fungi in composting tend to work more slowly than bacteria but are more effective at lignin degradation (Datta et al., 2017). Lignin is an aromatic organic compound found in plant cell walls, where it provides strength and rigidity (Datta et al., 2017). Plant cell walls are 10-25% lignin, 40-60% cellulose, and 20-40% hemicellulose (Datta et al., 2017). Lignin is biosynthesized in plant cells by the combination of three monolignols; *para*-coumaryl alcohol, coniferyl alcohol, found in softwood, and sinapyl alcohol, found in hardwood (Datta et al., 2017; Singh, 2006). Once polymerized these polymers are called *p*-hydroxyphenyl, guaiacyl, and syringl (Datta et al., 2017). Lignin is a water insoluble polymer, commonly found in nature, that is resistant to attack by most microorganisms (Singh, 2006). Due to lignin's recalcitrant nature, it is difficult to degrade by regular composting methods and this poses a challenge for dealing with organic waste. White-rot fungi are the most effective degraders of lignin in nature through the use of ligninolytic enzymes such as laccase, lignin-peroxidase (LiP), manganese-peroxidase (MnP), and versatile peroxidase(VP) (Datta et al., 2017). Lignin represents 30% of softwood and 20% of

hardwood, therefore it is a significant portion of the PMB at CBPPL which is made of all softwood pulp (Singh, 2006, p. 358).

Due to the composition of PMB being high in lignin and cellulose fungal inoculation can be effective in composting. Fungi are considered the most effective microorganisms at lignin degradation in nature and this has been studied in controlled situations as well (Abdel-Hamid et al., 2013; Brown & Chang, 2014; Cragg et al., 2015; Datta et al., 2017). The addition of biological organisms to composting has been studied with MSW consisting of mixtures of household organic waste and municipal plant waste such as lawn clippings, leaves, and branches (Awasthi et al., 2014; Parveen & Padmaja, 2011; Voběrková et al., 2017). Awasthi et al. (2014) studied composting of MSW in windrows with a fungal consortium of *Aspergillus niger, Aspergillus flavus* and *Trichoderma viride,* and different turning frequency. It was found that MSW inoculated with spore suspensions of the fungal consortium and turned weekly resulted in a faster maturity and low to no phytotoxicity while the control without the fungi and the same turning frequency showed low organic decomposition and stabilization (Awasthi et al., 2014). Parveen & Padmaja (2011) composted MSW with water hyacinth, an invasive plant in India, and a microbial consortium of fungi, *Paecilomyces variotti,* and *Chaetomium globosum*, and actinomycetes, *Streptomyces lavendulae,* and *Thermobifida fusca* in a small-scale lab trial. Results indicated that the cellulose content in MSW reduced from 50.91% to 26.84% in 90 days, while the control only reduced to 35.46%. It also produced compost with a lower C:N and phenol content, producing a more favourable end product compared to the uninoculated compost (Parveen & Padmaja, 2011). Voberkova et al. (2017) also studied the effects of composting MSW with fungal cultures of *Trametes versicolor, Fomes fomentarius,* and *Phanerochaete chrysosporium* individually and as a consortium against a control with no inoculum. *T.*

versicolor, F. fomentarius and the control resulted in more mature compost while MSW inoculated with *P. chrysosporium* and the fungal consortium resulted in phytotoxic compost that was not suitable for land application (Voběrková et al., 2017). The authors hypothesized that the low seed germination rate in these treatments may be due to the production of phenolic compounds, volatile organic acids and ammonia, which increased the pH (Voběrková et al., 2017). Negi & Suthar (2018) performed small scale lab trials with brown rot fungi *Oligoporus placenta* and *E. fetida* in PMB and manure. Results showed that trials with *O. placenta* reduced cellulose content 1.52 - 2.59 fold and trials with *O. placenta* and *E. fetida* together reduced cellulose content 2.2 – 3.08 fold (Negi & Suthar, 2018). These studies show that different species and combinations can produce better quality compost.

2.5 Background on selected species of fungi

White-rot fungi are considered the most effective lignin-degrading microorganisms, they are primarily Basidiomycetes and a few Ascomycetes that attack hardwood but can be found on softwood as well (Singh, 2006). White-rot fungi use enzymatic degradation to break down lignin, cellulose, and hemicellulose and mineralize them to CO_2 and water (Singh, 2006). *T. versicolor* is a white-rot species that is most commonly found on hardwood or deciduous tree species, it is rarely found on conifers (Aurora, 1986, p. 594). This species is part of a group of white-rot fungi that colonize the cell lumina and degrade the cell wall with the formation of cavities filled with mycelia, described as non-selective lignin-degrading fungi or simultaneous rot (Singh, 2006; Wong, 2009). This simultaneous degradation of lignin, cellulose, and hemicellulose makes *T. versicolor* a good candidate for degradation of PMB. Another group of white-rot fungi that degrade lignin leaving cellulose is white-pocket rot and *G. applanatum* can perform this type of rot and simultaneous rot in the same wood (Singh, 2006). *G. applanatum* has been observed

performing white-rot and brown-rot in the same wood, and though it is found most often on hardwood, it also grows on conifers making it a good candidate for degrading PMB (Aurora, 1986, p 576; McCoy, 2016, p 54-55).

Brown-rot fungi degrade cellulose and hemicellulose by altering lignin via demethylation, partial oxidation, and depolymerization (Singh, 2006). This group of fungi use Fenton's reaction ($Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot OH + {}^{-}OH$) to produce hydroxyl radicles that oxidize the lignocellulose then enzymes break down the cellulose and hemicellulose, without removing the lignin (Arantes et al., 2012; Shah et al., 2018; Singh, 2006). *F. pinicola* is a bracket-forming fungus that performs brown-rot on hardwoods and conifers that produces the enzymes endoglucanase, xylanase, β-glucosidase, chitinase, and acid peptidase that degrade cellulose (Shah et al., 2018). This versatile species is widespread in temperate regions, including boreal forests, and it grows on both hardwood and softwood making it a good candidate for degrading PMB. *F. betulina* (formerly *Piptoporus betulina*) is another widespread brown-rot species found primarily on birch trees and very common in NL. *F. betulina* degrades wood in the same way as *F. pinicola* altering the lignin to reach and degrade the cellulose it was chosen as an alternate brown-rot species due to its abundance in the area.

2.6 Vermicomposting process and effects on composting

Vermicomposting is the process of composting using earthworms to assist in degradation and aeration (Wright et al., 2016). Earthworms increase the surface area of organic matter which allows microorganisms to degrade more efficiently, thereby accelerating the decomposition process (Yang et al., 2014). The surface area is increased when the organic matter passes through the grinding gizzard of the worm and is fragmented into fine particles (Ndegwa & Thompson,

2001). Earthworms feed on the microorganisms that colonize the compost and as they pass the organic material through their gut it promotes the growth of more microorganisms (Ndegwa & Thompson, 2001). Earthworm fecal waste, also known as casts, has increased microbial activity which releases nutrients such as N, P, and K and makes them more available for plant uptake (Ndegwa & Thompson, 2001). One of the challenges of vermicomposting is that the substrate must remain below 35°C for the worms to survive therefore it does not reach the thermophilic stage required to kill pathogens. Therefore, a combination of thermophilic composting followed by vermicomposting has been most effective at producing a homogeneous and pathogen-free compost (Ndegwa & Thompson, 2001).

One of the most common species of worm used is the epigeic, or litter dwelling earthworm *E. fetida*, many studies have looked at their use of *E. fetida* for composting organic waste products including PMB (Boruah et al., 2019; Elvira et al., 1998; Karmegam et al., 2019; Mohapatra et al., 2019; Owojori & Reinecke, 2009; Sharma & Garg, 2019). *E. fetida* thrive in temperatures between 0°C and 35°C and a moisture level from 50-90% (Edwards, 2004), reproduce quickly with hatchlings reaching maturity in 40-60 days, cocoon incubation is 23 days, and mature worms can produce many cocoons with 1 to 7 hatchlings (Venter & Reinecke, 1988). *E. fetida* is the most widely used species of earthworm for vermicomposting as they are very common, often colonize organic waste naturally and have a wide range of temperature and moisture tolerance (Edwards, 2004). *E. fetida* have often been found to overtake other worm species and reproduce quickly making them very resilient (Edwards, 2004).

E. fetida have been studied for their ability to degrade PMB in previous studies when mixed with other substrates (Boruah et al., 2019; Elvira et al., 1998; Mohapatra et al., 2019). One study mixed PMB with dairy manure in 2 m^2 piles found that in 3 months the worms produced a

compost rich in P and N with good physical properties suitable as a soil conditioner or fertilizer (Elvira et al., 1998). Another study performed smaller-scale experiments with 2 kg of material, mixing PMB with sawdust and cow dung, and found that worms increased the rate of decomposition and released more nutrients than controls (Mohapatra et al., 2019). Similarly, another study was done with 250 g trials with 20 worms in PMB and citronella bagasse and found that the C:N was reduced by 91.1% with *E. fetida* and 70% in controls (Boruah et al., 2019). Trials with worms increased available P by 121.1% while controls only increased by 49.9% and overall, trials with *E. fetida* reported an increase in beneficial microorganisms as compared to controls (Boruah et al., 2019).

Vermicomposting has been widely studied and many authors have found similar changes in characteristics of waste after composting. Overall pH has been found to decrease after vermicomposting with the final pH tending to range between 6 and 8; this is thought to be due to the conversion of organic material into organic acids and the mineralization of N and P making the substrate more acidic as it degrades (Hait & Tare, 2011; Ndegwa & Thompson, 2000; Yadav & Garg, 2011). One study noted that the formation of ammonium during the decomposition of organic matter increases the pH, while humic acids lower the pH. The action of these two processes together stabilizes the pH resulting in a near-neutral pH upon the maturity of the vermicompost (Pramanik et al., 2007). Studies have also shown that vermicomposting increases the availability of essential plant nutrients such as N, P, K, and Ca (Garg & Kaushik, 2005; Hait & Tare, 2011; Kaviraj & Sharma, 2003; Khwairakpam & Bhargava, 2009; Le Bayon & Binet, 2006; Ndegwa & Thompson, 2000). Ca increases when worms mineralize bound Ca and convert it to biologically available forms (Garg & Kaushik, 2005). The increase in K is potentially due to the enhanced number of microflora in the gut of the worms (Kaviraj & Sharma, 2003). Similarly,

the increase in P is thought to be due to the enzymes in the worm gut that convert P into more available forms for microorganisms that convert the P in the fine particle worm castings (Le Bayon & Binet, 2006). The C:N was decreased due to a loss in C in the form of CO_2, and mineralization of C rich materials, while N increases due to the presence of N fixing microorganisms (Yadav & Garg, 2011). These changes can be used as a guideline to determine if our vermicompost has reached maturity.

2.7 Determining compost maturity with pH, EC, C:N, GI, and PLFA

Compost maturity must be determined before it can be used to avoid phytotoxicity, pathogens, and ensure a stable OM content (Bernal et al., 2009). Compost stability describes microbial decomposition and activity of microorganisms in the compost, while the terms stability and maturity are often used together or interchangeably, however they are not exactly the same (Bernal et al., 2009). To clarify the difference, mature compost is always stable, however sometimes stable compost may not be mature and could still be phytotoxic although these conditions are uncommon(McGill University, 2020). Immature composts can have significant negative effects such as anaerobic pockets that can become toxic or phytotoxic and can negatively impact the soil and plant life where they are used (Bernal et al., 2009). There are many methods to determine compost maturity, including pH, electrical conductivity (EC), carbon-nitrogen ratio (C:N), germination index (GI), and the phospholipid fatty acid profile (PLFA). Based on previous work, a combination of these analytical methods can determine if compost has reached a mature and stable state.

2.7.1 Physical and chemical methods for determining compost maturity

There are many ways to determine the maturity or stability of compost including physical characteristics such as colour, odor, and temperature (Bernal et al., 1998). Temperature evolution is a common way to determine compost maturity due to the thermophilic cycles occurring during the process. However, these observations alone cannot give adequate data on the maturity of compost and chemical analyses are more common. C:N is a common method for determining compost maturity, in general, a compost with a C:N over 25 is considered immature and a C:N under 25 is considered mature. Another method for determining compost maturity is a pH between 5.5 and 8.0 which is ideal for microbial activity and maintaining composts in this range helps avoid N losses by ammonia volatilization (Bernal et al., 2009). The seed germination test is considered a reliable method to assess the maturity of compost by determining phytotoxicity, this method is usually measured by the GI formula and the threshold for a successful compost is a GI of 60 - 80% or above, indicating a mature compost with no phytotoxicity (Gómez-Brandón et al., 2008; Goyal et al., 2005; Karmegam et al., 2019; Villar et al., 2016)

2.7.2 Microbial methods for determining compost maturity

PLFA is a method of analyzing the structure of the microbial community in soil or compost and determines changes throughout the process as an indication of maturity (Villar et al., 2016). PLFAs can be used as biomarkers to identify the abundance and succession of different groups of organisms such as bacteria, actinomycetes, archaea, and fungi (Jiang et al., 2018; Kato et al., 2005; Villar et al., 2016). The microbial structure throughout the composting process can vary depending on the original material and PLFA is a valuable method for characterizing the microbial community (Villar et al., 2016). PLFA is beneficial compared to

culturing and identifying individual species of microorganisms because it can identify the concentrations of whole microbial groups, both culturable and non- culturable, and the structure of the microbial community (Amir et al., 2008; Villar et al., 2016). Branched fatty acid methyl esters (FAMES) are regarded as biomarkers of gram-positive bacteria and some actinomycetes; these were observed, using FAMES, to increase gradually throughout the composting process by Kato et al. (2005) and remained stable during storage after composting poultry manure. Gram-positive bacteria are often tolerant of the high temperatures reached in composting which could indicate the reason for their increasing numbers (Kato et al., 2005). The increase to a constant value of gram-positive biomarkers coincided with compost maturity as defined by other physical parameters, making these biomarkers a good tool for determining compost maturity (Kato et al., 2005). Straight hydroxyl and saturated PLFAs (SOH-FAMES), specifically 2-OH 16:0, which are found in some gram-negative bacteria, were observed increasing during the initial stages of composting and then decreasing in later stages (Kato et al., 2005). The author reports that the SOH-FAMES could indicate an anaerobic stage during the aerobic composting and are negatively correlated with compost maturity parameters (Kato et al., 2005). Branched FAMES also showed a positive correlation with GI and negative correlations with NH_4^+, while SOH-FAMES showed the opposite correlations, negative and positive with GI and NH_4^+, respectively (Kato et al., 2005). Jiang et al. (2018) reported a negative correlation of bacterial PLFA 15:0 and fungi PLFA 18:1ω9,12 and GI while fungi PLFA 18:1ω9t had a positive correlation while composting pig manure, noting the potential variability of PLFAs with different substrates. Villar et al. (2016) also noted significant differences in microbial communities when composting 3 different waste products. Municipal sewage sludge had higher bacterial biomass overall with a decrease in biomass over time and gram-negative bacteria dominating the initial stages and

gram-positive bacteria dominating the maturation phase (Villar et al., 2016). Sewage sludge from the food industry had higher fungal biomass overall noting an increase in biomass initially followed by a decrease in the thermophilic stage and an increase again in the maturation phase (Villar et al., 2016). Hog manure was reported to have less microbial biomass than the other two substrates (sludge from the fishing industry and municipal sewage sludge) overall with a slight decrease in biomass over time with high levels of gram-positive and gram-negative in initial stages and gram-positive dominating the maturation phase (Villar et al., 2016). Jiang et al. (2018) also reported the highly negative correlation between fungi PLFA 18:1ω9t and changes in NH_4-N. Kato et al. (2005) reported proportions of 10Me PLFAs were reported low until an increase in the final stages of composting indicating actinomycetes were more active in later stages. Straight and saturated PLFAs, biomarkers for fungi, decreased during composting, indicating fungi were more active in initial stages and their population reduced over time, though the author notes that fungal biomass is generally greater than bacteria (Kato et al., 2005). The microbial community in outdoor trials could be significantly different in a colder climate like Canada.

2.8 Compost overwintering

Some research has been done investigating the benefits of overwintering organic waste or using a freeze-thaw pre-treatment for composting (Larney et al., 2000; Yu et al., 2019). Litter decomposition in cold climate areas naturally goes through freeze-thaw cycles which can aid in breaking down litter in the spring. Yu et al. (2019) looked at freeze-thaw as a pre-treatment for composting green waste drenched in water and found it promoted mineralization of total organic C by 2.73-8.01% and total N by 0.21-0.52% compared to the control. They found that lignin degradation was promoted by 3.52-3.73% and cellulose degradation by 13.23-14.26% compared to the control and it enhanced humus synthesis by 19.19-21.43% (Yu et al., 2019). Drenching

was found to increase the physical damage caused by freezing which increased decomposition as compared to the control (Yu et al., 2019). Another study looked at composting cattle manure in windrows in winter temperatures compared to summer in Alberta and found that both winter and summer composting were successful (Larney et al., 2000). Both actively turned and passive air injection processes were studied and it was found that winter composting was most successful with active turning and successfully reached the thermophilic phase at 65°C even with air temperatures below -30°C (Larney et al., 2000). The study performed by Larney et al. (2000) took place in Lethbridge, AB where average temperatures between November and February range from -1.2 °C to -6.0 °C with the coldest temperature recorded as -42.8 °C (Environment Canada, 2020). Average temperatures for Corner Brook, NL between November and February range from 2.3 °C to -6.8 °C, with the coldest temperature on record at -31.7 °C (Environment Canada, 2020). Winter composting also had a higher water content due to reduced evaporation, while summer had more volume reduction and the compost may require added water (Larney et al., 2000). This shows potential for winter composting in NL due to the climate similarities between the two research locations.

2.9 Bioremediation of soils

Bioremediation is defined as the use of biological organisms to breakdown or remove contaminants from soil (Kuppusamy et al., 2017). This method of remediation is considered to be safe, cost-effective, and environmentally friendly making it a favourable option for soil remediation (Kuppusamy et al., 2017). Many organisms are effective at contamination remediation including bacteria, fungi (mycoremediation), and plants (phytoremediation) (Robichaud et al., 2019). Compost amendment diversifies the microbial biomass of the soil including, bacteria, fungi, and archaea, and increases available nutrients in degraded and

contaminated soils which increases natural remediation of contaminants (Abioye et al., 2012; Bastida, 2016; Chen et al., 2015a; Kuppusamy et al., 2017; Robichaud et al., 2019; Taiwo et al., 2016; Wu et al., 2013). The addition of compost for bioremediation of soil is increasing in popularity due to its cost-effectiveness and efficiency as compared to physical strategies such as excavation and landfilling or incineration and chemical remediation strategies such as chemical oxidation (Chen et al., 2015a; Kuppusamy et al., 2017).

2.10 Soil contamination by petroleum hydrocarbons

Soil contamination by petroleum hydrocarbons is increasing worldwide due to the increased use of petroleum products (Abioye et al., 2012). The risk of oil contamination is common during exploration, refining, industrial process, transport, and during use which is an environmental concern globally (Bastida et al., 2016). Petroleum hydrocarbon contamination may contain heavy metals and polycyclic aromatic hydrocarbons (PAHs) which can cause hazards to human health including toxicity, mutagenicity, and carcinogenicity (Boonchan et al., 2000). Petroleum hydrocarbon contamination can also lead to reduced germination and growth of plants having negative effects on the greater ecosystem (Onwosi et al., 2017).

PAHs are composed of fused benzene rings and persist in soils due to their unique physical and chemical properties (Chen et al., 2015a; Sigmund et al., 2018). Due to PAHs hydrophobic nature, they bind to the soil making it more difficult for microorganisms to break them down (Boonchan et al., 2000). If not removed some of the PAHs can bind to particulates in soil and bioaccumulate in food chains, harming wildlife and humans (Boonchan et al., 2000). Compost amendment in soil introduces more diverse bacteria, fungi, and archaea communities which use hydroxylation and oxidation to degrade PAHs (Sigmund et al., 2018). In-situ bioremediation is the most efficient and environmentally friendly method as it allows the soil

structure to remain intact and, compost addition is an effective option for in-situ bioremediation as it stimulates the growth of indigenous microorganisms by supplying nutrients to the depleted soil (Hollender et al., 2003).

2.11 Compost for bioremediation

There are several remediation strategies that use compost including addition, bioaugmentation, bio-stimulation, direct composting (co-composting), and incorporation of a bulking agent which can be used individually or in combination with each other (Chen, et al., 2015).

Compost addition uses already composted organic matter and adds it to contaminated soil and is one of the most studied bioremediation strategies as it provides a degradation matrix and microorganisms to degrade the pollutants (Bastida et al., 2016; Chen et al., 2015a; Cipullo et al., 2019; Hussain et al., 2018; Marchand et al., 2016; Sigmund et al., 2018; Wu et al., 2013). The addition of compost to contaminated soil increases nutrients including N, P, and K which increases activity and density of indigenous microorganisms via bio-stimulation (Abioye et al., 2012; Bastida et al., 2016; Hollender et al., 2003; Taiwo et al., 2016). Bio-stimulation is the addition of nutrients to stimulate the activity of existing microorganisms (Kouzuma & Watanabe, 2011). Composting and compost addition can simultaneously increase soil organic matter and soil fertility while bioremediating which makes it an effective method (Chen et al., 2015a). Remediation with compost relies on two mechanisms, degradation by microorganisms and adsorption by organic matter (Chen et al., 2015a). Bastida et al. (2016) found that compost addition to petroleum-contaminated soil promoted the removal of 88% of both PAHs and alkanes in 50 days while removal was not significant in controls without compost. Another study

reported that in 8 months, compost addition was able to reduce PAHs in diesel contaminated soil by 90% (Wu et al., 2013). The authors also reported that higher rates of compost addition resulted in more successful PAH removal (Wu et al., 2013). Compost addition is one of the most effective and economic methods for soil bioremediation.

The method of direct composting or co-composting is when contaminated soil is actively composted, often with the addition of a bulking agent to provide organic matter and increase microbial activity (Atagana, 2004). As the microorganisms degrade the organic matter, they simultaneously adapt to degrade the contaminants resulting in rich soil with reduced or removed contaminants (Atagana, 2004). Co-composting diesel contaminated soil with bulking agents such as rice husks, sawdust, and wood chips was reported to remove 96.89%, 96.55%, and 90.01% of total petroleum hydrocarbons respectively (Onwosi et al., 2017). Similarly, PAH degradation was faster in soil composted with wood chips and poultry manure than in a control with wood chips and no manure and microbial populations were much higher with manure than without (Atagana, 2004).

Bioaugmentation is defined as the inoculation of microorganisms to increase degradation in soil or compost (Kouzuma & Watanabe, 2011). This process often happens naturally when compost or organic matter is added to soil, known as bio-stimulation, but sometimes specific microorganisms are added to increase the degradation of specific pollutants (Boonchan et al., 2000). Bioaugmentation with different combinations of bacteria and fungi in PAH-contaminated soil was reported to degrade some PAHs by 40 to 80% after 100 days (Boonchan et al., 2000).

Organic wastes from industrial processes, such as wastewater treatment, are considered an economical substrate to compost for bioremediation since they often end up in landfills or incinerated (Chen et al., 2015a). Using this waste product prevents further emissions via

incineration and removes thousands of tons of organic material from landfills, putting it to use creating healthy soils. The PMB from CBPPL is an effective option for bioremediation because of its high N content and availability, it could be used in direct composting or compost addition with the benefits of bio-stimulation or in-combination with bioaugmentation in contaminated soils in NL. This product would provide a good local source of organic matter on the island creating an economically viable solution to soil contamination.

3.0 Methodology

3.1 Isolating fungal cultures in compost experiments

Four known wood degrading fungi (*Fomitopsis betulina, Fomitopsis pinicola, Ganoderma applanatum,* and *Trametes versicolor)* were collected from forests around Corner Brook, NL. Inside a biosafety cabinet, fungal cultures of all 4 species were plated on malt extract agar (MEA) by taking small pieces of the fruiting body and placing it in the middle of a petri dish with sterile MEA. Once fungal growth was observed, pieces of the outer edge of the mycelia were cut out, using an aseptic technique, and placed on new sterile plates to create a pure culture with no contaminants. Cultures were expanded by cutting pieces off the mycelial edge of each pure culture and plating the pieces onto several new plates to produce more pure culture plates. Once adequate mycelia were produced, 2 or 3 pieces of myceliated agar were cut and placed in malt extract broth (MEB) in vented mason jars for liquid inoculation into compost experiments. Vented mason jars were prepared by drilling a hole in the lid and pulling a cotton ball tightly through the hole to act as a filter (**Figure 3-1)**. Inoculated MEB jars were stored at room temperature in regular day and night light and swirled every 2-4 days to incorporate air into the growing media, being careful not to get liquid on the cotton filter which could cause

contamination. Preliminary tests to determine the affinity of the fungi to PMB were performed by adding fungal cultures to sterilized grain, PMB, and bark in filter patch grow bags as shown in **Figure 3-2** and **Figure 3-3**.

Figure 3-1 Sterilized and inoculated malt extract broth (MEB) in vented mason jars

a  b 

Figure 3-2 Sterilized papermill biosolids (PMB), bark, and grain in filter patch grow bags colonized by *F. pinicola* (a) and *F. betulina* (b).

a  b 

Figure 3-3 Sterilized PMB, bark, and grain in filter patch grow bags colonized by *G. applanatum* (a) and *T. versicolor* (b).

3.2 Experimental design and set up of lab experiment for fungal and earthworm decomposition of PMB

Small-scale indoor trials consisted of 8 kg of the substrate, at its existing moisture level, with a ratio of 2 PMB:1 bark in 26 L (~42 x 33 x 31 cm^3) plastic bins with holes drilled on all sides for air circulation. A 2 L of liquid fungal inoculum in MEB was added to all treatments except to controls for which sterilized MEB was added as one control and water was added to a second control. White-rot species selected were *G. applanatum*, and *T. versicolor*, brown rot species selected were *F. betulina* and *F. pinicola* due to their local abundance and culturable nature. Bins were stored at room temperature and mixed by shaking every week to increase oxygen content and to break up mycelia encouraging faster fungal growth. After 20 days, each bin was separated into two equal sections (~21 x 33 x 31 cm^3) by a vertical plastic sheet secured with duct tape, and *E. fetida* were introduced to one side with moist newspaper as bedding as

shown in **Figure 3-4**. All treatments with *E. fetida* will henceforth be referred to as Ef+ while those without *E. fetida* will be referred to as Ef-. Worms immediately attempted to move from the Ef+ side to the Ef- side but were manually moved back to the Ef+ side for the first few days to encourage colonization on the Ef+ side. After the first-week, worms mostly remained on the Ef+ side, however complete separation was not accomplished. Samples were collected for all analyses on days 1, 20 (before worms were added), 50, and 96.

Lab Treatments replicated three times included:

1) HCTL: Control with 2 L tap water
2) BCTL: Control with 2 L uninoculated MEB
3) *T. versicolor*: 2 L MEB with *T. versicolor*
4) *G. applanatum*: 2 L MEB with *G. applanatum*
5) *F. betulina*: 2 L MEB with *F. betulina*
6) *F. pinicola*: 2 L MEB with *F. pinicola*
7) *T. versicolor & G. applanatum*: 1 L MEB each of *T. versicolor & G. applanatum*
8) *F. betulina & F. pinicola*: 1 L MEB each of *F. betulina & F. pinicola*
9) FC: 500mL MEB each of a fungal consortium of *T. versicolor, G. applanatum, F. betulina & F. pinicola*

   All treatments were split in half with *E. fetida* added to one half. 27 Ef+, 27 Ef- total

Figure 3-4 Compost bin divided, and *E. fetia* introduced on the right after 20 days with moist newspaper as bedding. Note the white fungal growth visible on the left side showing composting PMB without *E. fetida.*

3.3 Set up of outdoor compost experiment

Outdoor compost piles were made with approximately 2 PMB:1 bark and mixed by a loader operated by CBPPL staff (**Figure 3-5**). Once piles were formed, measurements were taken to estimate the volume and weight of the piles, based on the weight of known dimensions in the lab experiment, to determine the amount of urea needed to reach the desired C:N (**Table 3-1**). Six piles were made and 3 were inoculated with 2 L of cultures in MEB of each of the 4 species of fungi for a total of 8 L of inoculum per pile, the other 3 piles had no additives. Temperatures were taken manually from 3 locations in the pile approximately once a week until November when winter conditions made manual measurement too difficult, and thermographs were available. Thermographs were placed in the piles on day 77 (Nov 20) to see what temperature fluctuations occur over winter in the piles, and the temperatures were collected every hour.

Samples from 3 places in each pile were taken on days 1(Sept 5), 43(Oct 18), 77 (Nov 20), and 245 (May 6).

a  b 

Figure 3-5 Loader mixing the PMB and bark (a) ratio 2:1 and the 6 finished piles at CBPPL covered with tarps to hold in moisture (b).

Table 3-1 Estimated volume and weight of piles based on lab experiment of known weight, volume, urea added, and inoculation schedule.

Pile	Approximate Volume (m^3)	Estimated Weight (kg)	Urea Added (kg)	Fungi/ Control
1	4.03	1033.8	3.41	Fungi
2	5.30	1359.2	4.48	Control
3	3.68	942.82	3.11	Fungi
4	4.60	1179.5	3.89	Control
5	3.26	834.6	2.75	Fungi
6	5.00	1282.1	4.22	Control

3.4 DNA extraction and PCR analysis of compost

DNA was extracted as follows from cultures on MEA plates of all 4 cultured fungal species (*T. versicolor*, *G. applanatum*, *F. betulina*, *F. pinicola*) and from mushrooms of fungi found growing naturally in indoor lab trials. Samples weighing 100 mg of each fungal species were ground using a tissue grinder mortar and pestle. DNA extraction was performed with the DNeasy Plant Mini Kit® (Qiagen, Toronto, Ontario) as follows: for each sample, the following

steps were taken, 400 µL buffer AP1 and 4 µL RNase were added and vortexed for at least 20 s. Samples were then incubated for 10 min at 65°C and mixed 3 times during incubation by vortexing for 20 s to lyse the cells. Next 130 µL of buffer AP2 was added and incubated on ice for 5 min then centrifuged for 5 min at 14,000 rpm. Samples were then pipetted into the QIA shredder Mini spin column® (Qiagen, Toronto, Ontario) which was placed in a 2 mL collection tube and centrifuged for 2 min at 14,000 rpm. The flow-through lysate was pipetted, leaving the bottom cell debris, into a new 2 mL microcentrifuge tube, and 1.5 times the volume of buffer AP3/E was added and mixed by pipetting. 650 µL of this mixture was added to a DNeasy Mini spin column® (Qiagen, Toronto, Ontairo) placed in a 2mL collection tube and centrifuged for 1 min at 8000 rpm and flow-through was discarded. This step was repeated for the remaining mixture and flow-through was discarded. The DNeasy Mini spin column was then placed in a new 2 mL collection tube and 500 µL of buffer AW was added and centrifuged for 1 min at 8000 rpm, flow-through was discarded. Using the same collection tube another 500 µL of buffer AW was added to the spin column and centrifuged for 2 min at 14,000rpm to dry the membrane and flow-through was discarded. The DNeasy Mini spin column was placed into a 2 mL microcentrifuge tube and 100 µL of buffer AE was added and incubated for 5 min at room temperature and then centrifuged for 1 min at 8000 rpm to elute. This step was repeated, and the eluates were combined and stored in the freezer. DNA concentration was measured on the BioRad NanoDrop by placing 2 µL of each DNA extraction on the pedestal for reading. Polymerase chain reaction (PCR) primers, internal transcribed spacer (ITS)-4 and ITS-5, were prepared in 100 µM stock and a 1:10 dilution of each was used primers were kept frozen until use. The primer sequence for ITS-4 was TCCTCCGCTTATTGATATGC and for ITS-5 was GGAAGTAAAAGTCGTAACAAGG. Samples were prepared for PCR by maximizing DNA

concentrations and adding 12.5 µL Green Master Mix®, 2.5 µL bovine serum albumin (BSA), and 1 µL of each of the ITS-4 and ITS-5 primers for a total of 25 µL for each sample. Samples were run in small batches and frozen after PCR amplification. PCR amplification was carried out in 50 µL reaction tubes using BioRad C1000 Touch™ Thermal Cycler (Mississauga, Ontario) on ITS protocol. The initial denaturation and enzyme activation step was 10 min at 95°C followed by 36 cycles of amplification for 10 s at 95°C, 30 s at 52°C, and 1 min and 30 s at 72°C with a final extension of 7 min at 72°C. Agarose gel was prepared by adding 0.5 g of agarose powder to 50 mL of Tris/Borate/EDTA (TBE) and microwaved for a total of 90 s, swirling to mix halfway through. 0.3 µL of SyBR was added and swirled to mix and poured into the gel mold with a comb in place. Once the gel was set the comb was removed, the tank was filled with TBE, 10 µL of PCR mix was added to the wells, and electrophoresis was run for 40 min. Photos of each gel were taken with the Bio Rad Molecular Imager® Gel Doc™ XR+ with Image Lab™ Software (Mississauga, Ontario). Bands were cut out and cleaned up with the Promega™ Wizard® SV Gel and PCR Clean-Up System (Madison, WI). Each PCR band was added to a 1.5 mL microcentrifuge tube with an equal volume of Membrane Binding Solution and vortexed until it dissolved. The solution was then added to an SV mini column in a collection tube and incubated at room temperature for 1 min then centrifuged for 1 min at 12,000 rpm. Flow-through was discarded and the mini column was placed back in the same collection tube and 700 µL of membrane wash solution with ethanol was added and centrifuged for 1 min at 12,000 rpm. Flow-through was discarded and 500 µL of membrane wash solution was added and centrifuged for 5 min at 12,000 rpm. Flow-though was discarded and the empty mini column and collection tube were centrifuged for 1 min with the lid open to allow any residual ethanol to evaporate. For elution, the mini column was transferred to a clean 1.5 mL microcentrifuge tube, and 50 µL of

nuclease-free water was added and incubated at room temperature for 1 min, then centrifuged for 1 min at 12,000 rpm. The mini column was discarded, and the remaining DNA solution was stored at -20°C until further use. Samples were then prepared for sequencing by adding 50-100 ng of DNA with 1 µL each of the primers (ITS-4, ITS-5) and nuclease-free water to dilute. Samples were sent to The Centre for Applied Genomics (TCAG) in Toronto, Canada for sequencing, and results were identified using nucleotide Basic Local Alignment Search Tool (BLAST) at the National Institutes of Health, National Center for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/). Full sequences shown in Appendix 2.

3.5 Sample preparation for carbon, hydrogen, nitrogen (CHN) analysis

Samples were freeze dried and ground using the Mini Wiley Mill, and samples were weighed to 2-5 mg and encased in tin foil for analysis by combustion on the Perkin Elmer Series II CHNS/O Analyser 2400. Wet samples were also sent to the Soil, Plant, and Feed laboratory of the Government of Newfoundland and Labrador in St. John's, NL (NLSPF) for CHN analysis due to equipment malfunctions in the laboratory of the Boreal Ecosystems Research Facility (BERF) at Grenfell Campus.

3.6 Compost analysis

Compost analysis was performed at the NLSPF lab. Compost analysis consisted of total C, total N, pH, soluble salts, and nutrient analysis of P, K, Ca, Mg, Fe, Cu, Mn, Zn, Bo, and Na via Inductively coupled plasma – optical emission spectrometry (ICP-OES). This analysis was performed from samples taken on days 1, 20 (before *E. fetida* added), and 96 (Ef+ and Ef-). PMB and bark taken directly from CBPPL were sent to the NLSPF lab for initial characterization, and results are given in **Table 3-2**.

Table 3-2 Initial elemental composition, pH and electrical conductivity of bark and PMB before composting.

Nutrient	Bark	PMB
pH	6.27	6.80
Total N%	0.51	1.64
Total C%	50.44	45.70
C:N	111.71	28.00
Total P%	0.21	1.01
Total K%	0.18	0.37
Total Ca%	1.49	2.24
Total Mg%	0.13	0.23
Total Fe (mg L^{-1})	825.71	2010.00
Total Cu (mg L^{-1})	15.71	52.00
Total Mn (mg L^{-1})	1237.71	3350.00
Total Zn (mg L^{-1})	162.00	540.50
Total B (mg L^{-1})	23.29	59.50
Total Na (mg L^{-1})	261.71	505.50
EC (dS/m)	0.39	0.52
Water %	68.20	72.00

3.7 Germination test for compost maturity

Rudolph organic radish (*Raphanus sativus* L.) was chosen for the germination test because it is commonly used for phytotoxicity testing of composts in other studies (Luo et al., 2018). Due to delays in the shipping of the seeds the germination test was done with compost from day 123 after the other analyses were performed. The method was adapted from Oktiawan et al. (2018), a ratio of 1:10 (w/v) is common in literature and was used as follows; 20 ± 0.5 g of wet compost from each replicate was mixed with 200 mL ± 5% of tap water covered and kept for 72 h(Luo et al., 2018). Extracts were then filtered through filter paper and 10 mL of each was added to a 10 cm diameter petri dish with 20 seeds between 2 filter papers. Eight dishes of control were made with 10 mL of tap water and seeds were incubated in the dark for 4 days at

room temperature then germinated seeds were counted and radicle lengths were measured. Germination index (GI) was calculated using the formulas from Luo et al.(2018) as given in Eq 1 - 3.

$$\text{RSG (Relative Seed Germination)} = \frac{\text{Number of germinated seeds (sample)}}{\text{Number of germinated seeds (control)}} \quad \text{Eq.1}$$

$$\text{RRG (Relative Radicle Growth)} = \frac{\text{Total radicle length of germinated seeds (sample)}}{\text{Total radicle lenght of germinated seeds (control)}} \quad \text{Eq. 2}$$

$$\text{GI} = \text{RSG x RRG x } 100\% \quad \text{Eq. 3}$$

3.8 Phospholipid fatty acid analysis

PLFA analysis is used to determine the microbial communities present that can be an indication of compost maturity (Villar et al., 2016). For lipid extraction, lyophilized and ground samples were weighed to 0.5 g and 2 mL of 50 mM citrate buffer (31.52 g citric acid monohydrate in 400 mL deionized water pH adjusted with KOH to 4 then diluted to 1000 mL), 2.5 mL dichloromethane (DCM) and 5 mL methanol were added. Samples were shaken for 2 h and centrifuged for 10 min at 2500 rpm. The liquid phase was removed, and 5 mL of DCM and 5 mL high performance liquid chromatography (HPLC) grade water were added, shaken by hand, and left to separate overnight. The bottom phase was removed and evaporated under N_2 gas and stored at -20 °C until the next step.

For lipid fractionation, lipid classes were separated by solid-phase extraction (SPE) as follows. A 100 mg silica gel column was rinsed with DCM, samples were brought up with 1 mL DCM and loaded into the column, to elute undesired lipids 2.5 mL DCM and 5 mL acetone were run through the column into waste vials and 2.5 mL methanol was added to elute phospholipids

into clean vials. This phospholipid fraction was evaporated under N_2 gas and stored at -20 °C until the next step.

Transesterification was performed by adding to each vial, 0.2 mL transesterification reagent (0.561 g KOH dissolved in 75 mL methanol, and 25 mL toluene) vials were sealed with PTFE lined caps and incubated at 37 °C for 15 min. Then to each vial 0.4 mL, each of 0.075 M acetic acid and DCM was added. These were shaken by hand for 10 s and then allowed to separate. The bottom 0.3 mL was removed and added to clean 1 mL vials. The same volume of acetic acid and DCM were added shaken and separated again and the bottom 0.4 mL was removed and added to the same 1 mL vials. These were evaporated under N_2 gas just to dryness and samples were dissolved in 75 µL n-hexane transferred to gas chromatography vials and stored at -20 °C until analyzed. The analysis was performed on Thermo Scientific Gas Chromatography – Mass Spectrometer (GC-MS) Trace 1300 and TSQ 8000 equipped with an autosampler.

3.9 Fibre analysis of compost before and after

Fibre analysis was sent to Bureau Veritas Laboratory at 12622 Hwy 3, Webster, Texas, 77598, where acid detergent fibre (ADF) and acid detergent lignin (ADL) analysis were performed. The lab used the standard global method, AOAC 973.18 (Moller, 2008), in which samples are digested with the neutral detergent to first separate cell contents such as proteins, sugars, and starches from fibres, hemicellulose, cellulose, and lignin. The fibres are digested with an acid detergent to separate the hemicellulose from the cellulose and lignin, this is the ADF. ADF is then digested with 72 % sulfuric acid to separate the cellulose from the lignin, called ADL. Data was received for the ADF and ADL portions. Cellulose % was calculated by Eq. 4;

$$\text{ADF (fibre)} - \text{ADL (lignin)} = \text{Cellulose} \quad \text{Eq. 4}$$

3.10 Overall health of *E. fetida*

After taking samples on day 96, bins were sorted to determine the overall health of the *E. fetida* in each treatment. One bin of each treatment was selected randomly, and worms were sorted into adult, juvenile, and cocoons and weighed to estimate the overall proportions of each life stage from both Ef+ and Ef- treatments. Visual observations were recorded from all replicates such as particle size, texture, and moisture. These were recorded in Ef+ and Ef- treatments and were compared to bins in which worms were counted to estimate health in all bins.

3.11 Sample preparation for ICP-MS

Samples were collected from PMB, bark, and after the composting process from each treatment for contaminant analysis. Samples were lyophilized for 72 h then ground using the Thomas Scientific Wiley Mini Mill 3383-L10 until homogeneous. Digestion was performed on 0.5 g samples with acids added as follows: 10 mL of 1 HNO_3 (70%): 1 H_2O added and left to sit overnight, samples were then heated in a digestion block to 50°C for 30 min then the temperature was increased to 95°C for 30 min. Next, 5 mL of HNO_3 was added and held at 95°C for 30 minutes. A mixture of 2 H_2O: 3-30% H_2O_2 was added (drop by drop to avoid bubbling over) up to 2 mL. Once temperatures increased back up to 95°C another 2.5 mL of HNO_3 and 1 mL of HCL were added. Finally, 10 mL 30% H_2O_2 was added in small increments with samples maintained at 95°C in a block digester. Once samples were digested and acid was reduced, they were diluted to 50 mL with deionized water. Dilutions of 1:200 were made with deionized water and analyzed via inductively coupled plasma – mass spectrometry (ICP-MS).

3.12 Soil bioremediation experiment

Soil contamination is a concern worldwide and novel remediation methods are in demand. Compost has been used to remediate petroleum hydrocarbon contamination in previous studies (Bastida et al., 2016; Dados et al., 2015). In this section, an experiment was conducted to determine the ability of PMB compost to remediate 10W30 oil from a local NL soil in a lab setting.

Trials were set up in 1 L mason jars with holes punched in the lid for ventilation. Two concentrations (5 % and 10 % of soil mass) of 10W30 oil were tested against 4 ratios of compost (0 %, 5 %, 10 %, and 20 %) with 3 replicates for each treatment for a total of 24 jars. Soil was collected from Corner Brook NL in a vacant lot, air dried, and sieved through a 2 mm sieve. Soil was weighed to 200 g (dry mass) for each treatment and was thoroughly mixed with 10W30 oil. Ef+ treatments were mixed, and compost was collected and wet sieved through a 6.3 mm sieve and mixed with the soil at 0, 5, 10, and 20% of dry soil mass. Water was added to bring the mixture up to 50% of the field capacity (FC). FC was measured by placing the substrate in a cylinder and saturating it with water. Water was then drained and the mass was taken before placing the cylinder in the oven at 105 °C over night and dry mass was taken. FC was calculated by: water mass at FC / dry mass * 100. The average FC for treatments with 0, 5, 10 and 20 % compost added to soil were, 29.87, 36.33, 43.13, and 54.63 respectively. Treatments were mixed together and separated equally into 3 jars to ensure uniformity among replicates. Water loss was monitored every 3 to 4 days and water was added to maintain 50% field capacity.

3.13 Oil extraction and measurement

Samples were taken on days 0, 30, 60, and 90, and oil was extracted on the FOSS Soxtec™ 2043 Extraction System (FOSS, Hilleroed, Denmark) using Hexane to determine oil concentration. Samples were weighed to approximately 12 g wet mass into the thimbles and mounted on the Soxtec apparatus. The extraction cups were weighed then 20 mL of hexane was added with glass beads and they were placed in the Soxtec apparatus. Boiling was performed at 130 °C for 30 min and rinsing was performed for 40 min and solvent recovery for 15 min. Once cool the thimbles and extraction cups were removed, remaining hexane was allowed to evaporate at room temperature in a fume hood from both, and weights were recorded.

3.14 Statistical analysis

A repeated-measures ANOVA with sphericity assumed ($p > 0.05$) was performed on IBM© SPSS© Statistics Version 27.0.0.0 for compost analysis, and a post-hoc test with a Bonferroni correction for the difference over time in pH, EC, nutrients, and GI. PLFA and ICP-MS results were analyzed for significance on SPSS.

Remediation data were analyzed with ANOVA on SPSS statistical software. The oil remediated was determined by the change in oil weight each month from month 0 at the beginning of the experiment.

4.0 Results and discussion

4.1 Isolating fungal cultures and preliminary growth test

All cultures grew successfully on MEA and were successfully isolated. Preliminary growth tests on sterilized PMB and bark were successful with all species colonizing the substrate in bags. *T. versicolor* and *G. applanatum* are white-rot species that simultaneously degrade lignin, cellulose, and hemicellulose and this may explain their vigorous growth on the sterilized substrate, as shown in **Figure 3-3**, completely colonizing the substrate and forming thick mycelial mats. *F. betulina* and *F. pinicola* are brown rot species that modify lignin to access and degrade cellulose, these species were also successful on the sterilized substrate but not as vigorous, with thinner more delicate mycelial growth, colonizing more slowly as shown in **Figure 3-2**. This vigorous and thorough colonization was not observed in compost trials, likely due to the unsterilized substrate and competing microorganisms. The fungi selected do not naturally grow in compost and therefore are likely not accustomed to competing with the microorganisms found in the compost. It was hypothesized that wood degrading species of fungi would be effective due to the woody nature of the substrate but naturally occurring fungi may be more effective. Further research with other species should be conducted to determine the feasibility of fungi degrading PMB without sterilization. Fungal growth was observed in all trials as shown in **Figure 4-1** however, the growth observed may have been naturally occurring fungi in the substrate as identified in the DNA analysis; these could be the microorganisms that outcompeted the inoculated fungal species. Along with fungi, bacterial growth was observed, and other visible growth, based on appearance, may have been slime molds or other protist microorganisms. A variety of microorganisms are likely present in the PMB and bark because they are harvested from forested areas, stored outdoors where wildlife is present, and are

processed through the mill thus there are many opportunities for microorganisms to be introduced. The microorganisms may even vary from day to day as trees are harvested from different areas. To avoid the issues of competition, the existing microorganisms could be isolated, grown, and inoculated back into the substrate to further break down the compost rather than introducing other species.

Figure 4-1 Compost bin colonized by fungi and other microorganisms; possibly includes microorganisms that outcompeted the inoculated fungal species.

4.2 PCR and genetic sequencing

DNA extraction was successful and adequate DNA concentration was used for PCR and gel electrophoresis. PCR images were captured using Image Lab software and the SyBr Safe protocol. Examples of the results are shown in **Figure 4-2**.

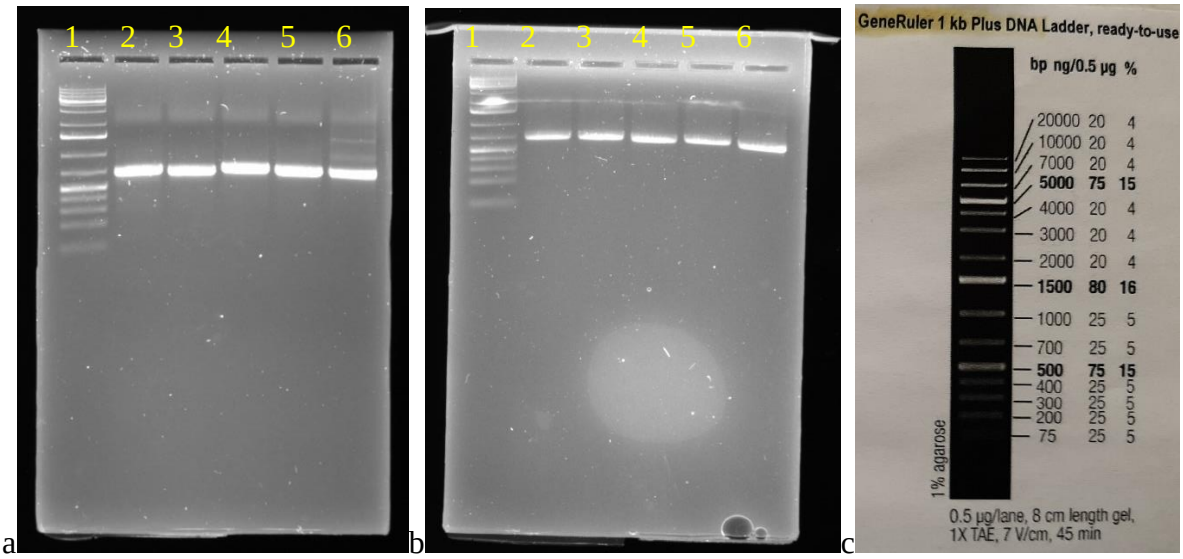


Figure 4-2 PCR gel electrophoresis next to the GeneRuler showing the bands in this set of samples is around 2000bp (base pairs). This confirms that DNA from the target species was present. a: loaded from left to right with (1) GeneRuler 1 kb, (2) *G. applanatum*, (3) *T. versicolor*, (4) *F. pinicola*, (5) species 1 (as discussed below), (6) sp. 1. b: loaded from left to right = (1) GeneRuler 1 kb, (2) *F. betulina*, (3) *F. betulina*, (4) *G. applanatum*, (5) *F. pinicola* (6) *T versicolor*. c: GeneRuler legend used to identify PCR bands.

Sequenced cultured samples that were sequenced confirmed the proposed identities of the fungi used in composting based on the BLAST analysis the species were *T. versicolor*, *G. applanatum*, *F. betulina*, and *F. pinicola.*

The unidentified fungi that grew in the compost were matched with the following species through the BLAST comparison. Species 1 (**Figure 4-3**) was matched to *Psilocybe coprophila*, *Psilocybe merdaria*, or *Deconica coprophila*. Species 2 (**Figure 4-4**) was matched to *Clitopilus scyphoides, Clitopilus hobsonii, Clitopilus passeckerianus* or *Omphalina mutila*. Since the DNA was extracted from mushrooms, not cultures the DNA analysis was broader, though the species

listed for each are similar to each other genetically and morphologically.

Figure 4-3 Species 1 identified as one of three genetically and morphologically similar species; *Psilocybe coprophila, Psilocybe merdaria,* or *Deconica coprophila*

Figure 4-4 Species 2 identified as one of four genetically and morphologically similar species; *Clitopilus scyphoides, Clitopilus hobsonii, Clitopilus passeckerianus*, or *Omphalina mutila.*

4.3 Carbon, hydrogen, nitrogen (CHN) analysis

Standards used at the BERF laboratory were cystine and acetanilide which were insufficient comparisons for determining the total C and total N of the compost and PMB samples. It was determined that it would be more cost effective to send samples to NLSPF for C, N and other nutrient analyses. All further CHN analysis was done by the NLSPF lab and is reported in the compost analysis section (4.4).

4.4 Compost analysis of lab composting experiment

Lab compost bin temperatures reached their highest point on day 2 with the highest temperature reaching 41 °C with an average temperature of 33.6 °C for all trials. Temperatures gradually decreased to room temperature by day 36 and remained between 20-25 °C for the remainder of the experiment, as given in **Figure 4-5**. The initial compost mix was coarse-textured, and the PMB and bark were easily distinguishable. C:N and other nutrient results are shown in **Figure 4-6** to **Figure 4-16** indicating the changes from day 0 until the end of the experiment. For the thermophilic stage to occur the bin size should be 37 L or have adequate insulation to retain the heat (Trautmann, 1996). The bins used were not adequate to retain the heat and therefore thermophilic composting was not achieved.

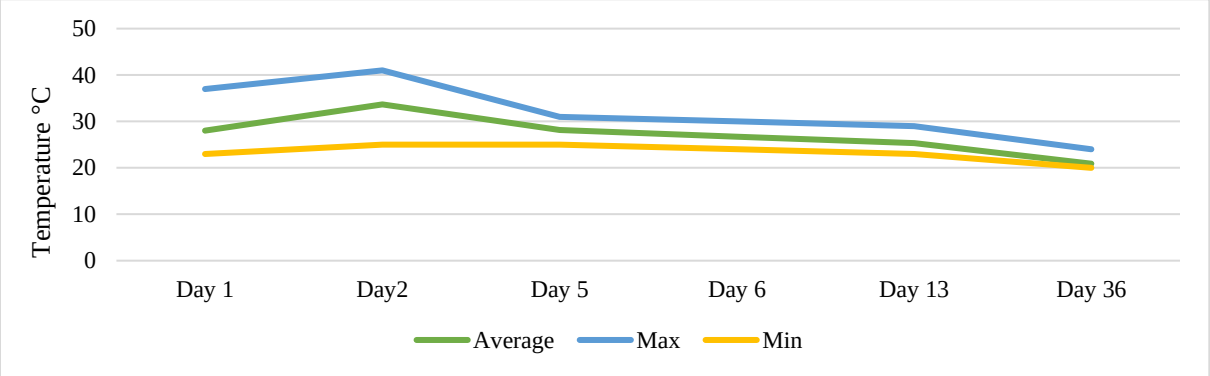


Figure 4-5 Temperature variations in the composting lab experiment observed in the first 36 days of composting.

The nutrient analysis determines the suitability of compost as a soil amendment by measuring plant nutrient levels. Each nutrient has an optimal range in which plants grow best, the data presented indicate which nutrients are within that optimal range and are, therefore, adequate for plant growth. Samples for nutrient analysis were taken on days 0, 20, before adding

E. fetida, and day 96. Day 96 samples were taken from both Ef- and Ef+ treatments to determine the effects of *E. fetida* on the composition. Nutrient levels, determined by the NLSPF lab, are given in **Figure 4-6** to **Figure 4-16** with optimal ranges marked where applicable. Changes in nutrient levels are explained after the respective Figures.

A repeated-measures ANOVA was performed on all nutrients over time with sphericity assumed ($p > 0.05$ by Mauchly's Test of Sphericity) and post-hoc tests with a Bonferroni correction revealing the difference seen over time. There was a significant difference in total N means over time (Ef-: ($F(2,36) = 20.875$, $p = 0.00$) , Ef+: ($F(2,36) = 23.694$, $p = 0.00$)) increase of .064 % in N over the first 20 days ($p = .015$) followed by a decrease of 0.105 % and 0.123 % in Ef- and Ef+ treatments respectively between days 20 and 96 ($p = 0.00$). Further analysis of forms of N via flow injection analysis, would help determine the N losses and compost maturity (Bernal et al., 2009). During the composting process, N is converted by bacterial fixation into more stable forms (NH_4^+ and NO_3^-) that are more available to plants and are less susceptible to volatilization, leaching, and denitrification which makes the compost a good source of N-rich organic matter for soil (Bernal et al., 2009). C losses are also more of a concern if raw, uncomposted matter is added to soil, while composted material is considered a better soil amendment overall as there is less nutrient loss to volatilization and leaching (Bernal et al., 2009). There was a significant difference between the means of C over time (Ef-: ($F(2, 36) = 30.008$, $p = 0.000$), Ef+: ($F(2, 36) = 38.039$, $p = 0.000$)). A post-hoc test revealed an increase of 1.381 % in C was observed between day 0 and 20 ($p = 0.000$) and a decrease of 2.681 % and 2.785 % between day 20 and 96 for Ef- and Ef+ treatments respectively. There were no significant differences in C between fungal treatments. Any significant differences between

fungal treatments in both Ef+ and Ef- treatments in the following nutrients are reported in Appendix 1.

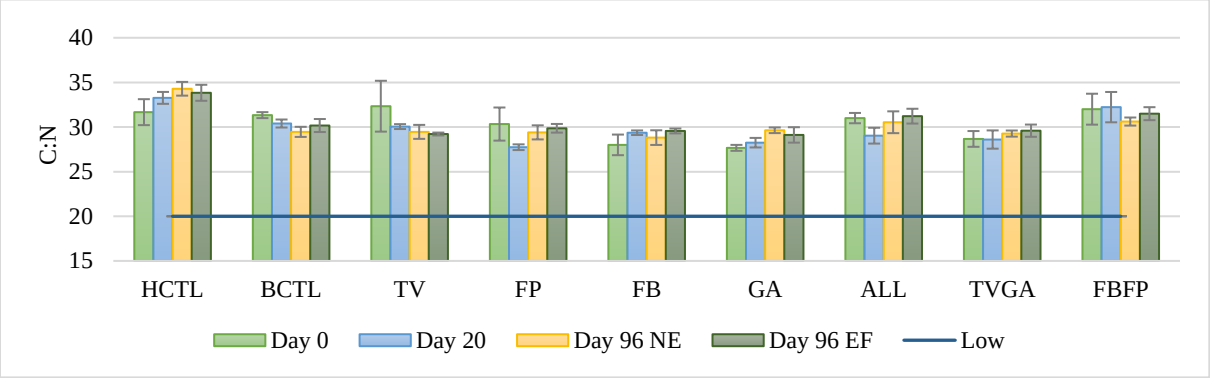


Figure 4-6 Mean C:N of compost over time ± standard error (SE), mature compost is recommended to have a C:N below 20 (A&L Laboratories, 2004). Treatment abbreviations are as follows, HCTL = water control, BCTL = MEB control, TV = *T. versicolor*, FP = *F. pinicola*, FB = *F. betulina*, GA = *G. applanatum*, ALL = fungal consortium of the 4 species listed, TVGA = *T. versicolor & G. applanatum*, FBFP = *F. betulina & F. pinicola*.

There was no significant difference over time of C:N means ($p = 0.575$) in Ef- nor Ef+ treatments. The recommended rate for a mature compost is a C:N below 20, due to the inability of plants to assimilate N at higher levels (Hait & Tare, 2011). Compost for agriculture is recommended to have a C:N of 15 or less therefore our compost has not reached an advanced degree of stabilization and is not mature enough for plant growth (Hait & Tare, 2011). Other studies have had more success composting sludge and obtaining the desired C:N in controlled conditions, such as in a compost drum or reactor (Dinel et al., 2004; Hait & Tare, 2011). The lack of temperature increase, size of the trials, or the original C:N of the material could explain the insignificant changes in C:N.

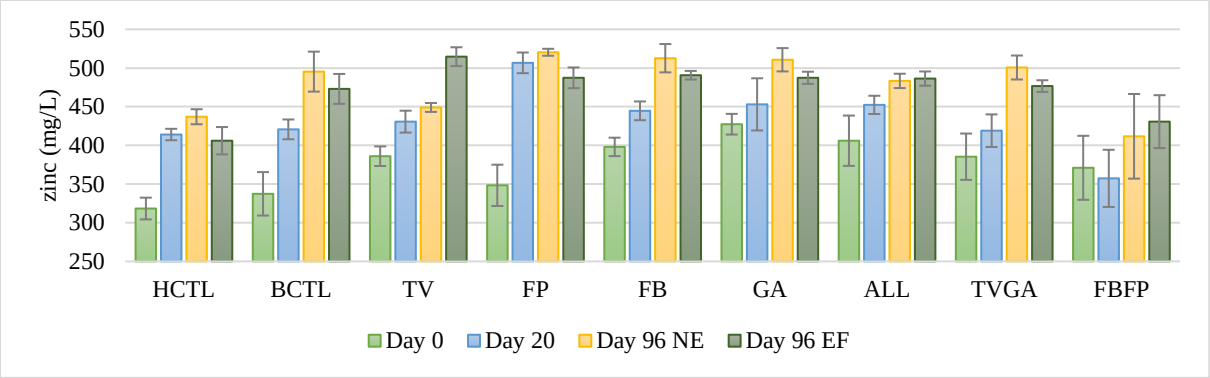


Figure 4-7 Mean zinc (Zn) levels ± SE, of the compost over time, guideline limit for compost of 700 mg/kg (CCME, 2005). Treatment abbreviations as stated in Fig. 4-6.

There was significant difference in Zn over time (Ef-: (F (2, 36) = 64.605, p = 0.0), Ef+: (F (2, 36) = 71.655, p = 0.0)) Zn means increased by 57.852 mg/L in the first 20 days and an additional 46.926 mg/L and 39.370 mg/L (p =0.0) between days 20 and 96 in Ef- and Ef+ treatments, respectively. Zn levels were below the Canadian Council of Ministers of the Environment (CCME).

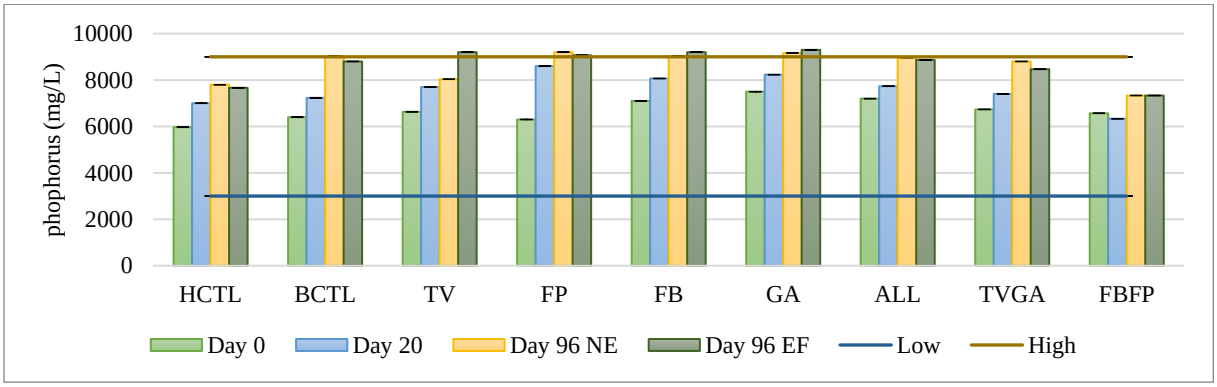


Figure 4-8 Mean phosphorus (P) levels ± SE, optimal range of P indicated between high and low lines. Treatment abbreviations as stated in Fig. 4-6.

P levels increased slightly over time in all treatments. Most treatments stayed between recommended levels of 3000 mg/L and 9000 mg/L while some went slightly over on day 96

(Sullivan et al., 2018). There was a significant difference over time in P (Ef-: (F (2, 36) = 71.828, p = 0.000), Ef+: (F (2, 36) = 97.106, p = 0.000)). There was an increase of .088% between day 0 and day 20 and another increase of 0.101 % and 0.107 % between day 20 and 96 of Ef- and Ef+ treatments respectively (p = 0.000). A more significant increase of P (30.1 – 86.1 %) was observed in another vermicomposting study which was hypothesized to be due to mineralization, and release of available P from organic matter through earthworm gut phosphates or by microorganisms (Hait & Tare, 2011).

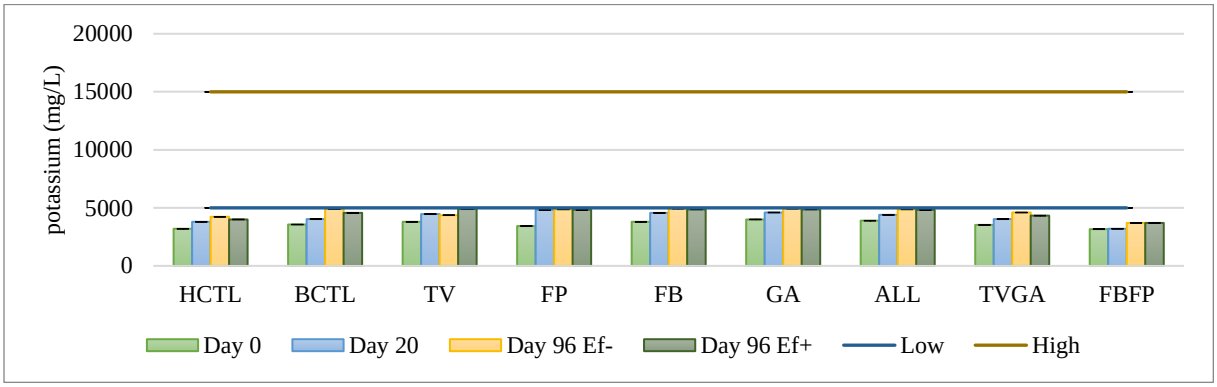


Figure 4-9 Mean potassium (K) levels ± SE of compost over time. Optimum K levels indicated between high and low lines. Treatment abbreviations as stated in Fig. 4-6.

K levels remained just below the recommended range for compost with some treatments just reaching the lower threshold (Sullivan et al., 2018). There was a significant difference in K over time (Ef-: (F (2, 36) = 88.171, p = 0.000) Ef+: (F (2, 36) = 104.690, p = 0.000)) with an increase in K by 0.061 % between day 0 and day 20 and a further increase of 0.039 % and 0.033 % in Ef- and Ef+ treatments respectively (p = 0.000). Other studies have also reported an increase in K during vermicomposting hypothesized to be due to increased microbial activity which increases acid production and mineralization rates that solubilize potassium making it more readily available in composts (Gupta & Garg, 2008; Hait & Tare, 2011; Khwairakpam &

Bhargava, 2009).

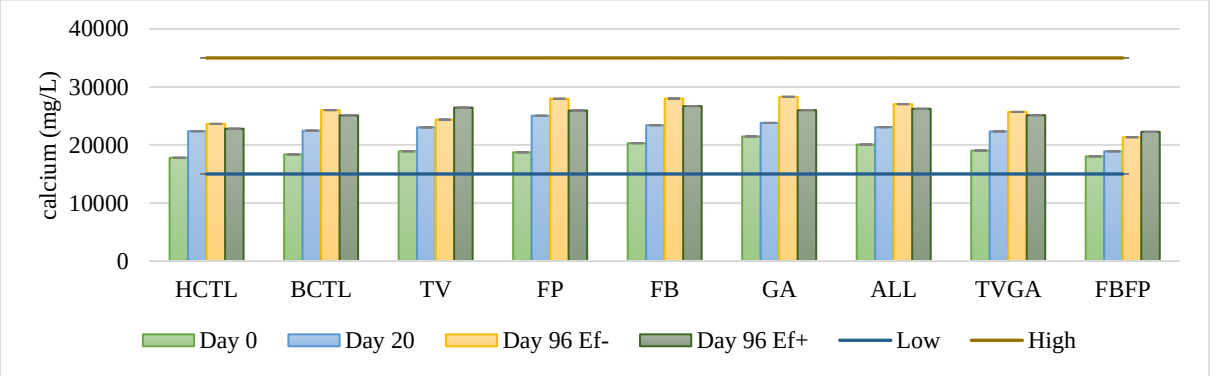


Figure 4-10 Mean calcium (Ca) levels ± SE of compost over time. Optimum levels indicated between high and low lines. Treatment abbreviations as stated in Fig. 4-6.

All Ca values increased slightly over time with the Ef- treatments reaching higher levels than the Ef+ treatments. All treatments remained within the recommended levels (Sullivan et al., 2018). Ca % showed a significant difference over time (Ef-: ($F(2, 36) = 136.769$, $p = 0.000$), Ef+: ($F(2, 36) = 149.112$, $p = 0.000$)) with an increase of 0.352 % between days 0 and 20 and a further increase of 0.310 % and 0.247 % between days 20 and 96 in Ef- and Ef+ treatments, respectively ($p = 0.0$). Mohapatra et al. (2019) hypothesized that in earthworms helped release Ca in their study composing PMB, however contrarily, our Ef- treatments had higher Ca levels than Ef+ treatments.

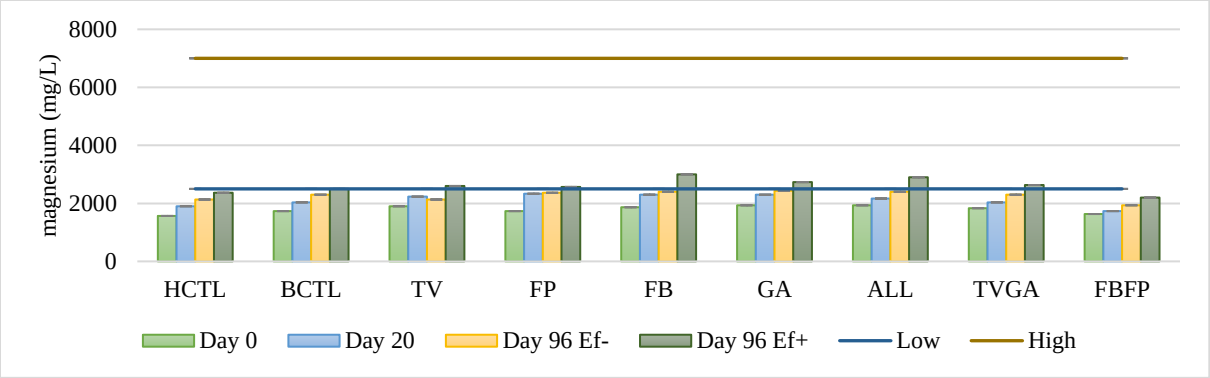


Figure 4-11 Mean magnesium (Mg) levels ± SE for compost over time, optimum levels indicated between high and low lines. Treatment abbreviations as stated in Fig. 4-6.

Mg levels remained between 1566 mg/L and 2733 mg/L with slight increases over time, and some Ef+ treatments reaching recommended levels by day 96 (Sullivan et al., 2018). There was a significant difference in Mg over time, (Ef-: (F(2, 36) = 105.490, p = 0.000), Ef+: (F (2, 36) = 205.431, p = 0.000)) with an increase of .032 % between days 0 and 20 and a further increase of 0.015 % and 0.50 % for Ef- and Ef+ treatments respectively (p = 0.000).

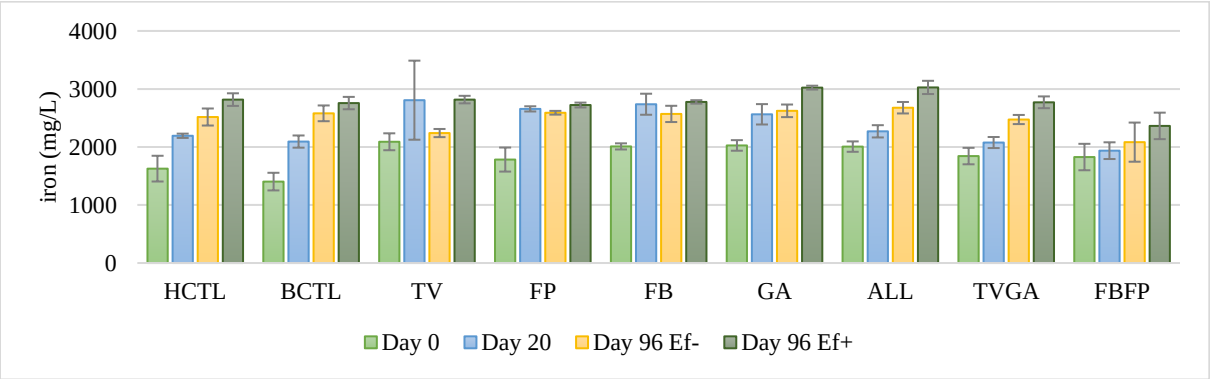


Figure 4-12 Mean iron (Fe) levels ± SE, there were no recommended levels found for Fe. Treatment abbreviations as stated in Fig. 4-6.

Fe levels increased from day 0 to 20 with *T. versicolor*, *F. pinicola*, and *F. betulina* decreasing on day 96 in Ef- treatments, while Ef+ treatments remained higher. Fe amounts (mg/L) differed significantly over time in Ef- treatments (Ef-: F (2, 36) = 26.457, p = 0.000), Fe means increased by 524.074 mg/L between days 0 and 20 (p = 0.000), however the increase of 113.333 mg/L, between days 20 and 96 in the Ef- treatment was not significant (p = .836). Mauchly's test of sphericity was not met by the Ef+ treatment (p = 0.002) so the Greenhouse-Geisser correction was applied to determine the significant difference of Fe over time, (Ef+: F (1.317, 23.702) = 57.245, p = 0.000), Fe means increased between days 0 and 20 by 524.074 mg/L (p =0.000) and further increased by 415.556 mg/L between days 20 and 96 (p = 0.001). Fe levels were comparable to those reported by Hazarika & Khwairakpam, (2018) who reported concentrations of 1982 – 4993 mg/L initially and after composting most treatments saw an increase to 3000 – 4891 mg/L which is explained by organic matter loss and mineralization. Elvira et al. (1998) reported higher levels of Fe between 5400 and 9600 mg/L but they also reported increases in all treatments.

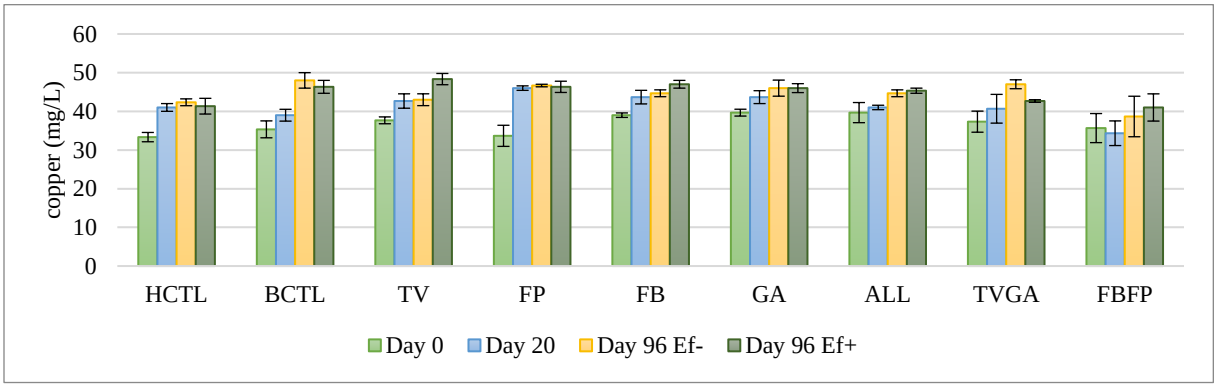


Figure 4-13 Mean copper (Cu) levels ± SE for compost over time. Treatment abbreviations as stated in Fig. 4-6.

Mean Cu levels were variable between treatments. All levels were below the CCME limit of 400 mg/L (CCME, 2005). There was a significant difference in the means of Cu over time in Ef- treatments (Ef-: F (2, 36) = 39.984, p = 0.000), a decrease in Cu means by 3.222 mg/L (p = 0.008) was observed in the first 20 days and a further decrease of 4.519 mg/L (p = 0.000) in Ef- treatments. Ef+ treatments did not meet Mauchly's test of sphericity (p = 0.023), so the Greenhouse-Geisser correction was applied and a significant difference over time in Cu was observed (Ef+: F (1.474, 26.526) = 13.479, p = 0.000) there was an increase of 3.593 mg/L (p = 0.0014) between days 20 and 96 in the Cu means of Ef+ treatments. Cu levels were comparable to other studies composting PMB that have reported concentrations between 22 and 84 mg/L, and the increase in Cu over time was explained due to loss of organic matter and mineralization over time (Elvira et al., 1998; Hazarika & Khwairakpam, 2018).

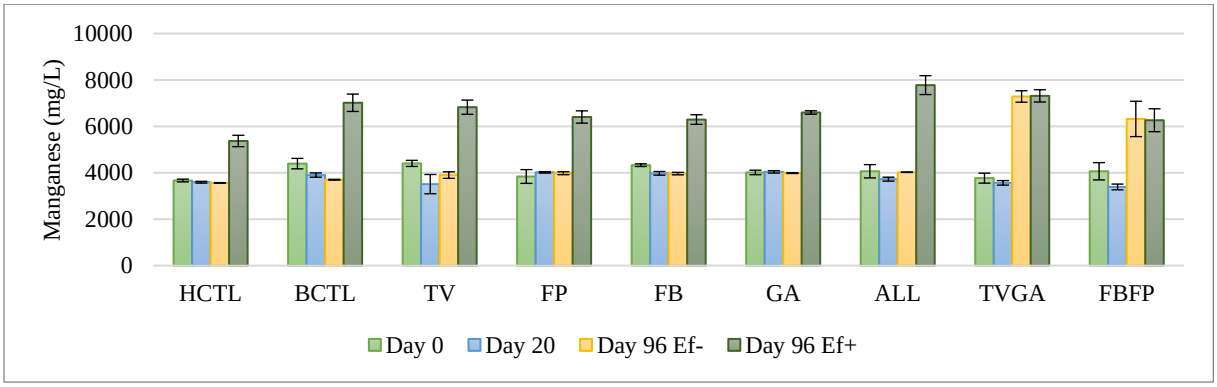


Figure 4-14 Mean manganese (Mn) levels ± SE for compost over time. Treatment abbreviations as stated in Fig. 4-6.

Mean Mn values remained between 3000 and 5000 mg/L except for a significant increase in *T. versicolorG. applanatum* (Ef-) and *F. betulinaF. pinicola* (Ef-) on day 96, and all Ef+ treatments on day 96. No recommended limit for Mn was found. A significant difference was observed in Mn over time, (Ef-: (F (2, 36) = 31.421, p = 0.000), Ef+: (F (2, 36) = 552.275, p =

0.000)) with a mean decrease of 312.963 mg/L (p = 0.001) in the first 20 days and an increase of 777.037 mg/L and 2904.074 mg/L (p = 0.000) between days 20 and 96 for Ef- and Ef+ treatments respectively. The Mn concentration observed here, particularly in vermicompost treatments is significantly higher than those observed by other studies with PMB, and increases are explained by organic matter loss and mineralization during composting(Elvira et al., 1998; Hazarika & Khwairakpam, 2018).

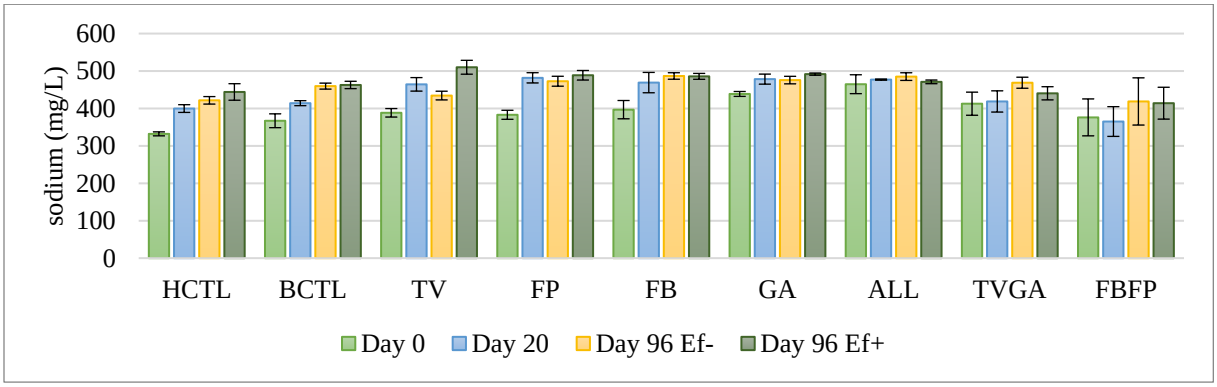


Figure 4-15 Mean sodium (Na) levels ± SE for compost over time. Treatment abbreviations as stated in Fig. 4-6.

Na values were variable over time with most treatments showing an increase between days 0 and 96. All treatments remained below the phytotoxicity limit of 6000 mg/L(A&L Laboratories, 2004; Sullivan et al., 2018) There was a significant difference in Na means over time (Ef-: (F (2, 36) = 54.626, p = 0.000), Ef+: (F (2, 36) = 71.848, p = 0.000)) and increase in means of 45.407 mg/L was observed in the first 20 days (p = 0.000), and an additional 17.259 mg/L (p = 00.027) and 26.704 mg/L (p = 0.000) between days 20 and 96 in Ef- and Ef+ treatments respectively. Na increases have been explained due to the loss of overall dry mass during the composting process(Hazarika & Khwairakpam, 2018).

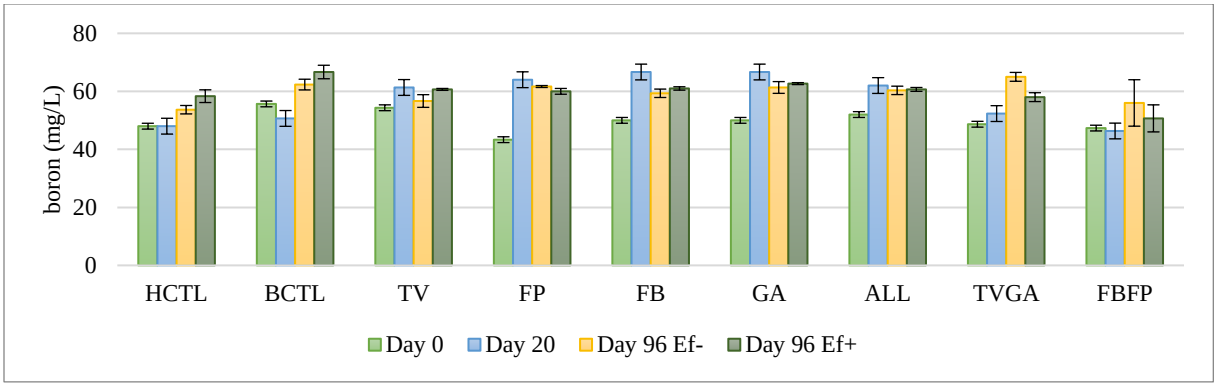


Figure 4-16 Mean boron (B) levels ± SE of compost over time. Treatment abbreviations as stated in Fig. 4-6.

Mean B levels were variable on day 20 with *T. versicolor & G. applanatum*, *F. betulina & F. pinicola*, and Controls remaining between 45 and 50 mg/L and all other treatments increased to 60-70 mg/L. No optimal range could be found for B. There was a significant difference in means observed over time in B (Ef-: ($F(2, 36) = 33.377$, $p = 0.0$)), B data for Ef+ treatments did not satisfy Mauchly's test of sphericity so the Greenhouse-Geisser correction was performed, there was a significant difference in B means over time (Ef+: ($F(1.358, 24.449) = 42.392$, $p = 0.0$)), an increase of 7.630 mg/L was observed in the first 20 days with a further increase of 2.296 mg/L between days 20 and 96 in Ef+ treatments, however, the mean increase of 2.037 mg/L ($p = 0.0$) between days 20 and 96 in Ef- treatments was not significant ($p = 0.350$). Similar to results by Fornes et al., (2012) B levels were higher in Ef+ treatments than Ef-.

4.5 Germination, pH, and electrical conductivity (EC)

The GI threshold of 60 % is a standard in the literature (Gómez-Brandón et al., 2008; Oktiawan et al., 2018). The mean GI was above the threshold for 100 % of Ef+ treatments while the Ef- treatments had only 33.3% above the threshold of 60 % as shown in **Figure 4-17**. Error bars

show a significant variation between the replicates of each treatment. Based on these results, the Ef- compost may be considered phytotoxic while the Ef+ compost is safe for plants. The reduced germination rate could be due to the high EC recorded in both treatments. In **Figure 4-18** the increase in soluble salts or EC is clear for both Ef- and Ef+ treatments which could explain the lower GI of some of the final composts. A multivariate analysis determining between subject effects found a significant effect between Ef+ or Ef- and GI ($p = 0.001$) and an effect with EC ($p = 0.003$). An effect between fungal treatment and EC was also significant ($p = 0.000$). However, fungal treatment did not have a significant effect on GI ($p = 0.387$). A repeated-measures ANOVA was performed and the Mauchly's test of sphericity was not met ($p < 0.05$) therefore a Greenhouse-Geisser correction was applied. There was a significant difference between the means of EC over time (Ef-: ($p = 0.000$), Ef+: ($p = 0.000$)). There was a significant increase in EC in the first 20 days of 0.185 ($p = 0.000$) and a further increase of 1.599 dS m^{-1} and 1.974 dS m^{-1} in Ef- and Ef+ treatments respectively ($p =0.000$). A univariate analysis of worm treatments and GI indicated there was a significant difference between GI means, with Ef+ treatment GI being 40.54 % higher than Ef-. Other studies have documented a significant trend of increasing EC in vermicomposts as compared to the same substrates without worms (Biruntha et al., 2020; Yadav & Garg, 2011). Ef+ treatments had higher EC than Ef- treatments in all except for BCTL which had a slightly higher EC in the Ef- treatment. The increase in EC may have been due to the mineralization of soluble salts and the release of mineral ions (Biruntha et al., 2020; Yadav & Garg, 2011). However, this does not explain the increased phytotoxicity of Ef- treatments compared to Ef+ treatments. Gómez-Brandón et al. (2008) recommend EC values not exceed 3.0 dS m^{-1} for compost produced for soil application. Based on this standard 4 Ef- treatments (BCTL, *T. versicolor*, *F. pinicola*, *F. betulina*) and 2 Ef+ treatments (BCTL, *F. betulina*) would not be

considered suitable, however, both Ef+ treatments and Ef--BCTL were above the GI threshold of 60 % (Gómez-Brandón et al., 2008). Meanwhile, 3 of the Ef- treatments that did not meet the GI threshold, had an EC below 3.0 (*G. applanatum, T. versicolor, G. applanatum, F. betulina, F. pinicola*). Therefore, it is likely a combination of factors, including EC, that may have influenced the GI.

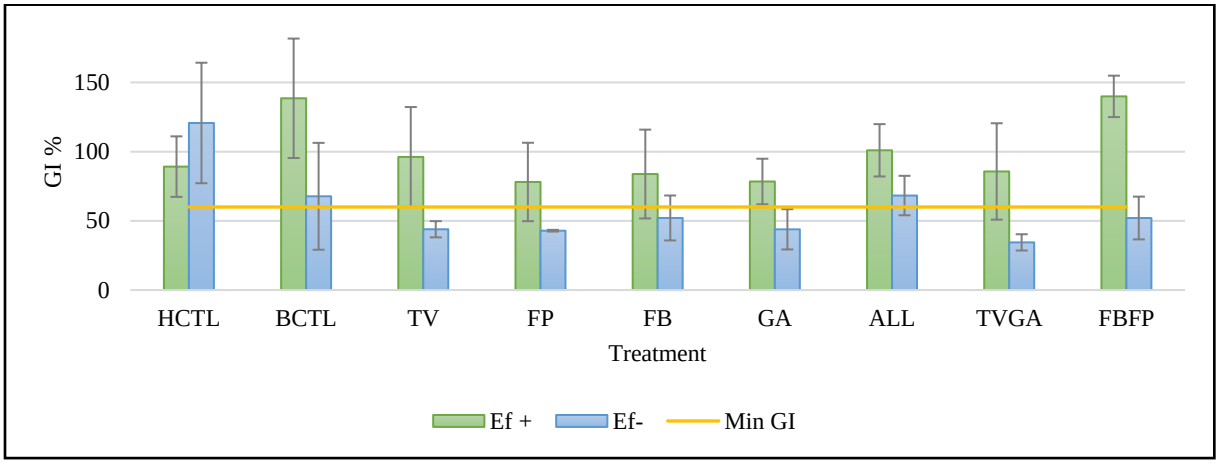


Figure 4-17 Mean germination index (GI) % of each treatment ± SE with the horizontal line showing the threshold germination index for phytotoxicity. Fungal treatment abbreviations as stated in Fig. 4-6.

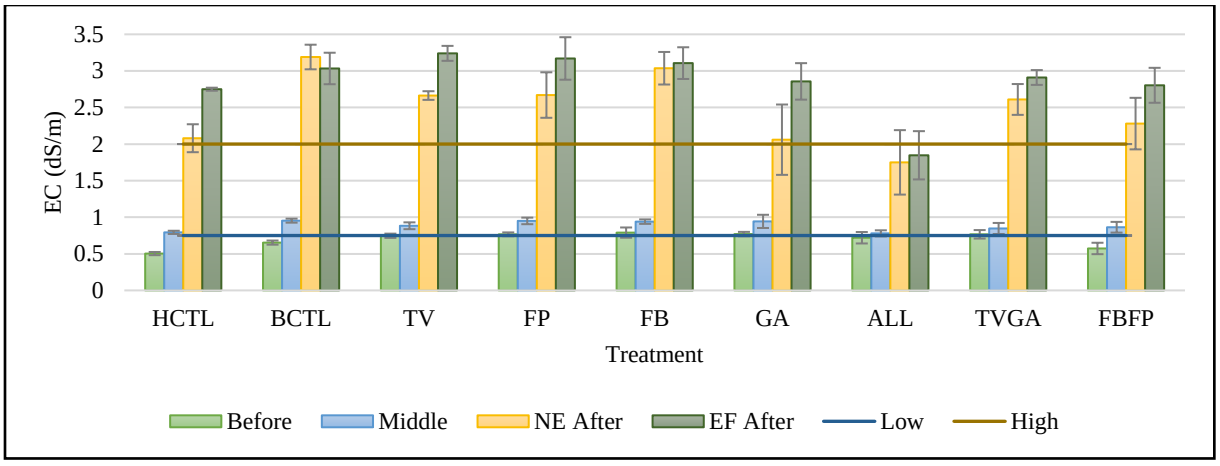


Figure 4-18 Electrical conductivity (EC) of compost treatments ± SE "before" indicating the initial product at the beginning of the experiment, "middle" indicating samples taken before *E. fetida* was added, and "Ef+ After" and "Ef- After" indicating the treatments with and without worms after composting was complete. Optimal range indicated between high and low lines (A&L Laboratories, 2004; Gómez-Brandón et al., 2008). Treatment abbreviations as stated in Fig. 4-6.

Another potential cause of the low GI in Ef- treatments is the plant species used, Rudolf organic radish (*Raphanus sativus*), which was used for the germination test grows best at a pH of 6.0-7.0. This plant species was chosen due to the abundance of its use in previous studies, showing that it germinates quickly and has been a good indicator of phytotoxicity(Luo et al., 2018). As shown in **Figure 4-19** pH decreased in all treatments to below 6.0 except for the treatment with all 4 fungal species. Adding some fly-ash to the compost could help raise the pH in future studies. This slightly more acidic pH may have influenced the germination of seeds. There was a significant difference between the pH of the treatments, and the pH of the controls used in the germination tests ($p = 0.000$), except for the treatment with all 4 fungal species. A three-way ANOVA was performed (SPSS) and a significant effect was observed for worm

presence or absence ($p = 0.002$), time ($p = 0.000$), and fungal treatment ($p = 0.000$). There was also a significant effect of time on treatments ($p = 0.005$) however, no significant effect was observed between worm presence and fungal treatments nor the three factors (worm, fungi, time) together ($p > 0.554$). Correlation analysis was performed on Excel and there was a positive correlation between pH and GI of 0.02 in both Ef+ and Ef- treatments indicating that composts with a higher pH had higher germination rates. A more pH tolerant plant may improve the GI significantly. A pH range of 6.0 – 7.5 is recommended for bacteria, 5.5 – 8.0 for fungi, and 5.0 – 9.0 for actinomycetes (Gómez-Brandón et al., 2008). The lowering pH of the compost over time may have reduced the activity and abundance of microorganisms, particularly bacteria, required to complete the composting process. Fang et al. (2018) hypothesized that a reduction in pH may be due to the production of organic acids by fungi as they degrade lignocellulosic materials, based on the findings of Chen et al. (1983) however further research must be done to determine the accuracy of this theory. Other studies have also observed a decrease in pH during composting and as such, a potential solution to the acidity in the PMB compost could be to adjust the pH of the initial product. Raw PMB had an initial pH of 6.8 making it already slightly acidic, however, the raw bark had a pH of 4.9 – 5.0 reducing the overall pH of the substrate and making it quite acidic before composting. Due to the high C:N and low pH of the bark, further research should be done to determine the success rate of composting PMB on its own.

A repeated measure ANOVA was conducted with sphericity assumed ($p > 0.05$) showed that pH differed significantly over time in both Ef- and Ef+ treatments ($F(2,36) = 261.21$, $p = 0.000$, $F(2, 36) = 591.38$, $p = 0.000$, respectively). A post-hoc test using the Bonferroni correction revealed that the pH reduced by 0.389 in the first 20 days and another 0.522 and 0.648 between days 20 and 96 in Ef- and Ef+ treatments respectively. A post-hoc Tukey HSD was

performed between treatments and there was only a significant difference between the ALL treatment (ALL > others) and all other treatments except HCTL in the Ef+ group ($p = 0.250$) and between ALL > *F. betulina* in the Ef- group ($p = .025$).

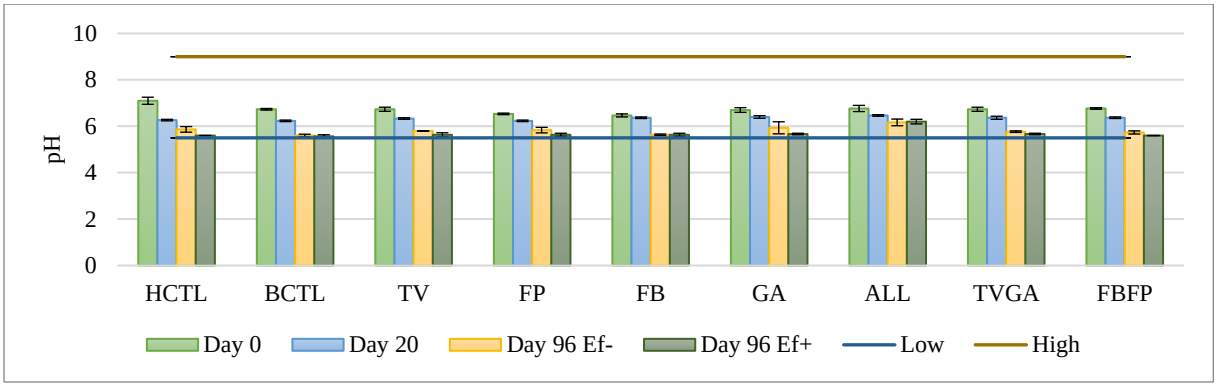


Figure 4-19 Mean pH changes over time ± SE of each compost treatment. "Before" indicating the initial product at the beginning of the experiment, "middle" indicating samples taken before *E. fetida* was added, and "Ef+ After" and "Ef- After" indicating the treatments with and without worms after composting was complete, recommended pH range indicated by lines (A&L Laboratories, 2004). Treatment abbreviations as stated in Fig. 4-6.

A decrease in pH was observed in all treatments with most of the Ef+ treatments finishing more acidic than Ef- treatments except for the treatment with all 4 fungi in which the Ef+ had a slightly higher pH and the *F. betulina* treatment was equal. Boruah et al. (2019) observed pH decreases with vermicomposting and reported that earthworms can neutralize substrate and that the pH decreases in vermicomposting are likely due to the production of CO_2 and NO_3^-, and the mineralization of P and N. This could explain the pH changes observed.

4.6 Compost maturity of the lab experiment

Temperatures in all lab experiments never exceeded 41 °C, as given in **Figure 4-5**, and therefore never reached the thermophilic stage in which pathogens and weed seeds are destroyed. A reason for this lower temperature could be that the bins were not large enough to facilitate the microbial activity needed to increase to the desired temperatures or inadequate insulation material. Goyal et al., (2005) composted 10 kg of different mixtures and found the temperature only increased to a maximum of 46 °C in one treatment. Similarly, the 8 kg mixtures only reached a maximum temperature of 41 °C indicating a relationship between the amount of material and temperature achieved. The University of Arkansas Division of Agriculture Research and Extension service suggests compost bins should be at least one cubic yard (0.765 m^3 = 765 L) for thermophilic composting so the 26 L lab experiments did not reach this limit (University of Arkansas, n.d.). The PMB and bark may not have had adequate diversity of degradation microorganisms because of the water treatment process it had undergone before use. The microorganisms used in the water treatment are adapted for living in water while the microorganisms needed for composting are soil-dwelling this could have made it difficult for the existing microorganisms to properly degrade. Further research on the microbial community of the PMB should be conducted to determine the existing ability to degrade and determine which, if any, microorganisms should be added to assist in degradation.

Another indication of incomplete composting is the C:N. There was little change in the C:N between the beginning and end of the experiment indicating that complete composting may not have occurred as given in **Figure 4-6**. Though N was added to all treatments to reach the initial C:N, inadequate microbial activity appears to have inhibited the reduction in C expected in successful composting. A C:N of mature compost should be at least less than 20 but a C:N of

less than 10 is ideal (Bernal et al., 2009). Other studies have had success mixing PMB with other N-rich substrates such as dairy sludge or cattle manure (Elvira et al., 1998; Hazarika & Khwairakpam, 2018). An addition of a similar N-rich substrate to PMB may lead to a more mature compost.

The mean GI was >60% for all of the Ef+ treatments and 33.3% for Ef- treatments. GI data is shown in **Figure 4-17**. Further field trials with the compost should be performed to determine the phytotoxicity of the final Ef- compost. Due to time constraints, the compost could not be left longer to determine if a more stable product would develop over time. Based on the data obtained from this experiment it is hypothesized that PMB has the potential to become a stable compost with a few changes to the methods. Some method changes may include larger scale compost trials to reach the minimum pile or bin size, removing the bark from the compost, adding some fly-ash from CBPPL to increase the pH and make a more suitable compost, and performing large scale trials with vermicomposting to determine the effect of *E. fetida* on large scale composting.

Ef+ treatments resulted in darker, homogeneous, granular textured compost compared to Ef- treatments which were drier and chunkier. These differences in texture were also reported in a study comparing thermophilic and vermicomposting processes where vermicompost had a reported higher water content, lower air volume, and higher water holding capacity than thermophilic compost (Fornes et al., 2012). Thermophilic composting depends on microorganisms and subsequent temperature increases to perform the degradation and sanitation of the substrate while vermicomposting utilizes the worms digestion and requires higher humidity and lower temperatures (Fornes et al., 2012). For best results, the combination of

thermophilic composting with vermicomposting should ensure that temperatures rise to at least 55 °C for pathogen elimination before the worms are introduced (Fornes et al., 2012).

4.7 Phospholipid fatty acid (PLFA) analysis results

Due to unforeseen circumstances related to the Covid-19 pandemic, the PLFA analysis of the compost both during and after composting was unsuccessful. Results from the PLFA analysis before composting indicate that biomarkers for fungi and gram-negative bacteria were present in the substrate, however without the analysis of sample after there is no way to determine the maturity based on the microbial evolution of the compost at this time. Overall average concentrations of PLFAs present are shown in the two graphs of **Figure 4-20**. The high variation between and within treatments is shown, a MANOVA performed indicated that there was no significant effect of fungal treatment on the PLFAs (Pillai's Trace $p > 0.05$, Wilk's Lambda $p > 0.05$). These results may not be an accurate representation of the PLFA biomarkers present in the compost.

A study was performed on different storage conditions of PLFA samples throughout the analysis process and it was found that samples, should be immediately lyophilized and stored at -80 °C (Veum et al., 2019). Samples that were stored at room temperature, like my samples, showed significant loss of PLFA biomarkers which affected the overall ratios of microbial biomarkers in the samples (Veum et al., 2019). Particularly gram-negative bacteria and fungi were significantly affected by the storage handling procedures (Veum et al., 2019). Fungi biomarkers 16:1w5cis and 18:2w6,9cis have shown a 13-55% decline in samples stored at room temperature and oven-dried samples saw an 86% reduction in fungal biomarkers (Veum et al., 2019). Total PLFA biomarkers were reduced by 11-16% in room temperature storage and by

38% during oven drying (Veum et al., 2019). Based on the method performed there could have been significant degradation of PLFAs in the samples from these compost trials due to storage in a -20 °C freezer rather than a -80 °C freezer, and once samples were lyophilized they were stored at room temperature, unknowingly degrading the PLFA biomarkers. Further, due to limited lab access due to the COVID19 pandemic processed samples were run on the GC-MS in a large batch that sat at room temperature over the weekend and likely would have degraded a significant portion of the desired biomarkers. Also due to the pandemic, I was not able to obtain the bacterial acid methyl ester (BAME) standard in time for analysis and therefore did not see a full range of PLFA biomarkers as desired. It is hypothesized that the PLFA biomarkers found in this study are not accurate representations of the microbial community in the compost. Further research should be performed with small batches of samples stored at -80 °C throughout the process to determine an accurate representation of the microbial biomarkers present.

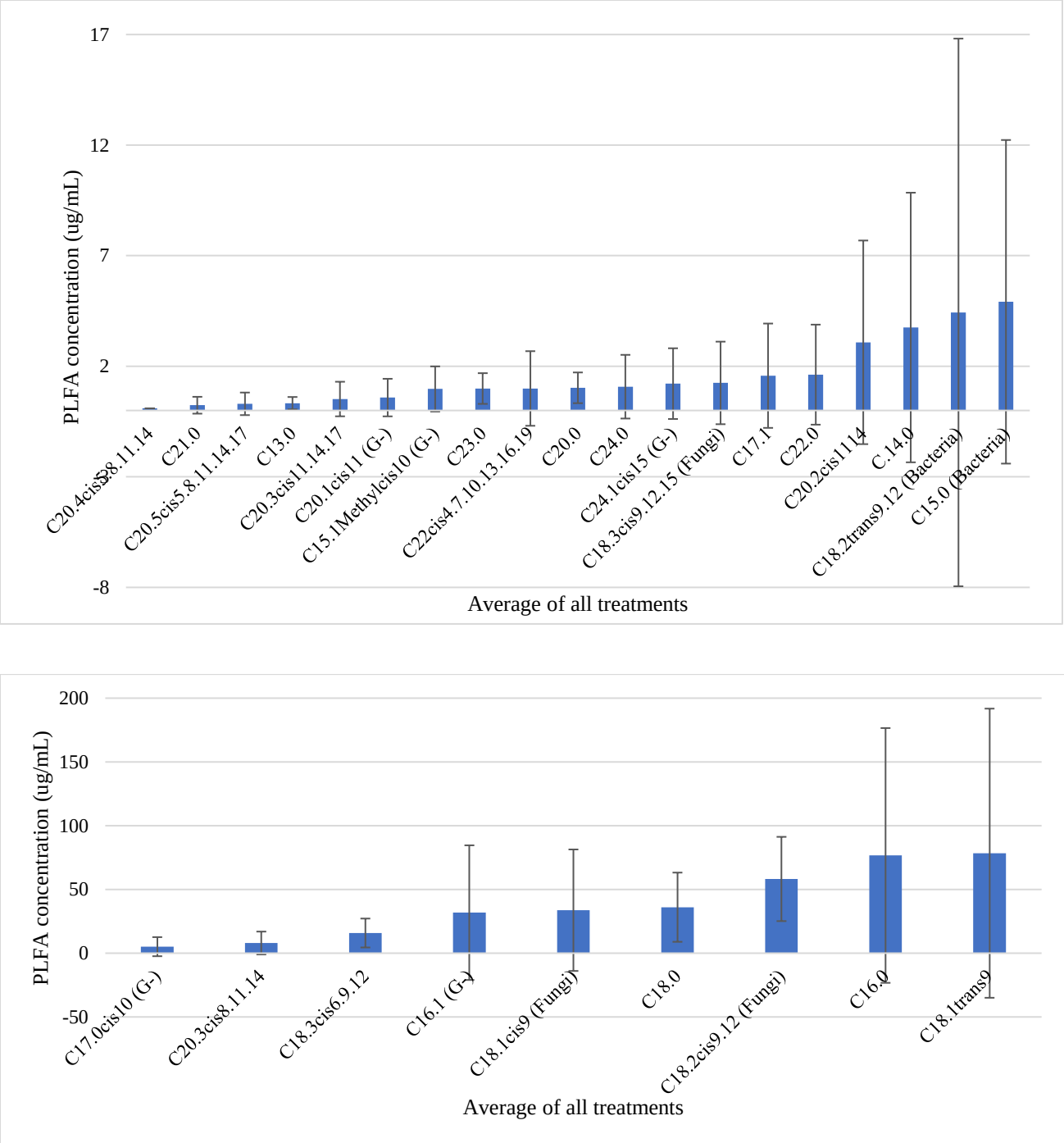


Figure 4-20 Mean PLFA values ± SE in µg/mL of all treatments separated into two graphs for lower and higher concentrations. PLFAs that are biomarkers for microorganisms are noted in parentheses.

4.8 Overall health of *E. fetida* in the lab experiment

Due to the migratory nature of *E. fetida*, the worms migrated to the Ef- treatments despite the plastic barrier taped in place. Upon visual observation of the compost, it was evident that most worms stayed on the Ef+ side until the material was fully degraded and then migrated over to the Ef- side in search of more food. Earthworms often show collective movement when habitats are no longer favourable, particularly O_2 levels, and populations will seek out new environments (Zirbes et al., 2010). *E. fetida* commonly show avoidance behaviour when undesirable conditions are present in the soil. *E. fetida* have shown avoidance behaviour in soils with an EC of 1.03 dS m^{-1}or higher and the EC in all treatments was higher than this threshold (Owojori & Reinecke, 2009). With the Ef+ side of the bins having higher EC and wetter denser consistency than the Ef- side, it is hypothesized that worms migrated to the Ef- side in search of a less saline and possibly more O_2 rich habitat. There was an easily distinguishable difference in the appearance of compost with and without worms due to the texture of the substrate. Successful vermicomposting results in a more homogeneous product and this was achieved in this experiment (Hait & Tare, 2011). When the analysis of worm health was conducted, many of the adult worms had moved over to the Ef- side and on average the Ef- side had higher biomass of adult worms but lower biomass of juvenile worms and cocoons than the Ef+ side as shown in **Figure 4-21**. The Ef- side was also visibly less degraded than the Ef+ side often appearing dryer. Ef- treatments had an average moisture content of 72-75% and a coarser texture. The Ef+ side was visibly moist and fine-textured, with a slightly higher average moisture content of 75-77% appearing almost like mud as shown in **Figure 4-22**. Vermicomposting has been described as a more efficient way of recycling organic nutrients and a more cost-effective solution compared to thermophilic composting of organic sludges (Hait & Tare, 2011).

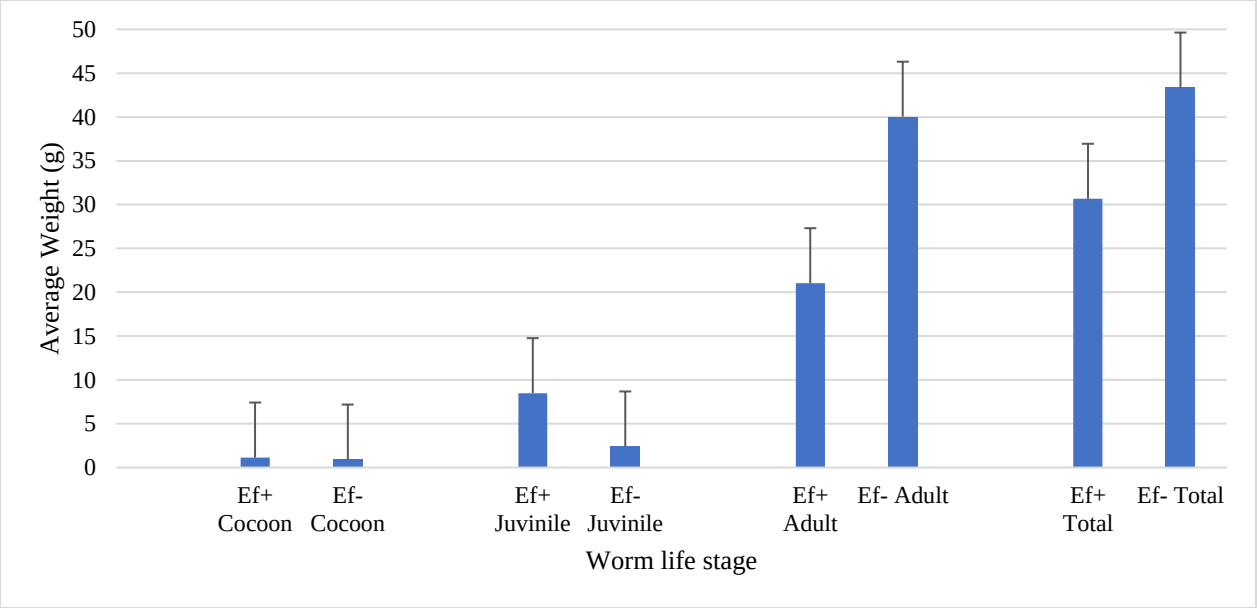


Figure 4-21 Mean abundance of *E. fetida* life stages ± SE, and combined total, as found in Ef+ and Ef- treatments after compost completion.

Figure 4-22 Texture difference between the side of a compost bin colonized Ef+ (left) and Ef- (right) at the end of composting.

4.9 Compost analysis and maturity of the outdoor trial

The piles made with bark and PMB outdoors were larger than the recommended size of 0.765 m^3 and temperatures still did not significantly surpass air temperatures nor reach the thermophilic stage, with the highest temperature recorded being 41.67 °C (**Figure 4-23**) four days after starting. Temperatures peaked on day 5, showing the biggest difference between the pile and air temperature, and decreased until the onset of winter when records were switched to thermographs shown in **Figure 4-24**. As shown in **Figure 4-24** some thermographs malfunctioned, no reading was received from pile 6 and only partial readings were available for piles 2, 4, and 5. We can assume that all piles followed a similar temperature pattern as piles 1 and 3 because there was no significant temperature variation between piles before thermographs were placed nor in lab trials. The lack of temperature increase in the large piles could indicate an inadequate supply of nutrients, available C, and microorganisms to attain thermophilic conditions. Lack of temperature increase could also be related to the cold air temperatures since the outdoor trial was delayed until the fall and winter months. Seasonal variations affect the microbial community in soil and can affect the abundance and diversity of N_2O producing microorganisms over winter months (Tatti et al., 2014). Freeze-thaw events in the fall and spring can lead to the establishment of cold-resistant microorganisms (Tatti et al., 2014). Mixing with a front loader may be an insufficient method of homogenizing and piles mixed this way have previously required a longer time to compost fully, and therefore our piles may have reached maturity had time been allowed (Evanylo & Daniels, 1999). Further research must be done to determine the missing elements of the PMB required for proper thermophilic composting and the microbial dynamics of overwintering PMB. An outdoor compost trial in the summer months may produce different results.

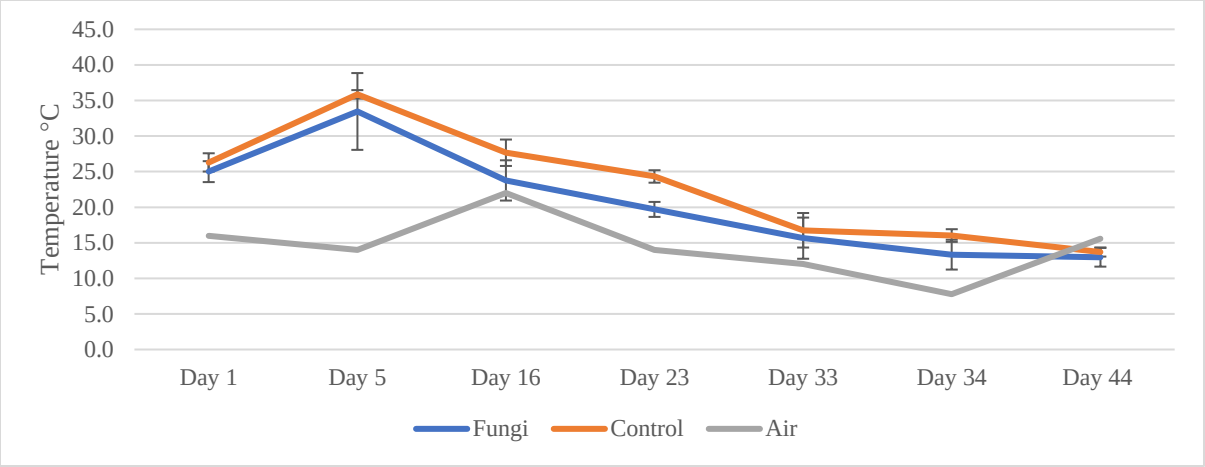


Figure 4-23 Temperature variation ± SE in outdoor compost piles during the first 44 days of field experiment and outdoor temperature.

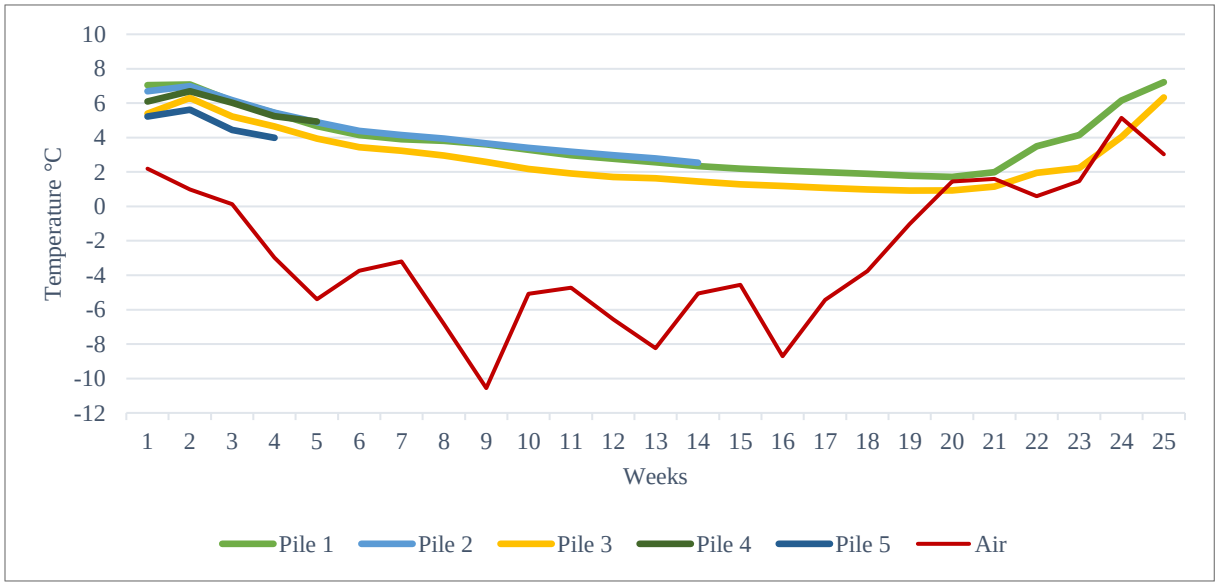


Figure 4-24 Outdoor compost piles internal temperature variation from thermograph readings beginning in late November (week 1) about 3 months after starting the experiment and ending in mid-May (week 25).

Compost nutrient levels were tested with a two-tailed t-test for significant difference between means, before and after composting, with a null hypothesis that there was no difference

between the means at a confidence interval of 95%, and an alternate hypothesis that there is a significant difference between the means before and after composting. C:N began higher than the recommended value and both treatments lowered to an average of just below 40 showing insufficient carbon degradation as shown in **Figure 4-25**. The difference between C:N before and after composting was not significant and had a p-value of 0.22. Nutrient data is displayed in **Figure 4-26** to **Figure 4-29** with micronutrients displayed in **Table 4-1**. Mean P (**Figure 4-26**) values remained within the recommended levels, both treatments showed a slight increase over time and there was a significant difference between the P % before and after composting with a p-value of 0.008. Mean K (**Figure 4-27**) values were below the recommended levels and showed little change from start to finish, showing no significant difference in means before and after with a p-value of 0.53. High Ca levels, shown in

Figure 4-28, in the outdoor trials indicate a difference in the composition of these compost piles and the lab trial material. The mean Ca (**Figure 4-28**) level before was 6.6 % and after composting the mean was 7.5 % with the difference between the means not significant from a t-test result with a P-value > 0.05, therefore, accepting the null hypothesis that there is no significant difference between the means before and after composting. Ca levels were over the recommended limit throughout the composting process for both treatments. There was a high variability of Ca in all piles and a t-test found no significant difference between the means before and after composting, with a p-value of 0.13. Mean Mg levels (**Figure 4-29**) remained between the recommended levels both treatments showing an increase from start to finish but the control finishing with higher levels overall. There was variability between piles before and after, and there was no statistically significant difference between the means before and after composting, with a p-value of 0.27.

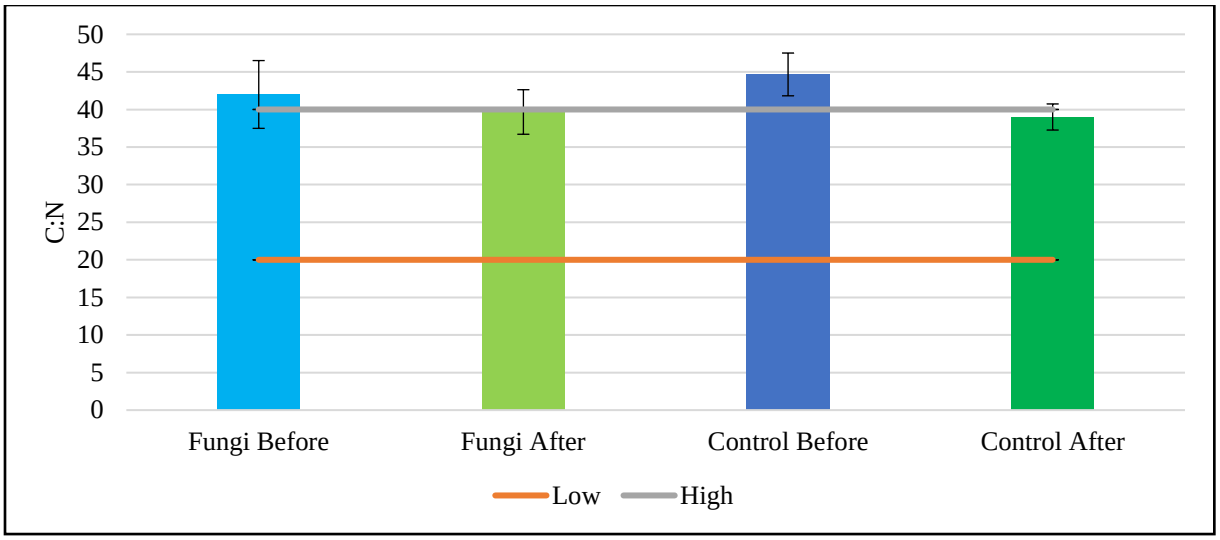


Figure 4-25 Mean C:N levels ± SE of outdoor compost piles. Optimal range indicated between high and low lines. Fungi treatment with 4 species, *T. versicolor, G. applanatum, F. pinicola, F. betulina,* control with no additives.

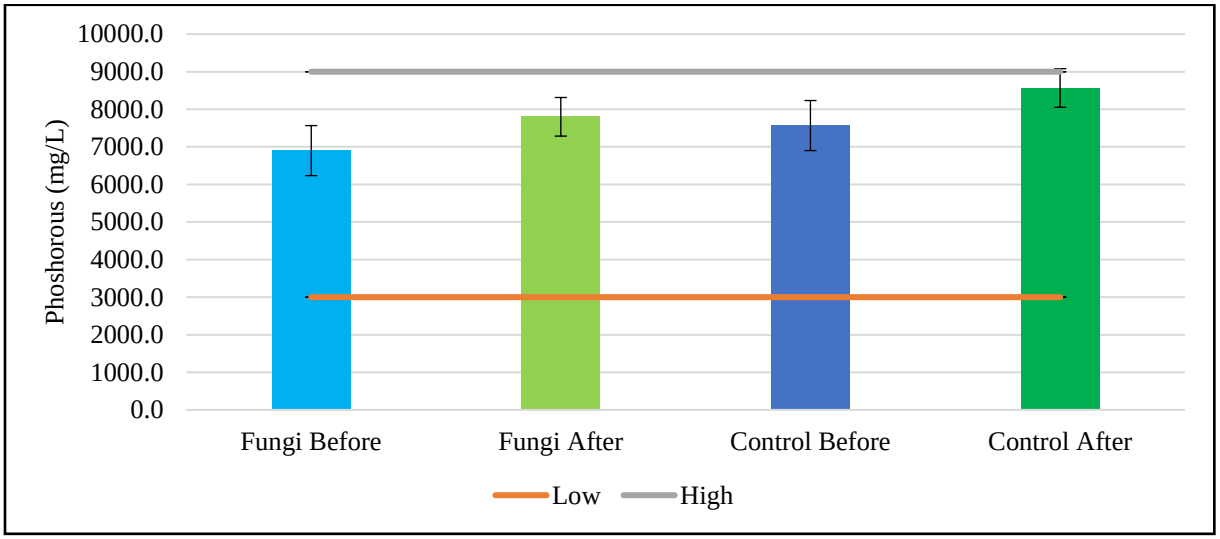


Figure 4-26 Mean P values ± SE of outdoor compost piles before and after composting of both fungi and control treatments. Optimal range indicated by the high and low lines. Treatments as stated in Fig 4-25.

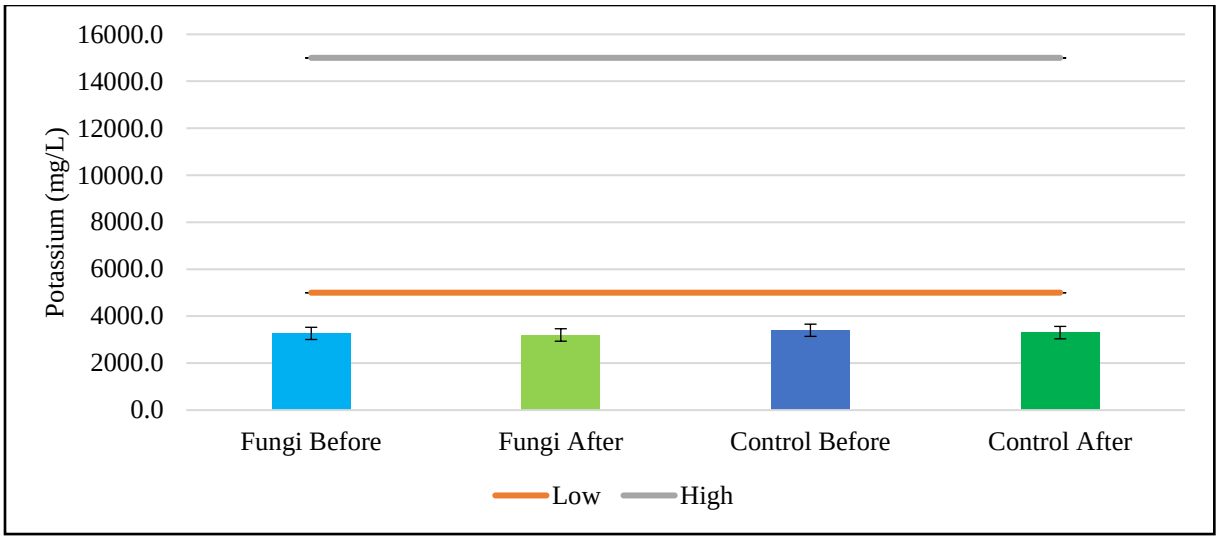


Figure 4-27 Mean K levels ± SE of outdoor compost piles with the optimal range indicated between high and low lines. Treatments as stated in Fig. 4-25.

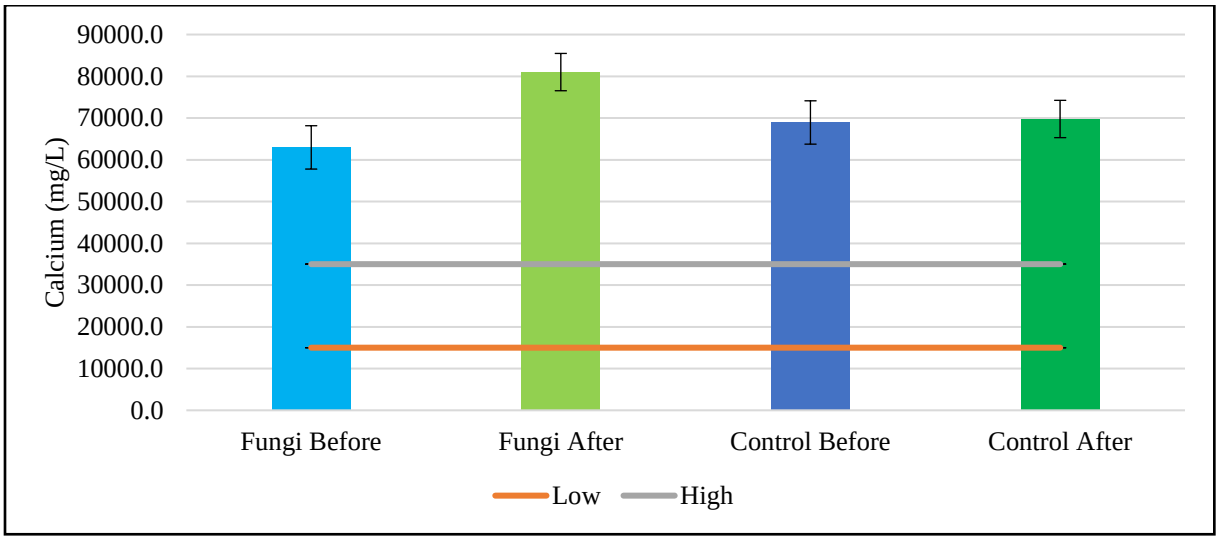


Figure 4-28 Mean Ca levels ± SE of outdoor compost piles before and after composting, with optimal range indicated between high and low lines. Treatments as stated in Fig. 4-25.

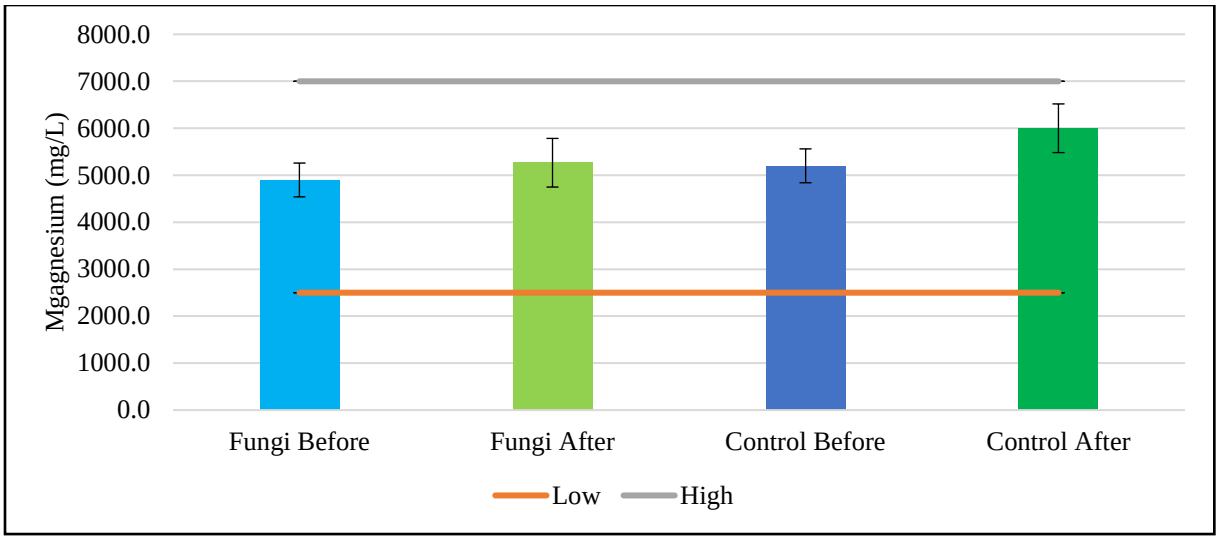


Figure 4-29 Mean Mg levels ± SE of outdoor compost piles before and after composting, with optimal range indicated by high and low lines. Treatments as stated in Fig. 4-25.

Table 4-1 Micronutrient mean values (mg/L) of outdoor compost piles before and after addition of fungi to PMB. Control measurements are shown for comparison. Treatments as stated in Fig. 4-25.

	Fungi Before	Fungi After	Control Before	Control After
Fe	3043.3	3306.7	3280.0	3360.0
Cu	51.0	58.0	54.3	61.7
Mn	3083.3	4200.0	3350.0	4620.0
Zn	493.0	566.3	536.0	621.7
B	65.0	68.0	68.0	73.0
Na	593.0	550.7	602.0	519.0

All nutrients were present at acceptable levels for compost. There was a statistically significant difference in the means before and after of Cu, Mn, Zn, and Na with p-values < 0.05, while Fe and B had no significant difference with p-values > 0.05. Despite attempts to decrease the C:N with the addition of urea fertilizer the outdoor piles began with C:N higher than the recommended starting rate for composts of 20 to 30 as given in

Figure 4-25. Due to delays in the setup of the outdoor trials, and the estimation of the pile size and weight, the amounts of urea added to the piles to reach the intended target C:N were underestimated and the C:N did not start at the recommended level. There was no significant difference between the means before and after as determined by a t-test with the p-value > 0.05.

Similar to my experiment, the study by Gong et al., (2017) added urea to adjust the C:N and water to adjust the moisture content, however, the authors also reported adding brown sugar to promote the growth of microorganisms, and fungal inoculation was done with cultures in potato dextrose broth twice throughout the experiment, on days 0 and 14 (Gong et al., 2017). The brown sugar may have been the missing ingredient for our compost as it would provide more available C. These changes may indicate reasons why my experiment did not reach the thermophilic stage and maturity levels of composting (Gong et al., 2017). The authors reported a steady decrease in C:N, an increase to >80 % GI in all treatments, significant lignin and cellulose degradation in fungal inoculated treatments when compared to the control (Gong et al., 2017). While my data shows nutrient increases, my GI and fibre analyses were not corresponding to those of Gong et al. (2017).

The thermograph for pile 6 failed and piles 2, 4, and 5 stopped recording part way through the winter as indicated by their lines ending in **Figure 4-24**. Temperatures did not drop below 0 °C despite air temperatures in Corner Brook dropping below 0 °C for significant portions of the winter months (Environment Canada, 2020).

4.10 Outdoor pH and EC

EC values remained between recommended levels both treatments showing an increase from start to finish and piles 1 and 2 have higher values than the other piles(A&L Laboratories, 2004). Piles 1 – 6 followed similar trends before and after with all after values being higher than before. The mean EC before composting was 0.92 dS/m with a variance of 0.07 and after composting mean EC was 1.47 dS/m with a variance of 0.10 as given in **Figure 4-30**. A t-test was performed indicating that the difference in the means before and after is significant with a p-value < 0.05 and the t value < t critical in both one and two-tail tests. EC levels in the outdoor trial did not exceed 2.5 and therefore did not reach the high levels seen in the indoor trials. This could be due to the lack of worms in the outdoor trials or could be due to native microorganisms in the soil and air in the outdoor environment.

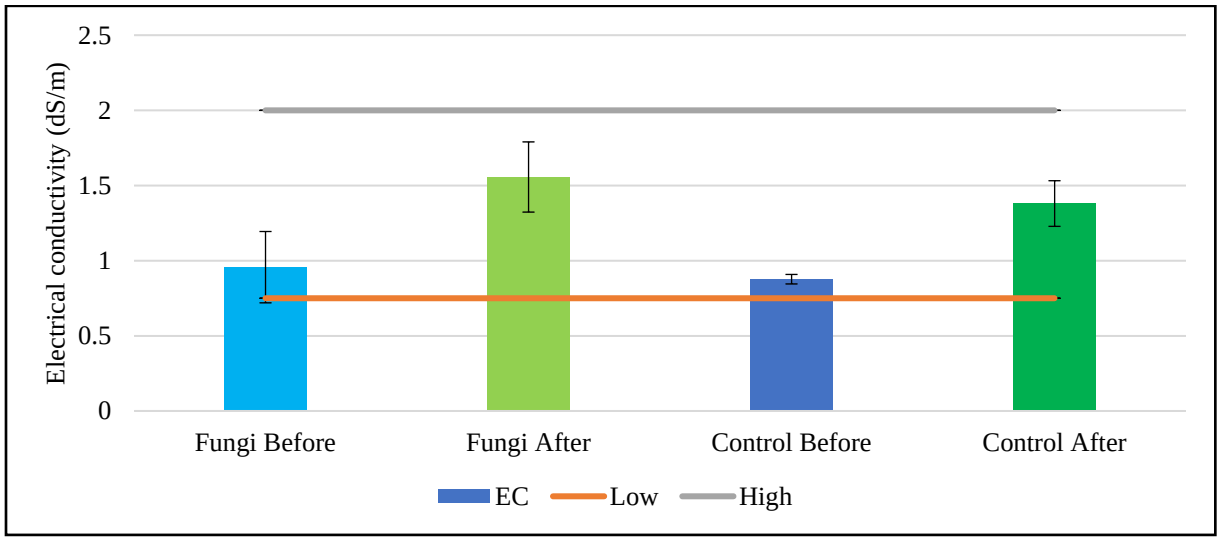


Figure 4-30 Mean electrical conductivity values ± SE of outdoor compost piles before and after composting, with optimal range indicated by high and low lines. Treatments as stated in Fig. 4-25.

There is a significant difference between the pH before and after composting in the outdoor trial as given in **Figure 4-31**. pH values began within the appropriate range for compost but both treatments pH lowered to a slightly acidic pH which may have negatively affected the composting process(A&L Laboratories, 2004). Piles 1 and 2 had lower pH than the other piles and pH lowered for all treatments over time. With the mean pH before at 6.9 with a variance of 0.12 and pH after at 6.3 with a variance of 0.05. A t-test revealed that the t stat of 4.47 is larger than the two-tailed t critical of 2.57 with a p-value of 0.006 indicating a rejection of the null hypothesis that there is no significant difference between pH before and after. As stated in the lab experiment section pH is expected to decrease during the composting process and the piles remained at a near-neutral pH between 6 and 7, slightly less acidic than the lab trials.

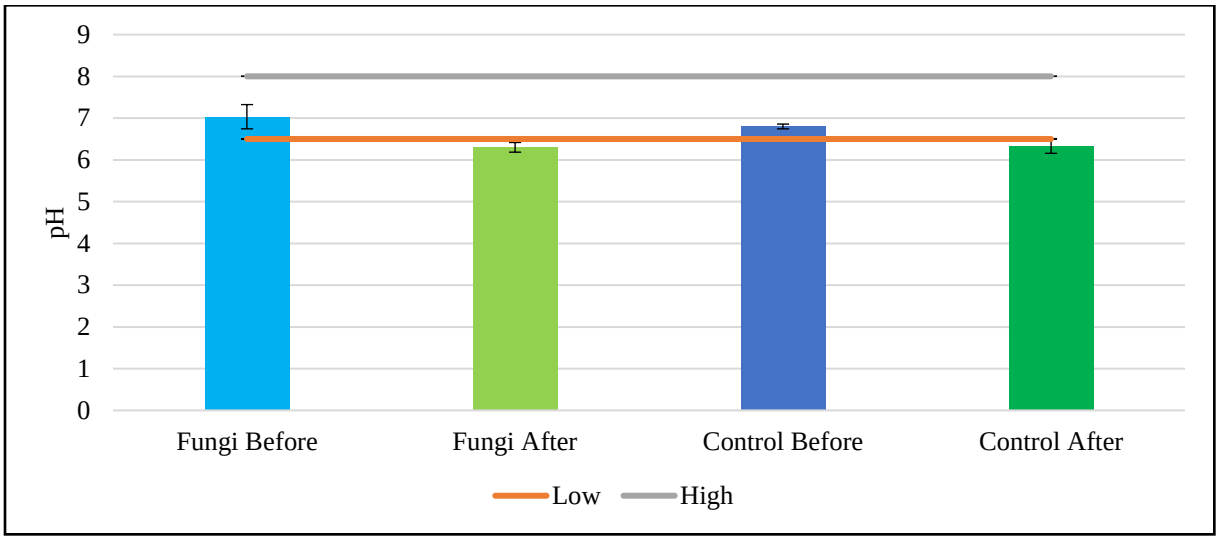
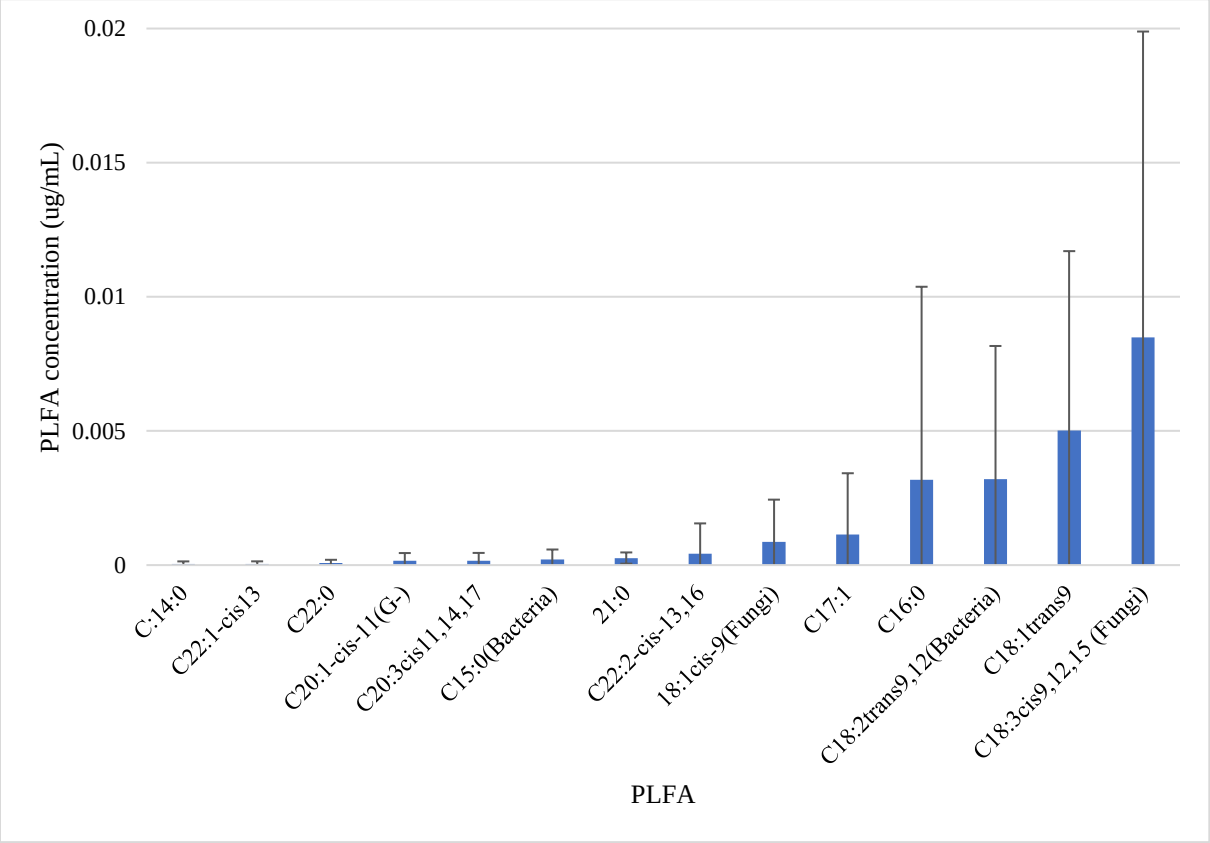
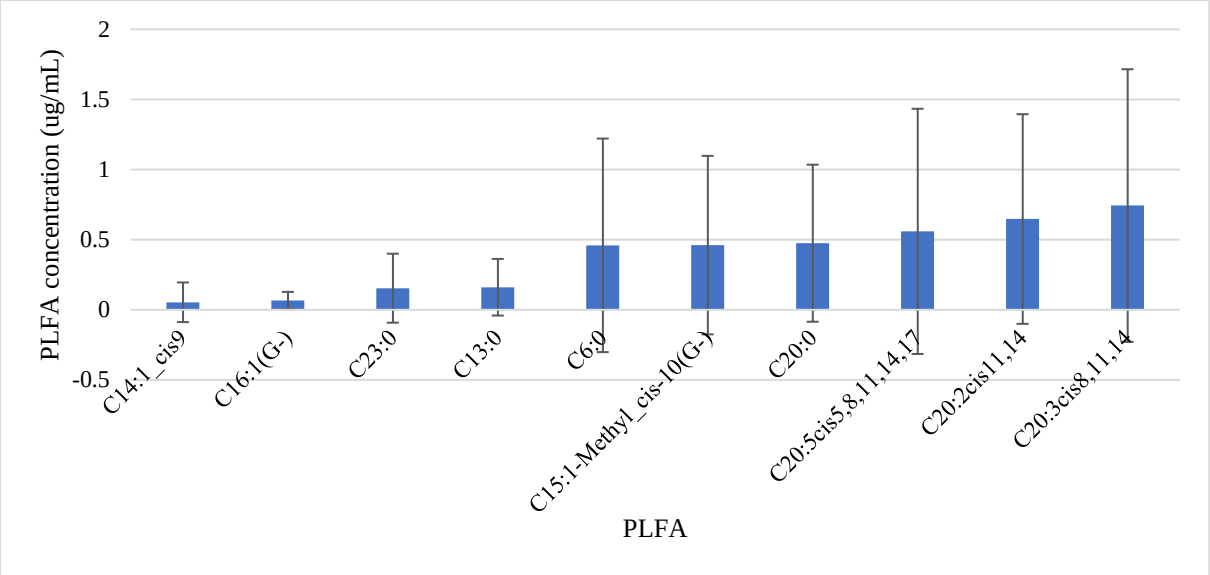


Figure 4-31 Mean pH levels ± SE of outdoor compost piles before and after composting, with the optimal range indicated by high and low lines. Treatments as stated in Fig. 4-25.

4.11 Outdoor PLFA

As with the lab trial results, PLFA results are limited to the initial before values. There was no significant difference between the PLFA of treatments with the fungal consortium and the control ($p > 0.05$). Results are shown in 3 graphs divided by concentrations for clearer depiction in **Figure 4-32**. These results may not be an accurate representation of the PLFA biomarker present in the compost. As described in the lab trial PLFA section, it is likely that improper storage and handling, partly due to the COVID19 pandemic, resulted in significant degradation of some PLFA biomarkers (Veum et al., 2019).





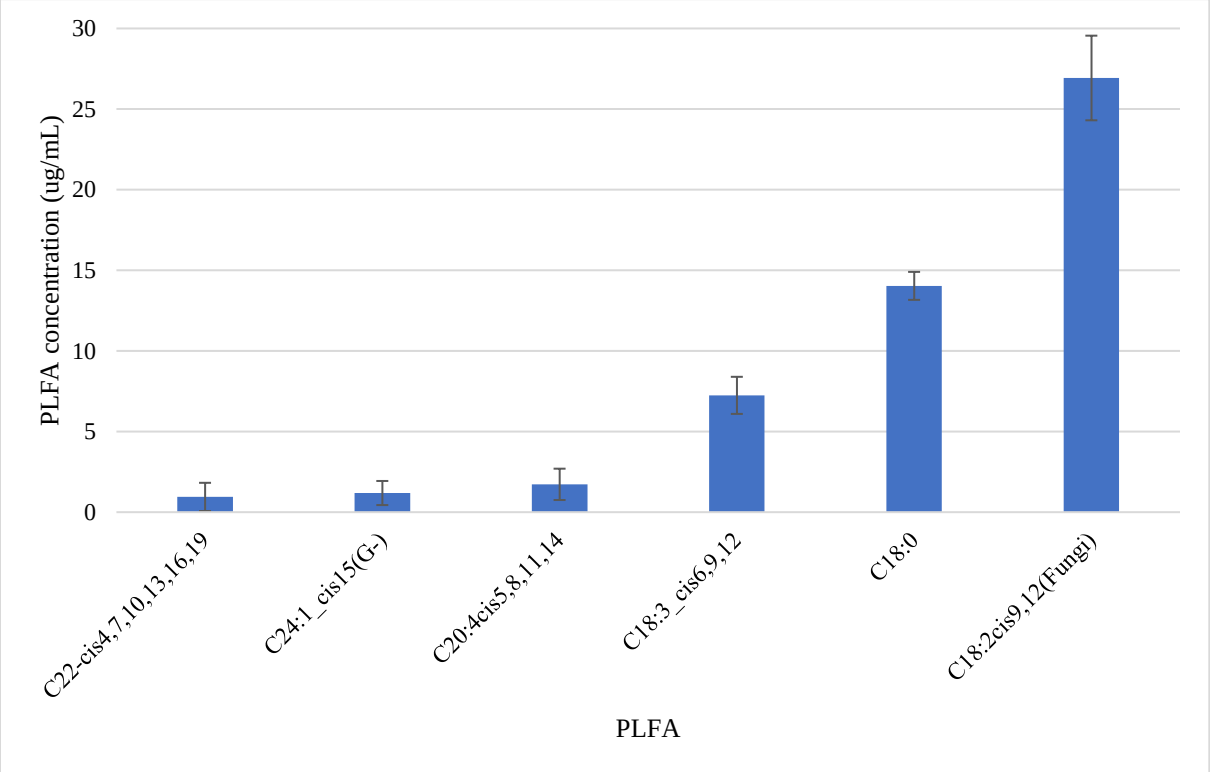


Figure 4-32 Mean PLFA profiles ± SE of outdoor compost trails, three graphs are displayed to include the wide range of concentrations found. PLFAs are displayed from smallest to largest concentrations in each graph.

4.12 Compost fibre analysis

Air dried samples from lab trials were sent to Bureau Veritas Laboratories for fibre analysis via acid detergent fibre (ADF) and acid detergent lignin (ADL). Lignin and cellulose were represented as a percentage of the total dry mass of each sample. Due to the high costs of the analysis, this was only performed for all 3 replicates of 4 of the Ef- treatments; HCTL, ALL, *T. versicolor & G. applanatum*, and *F. betulina & F. pinicola* as well as some raw PMB without additives for a total of 15 samples. Results of the ADL and were highly variable with 7 compost samples (1 HCTL, 3 ALL, 2 *T. versicolor & G. applanatum*, 1 *F. betulina & F. pinicola*) showing a lignin increase ranging from 1.8 % to 11.2 % and 5 samples (2 HCTL, 1 *T. versicolor & G. applanatum*, 2 *F. betulina & F. pinicola*) showing a decrease in lignin ranging from 0.3 % to 8.1 % after composting. Cellulose increased in 7 samples (2 HCTL, 2 *T. versicolor & G. applanatum*, 3 *F. betulina & F. pinicola*) with increases ranging from 0.7 % to 9.3 % and 5 samples (1 HCTL, 3 ALL, 1 *T. versicolor & G. applanatum*) reported a decrease in cellulose ranging from 0.6 % to 4.7 %. Differences before and after are shown for all treatments in **Figure 4-33**. The lignin in PMB ranged from 24.5 % to 37.6 %, cellulose ranged from 13.8 % to 32.0% and total fibre ranged from 51.4 % to 58.3 %. In the compost samples, total fibre before composting ranged from 55.4 % to 59.8 %, and after composting ranged from 58.3 % to 62.6 % indicating an increase in the proportions of ADF overall after composting. All ADF % increased from before to after composting except for treatment 2 HCTL. No further details were obtained from the lab concerning the apparent increases. Contrary to the hypothesis that the white-rot mushrooms would degrade lignin faster the 2 treatments with an average increase in lignin were those including white-rot species (ALL and *T. versicolor & G. applanatum*). Means were compared between pairs of treatments and between lignin before and after composting and all t-

tests failed to reject the null hypothesis that there was no significant difference between the means. The explanation for this may be that white-rot fungi degrade both lignin and cellulose and could have more vigorously degraded the cellulose leaving a larger proportion of lignin in the final product. Fang, et al (2018) reported that *T. versicolor* demonstrated a stronger degradation of cellulose than lignin which could explain the results found here. As described by Have & Teunissen (2001) white-rot fungi presumably degrade lignin of the cell wall to access the cellulose and hemicellulose inside the cell. It is reported that the production of lignin-degrading enzymes is highly dependent on the presence of H_2O_2 and O_2 which may be another explanation for the reduced degradation seen here (Have & Teunissen, 2001). The lignin: cellulose ratio is highly correlated with the hydrolysis of lignocellulose, a lower ratio results in higher levels of hydrolysis which increases biodegradability (Scharer & Moo-Young, 1979). The authors report that the lignin: cellulose ratio of wood in their study was 0.3 – 0.6 which had a hydrolysis rate of 0 – 40 %, while newsprint had a ratio of 0.34 – 0.43 and a hydrolysis rate of 23 – 37 % (Scharer & Moo-young, 1979). Lignin: cellulose ratios of the compost samples submitted ranged from 0.77 – 2.08 before composting with 7 samples increasing after composting and 5 samples decreasing. After composting lignin: cellulose ratios ranged from 1.11 – 1.35. Averages of these ratios show a decrease in the HCTL and *F. betulina & F. pinicola* treatments, indicating more lignin degradation while the ALL and *T. versicolor & G. applanatum* treatments increased, indicating more cellulose degradation, contrary to predictions. Raw PMB lignin: cellulose ratios ranged from 0.77 – 2.72 indicating considerable variability between samples. Both treatments show a decrease which may be used as indicators of maturity.Another composting study with green waste inoculated with *T. versicolor* and *P. chysosporium* reported an increase in lignin and cellulose degradation in both treatments compared to a control (Gong et al., 2017).

Although samples were taken from the side without *E. fetida* added the presence of worms is still likely in all treatments which may have affected the proportions of cellulose and lignin degraded in the lab trials. Biruntha et al., (2020) reported a 30 -50 % decrease in the cellulose: N ratio stating that the degradation of cellulose played a major role in the reduction of the C:N. In our study, all samples, except 3, had an increase in cellulose: N ratio (**Figure 4-34**). Biruntha et al., (2020) additionally reported that cellulose: lignin ratios remained within 1.0 indicating maturity (**Figure 4-34**). While our results before composting ranged from 0.48 to 1.29, and after composting the cellulose: lignin ratio was between 0.74 and 0.90 indicating degradation of cellulose and lignin. High variability is seen between lignin and cellulose amounts in different treatments.

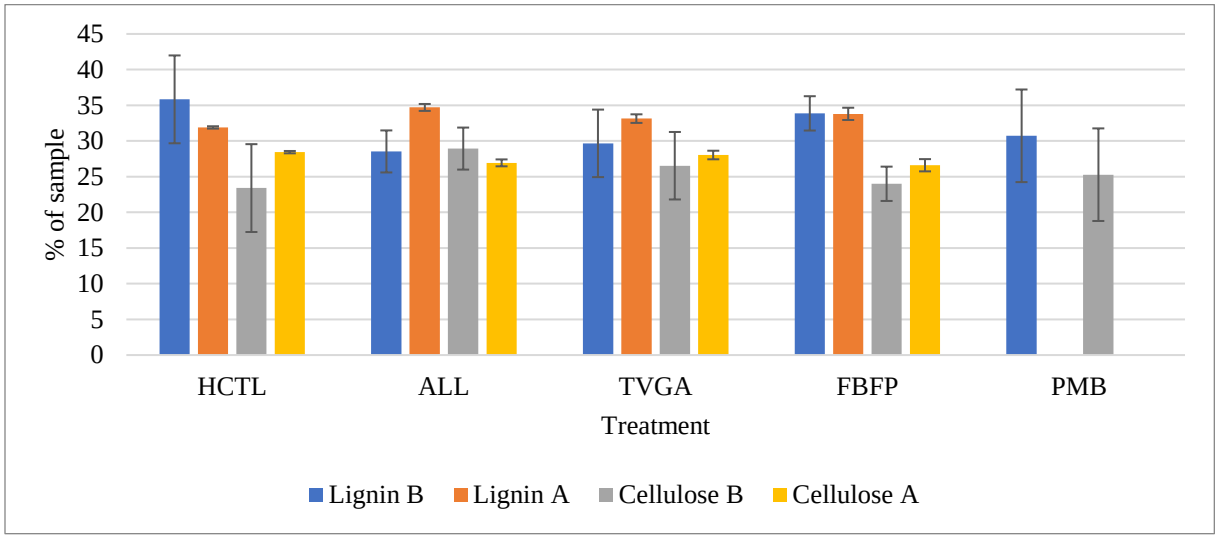


Figure 4-33 Mean Lignin and cellulose as a % of the total sample ± SE B = before composting, A = after composting. Treatment abbreviations as follows, HCTL = water control, ALL = fungal consortium of 4 species, *T. versicolor, G. applanatum, F. betulina* and *F. pinicola*, TVGA = *T. versicolor & G. applanatum*, FBFP = *F. betulina & F. pinicola*

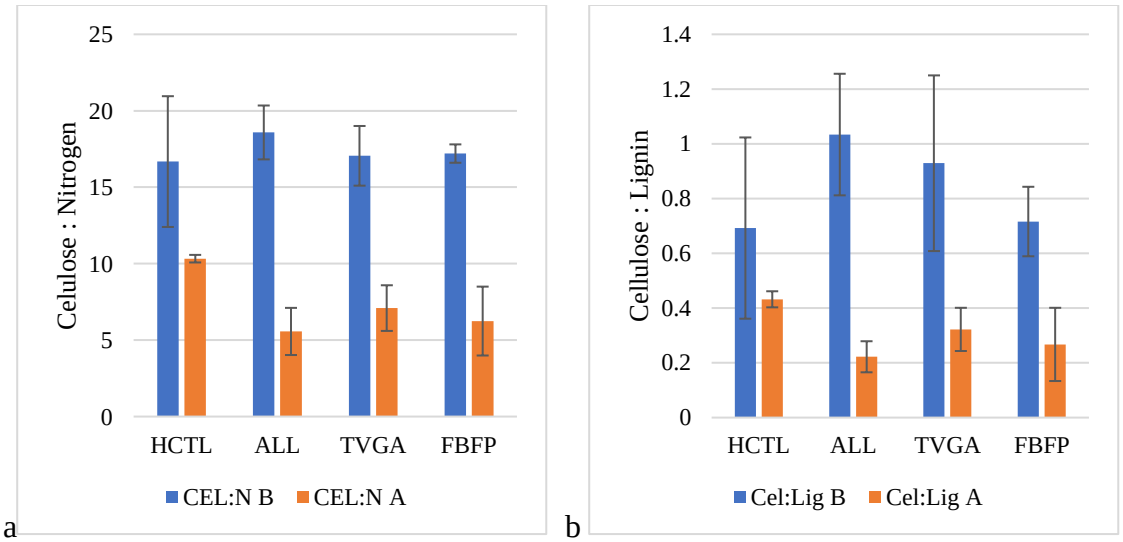


Figure 4-34 Mean Cellulose: Nitrogen ratio (a) and Cellulose: Lignin ratio (b) ± SE Treatment abbreviations as stated in Fig. 4-33.

4.13 ICP-MS analysis

All elements in the final compost were below the Canadian Council of Ministers of the Environment (CCME) guidelines for compost quality(CCME, 2005). Based on these results the compost can be safely used in agricultural, horticultural, and residential environments. Results of CCME regulated elements analyzed by ICP-MS are shown in **Table 4-2**. As hypothesized, none of the elements of concern were over CCME limits and most elements were well below the limits. For those elements with our a CCME limit, there are no regulations or thresholds. Al becomes toxic for plants in soils with a pH below 5.5 which could have affected the GI in those samples with a pH lower than 5.5 (Delhaize & Ryan, 1995).

Table 4-2 Mean (± standard error) elemental composition of composted PMB with and without *E. fetida* measured in mg/L by ICP-MS with CCME limits shown where applicable.

Element	Mean concentraion mg/L Ef+ Avg	Mean concentration mg/L Ef- Avg	CCME Limit mg/L
Ni	2.43 ± 0.53	1.01 ± 0.25	62
As	0.19 ± 0.02	0.11 ± 0.01	13
Pb	2.74 ± 0.42	1.35 ± 0.10	150
Se	0.19 ± 0.11	0.19 ± 0.12	2
Al	1086.55 ± 69.38	627.00 ± 42.46	
Rb	0.16 ± 0.01	0.12 ± 0.01	
Ga	41.37 ±2.44	31.03 ± 1.27	
Sr	17.07 ± 0.99	12.44 ± 0.45	
Co	0.61 ± 0.04	0.39 ± 0.02	34
Mo	0.53 ± 0.05	0.35 ± 0.04	5
Cd	0.51 ± 0.03	0.42 ± 0.02	3
Cs	0.00 ± 0.00	0.001 ± 0.00	
Ba	127.10 ± 7.22	94.16 ± 3.58	
Hg	0.51 ± 0.00	0.55 ± 0.01	0.8
U	0.40 ±0.02	0.29 ± 0.01	
Tl	0.18 ± 0.00	0.17 ± 0.001	
V	2.16 ± 0.12	1.30 ± 0.06	
Be	0.37 ± 0.03	0.34 ± 0.02	
Fe	2785.93 ± 92.10	2483.70 ± 0.01	
B	59.85 ± 1.51	59.59 ± 2.25	
Cu	44.93 ± 1.47	44.56 ± 1.66	400
Mn	6651.11 ± 293.02	4524.07 ± 147.19	R 3000 - 5000
Zn	472.52 ± 14.10	480.07 ± 17.63	700
Na	467.56 ± 15.46	458.11 ± 16.62	R 6000

4.14 Bioremediation experiment

Compost was effective at remediation of 10W30 motor oil from the soil with a positive relationship observed between the amount of compost added and oil degradation. While oil reduction was seen in all treatments, a significant reduction was observed in treatments with 10 and 20% compost added. After 3 months treatments with oil added at 5 % of soil dry weight showed a reduction in oil by 9.09 %, 16.45 %, and 19.92 % in treatments with 5%, 10 % and 20 % compost respectively, while no remediation was observed in the control. In treatments with 10 % oil added, a reduction of 3.73 %, 8.93 %, and 10.79 % was observed after 3 months, in 5%, 10 % and 20% compost respectively **Figure 4-35** shows a positive relationship is seen between oil remediated and compost amounts over time. With no oil remediated from the control with 0% compost while 10 and 20% compost treatments show the highest amount of oil remediated. **Figure 4-36** shows a positive relationship is seen between the amount of compost added and the amount of oil remediated, with 10 and 20 % compost remediating the most oil. Compost acts as a bio-stimulant in the soil, increasing the microorganisms that use petroleum hydrocarbons as a carbon source and break it down to less harmful compounds (Onwosi et al., 2017). Compost also provides more nutrients for these microorganisms to increase their activity, thereby increasing their consumption of the oil (Onwosi et al., 2017). Based on this data, the potential for using composted PMB from CBPPL for soil remediation on the island of Newfoundland is a good local solution. Results were evaluated with SPSS software and ANOVA was conducted for one, two, and three-way interactions. There was a significant decrease in mg/kg of oil with increasing compost concentrations ($p = 0.000$). A significant decrease in oil (mg/kg) was also observed between month 0 and month 1 (2.66 mg/kg, $p = 0.008$), month 2 (2.78 mg/kg, $p = 0.005$) and month 3 (4.10 mg/kg, $p = 0.000$). There was a statistically significant interaction between the

amount of compost and oil added (F = 27.907, p = 0.000) and between the amount of compost added and the month the sample was taken (F = 4.524, p = 0.000) however there was no significant interaction between the month and oil added (F = 1.243, p = 0.302). There was no significant three-way interaction between the month, compost, and oil added (F = 0.992, p = 0.456). A Tukey HSD test revealed that there was a significant difference between all mean levels of compost with mean differences ranging from 0.1200 mg to 0.3675 mg and a p-value of 0.0 for all, while the only significant difference between means of months was between month 1 and 3 with a mean difference of 0.0312 mg and a p-value of 0.01, all other months had p values above 0.05.

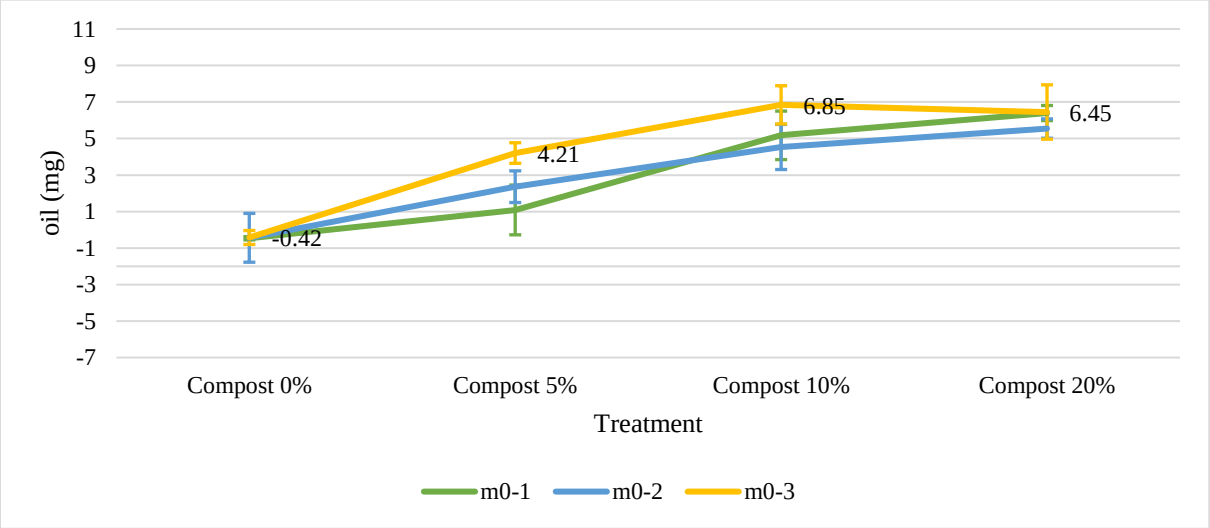


Figure 4-35 Amount of oil remediated ± SE from treatments with 5 % (10 g) oil added.

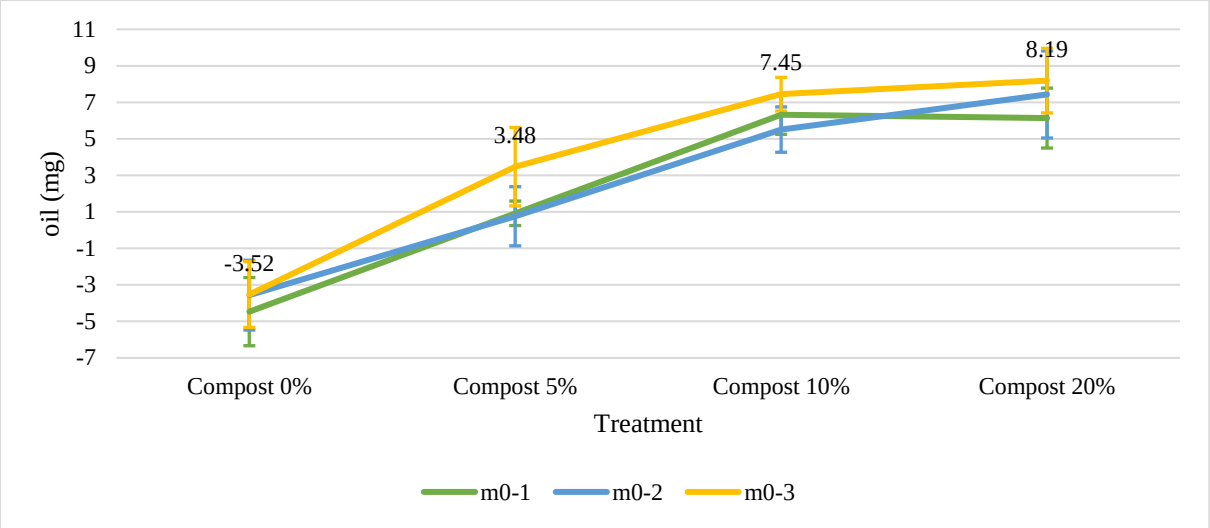


Figure 4-36 Amount of oil remediated ± SE from treatments with 10 % (20 g) oil added.

5.0 Conclusions and future recommendations

5.1 Compost potential of PMB

Though the compost trials performed in this work were not all considered mature, based on C:N, and GI, PMB showed potential for its ability to become a nutrient-rich compost under modified conditions. The potential of PMB alone as a compost substrate should be explored, as the C:N for PMB was ideal for composting before the addition of bark. Bioaugmentation should be considered to increase the microorganism population required for degrading the PMB completely, specific microorganisms could be isolated from existing piles of PMB on-site and may be effective at the degradation of PMB. PLFA analysis of raw PMB will increase the knowledge of existing microbial communities and that could lead to further bioaugmentation success. Further research should be performed with PMB on its own in a large-scale outdoor composting trial to determine the feasibility of this method on a large scale as required by CBPPL. The potential of PMB as a soil amendment in its raw form and its effects on plants need

to be explored. The results of this study indicate the safety of PMB with all contaminant levels below regulated thresholds. The potential of *E. fetida* to increase the rate of decomposition and produce a more homogeneous product was significant and future research should determine the feasibility of using *E. fetida* in large-scale outdoor trials in summer months. PMB shows potential for a successful compost and as a soil amendment on its own.

5.2 Remediation potential of PMB

This work has demonstrated the potential of composted PMB for remediating petroleum hydrocarbons from the soil. The maturity of the compost is likely not inhibitory to its remediation capabilities due to the immature nature of some of our final composted products and further research should be performed to determine if composted PMB could be used on a larger scale and for *in-situ* bioremediation of contaminated soil in NL. Further research should also be performed to determine if raw PMB has the same remediation capabilities as the composted mixture as this would remove the composting step and allow the raw PMB to be taken directly from CBPPL to the contaminated site. This potential use of PMB could create a secondary revenue stream for CBPPL especially if no composting preparation is required.

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Appendix

5.3 Appendix 1: Nutrient differences between treatments

Measure	Worm Treatment	Fungi Treatments	Mean Difference	P-value
N %	Ef-	*F. betulina* > *F. betulinaF. pinicola*	0.19	0.015
N %	Ef-	*F. betulina* > HCTL	0.2233	0.003
N %	Ef-	*G. applanatum* > *F. betulinaF. pinicola*	0.1867	0.006
N %	Ef-	*F. pinicola* > *F. betulinaF. pinicola*	0.1622	0.049
N %	Ef-	*G. applanatum* > HCTL	0.22	0.004
N %	Ef-	*T. versicolorG. applanatum* > HCTL	0.1667	0.04
N %	Ef+	ALL > HCTL	0.1533	0.02
N %	Ef+	BCTL > HCTL	0.1478	0.027
N %	Ef+	*F. betulina* > *F. betulinaF. pinicola*	0.1511	0.023
N %	Ef+	*F. betulina* > HCTL	0.2044	0.001
N %	Ef+	*G. applanatum* > *F. betulinaF. pinicola*	0.1707	0.006
N %	Ef+	*F. pinicola* > HCTL	0.1833	0.004
N %	Ef+	*G. applanatum* > HCTL	0.23	0
N %	Ef+	*T. versicolorG. applanatum* > HCTL	0.1778	0.005
N %	Ef+	*T. versicolor* > HCTL	0.1533	0.02
C:N	Ef-	HCTL > *F. betulina*	4.3444	0.014
C:N	Ef-	HCTL > *F. pinicola*	3.9222	0.032
C:N	Ef-	HCTL > *G. applanatum*	4.5667	0.009
C:N	Ef-	HCTL > *T. versicolorG. applanatum*	4.2444	0.017
C:N	Ef+	HCTL > *F. betulina*	3.9556	0.01
C:N	Ef+	*F. betulinaF. pinicola* > *G. applanatum*	3.5778	0.023
C:N	Ef+	HCTL > *F. pinicola*	3.6222	0.021
C:N	Ef+	HCTL > *G. applanatum*	4.6	0.002
C:N	Ef+	HCTL > *T. versicolorG. applanatum*	3.9889	0.009
P	Ef+	*G. applanatum* > *F. betulinaF. pinicola*	0.16	0.035
K	Ef+	ALL > *F. betulinaF. pinicola*	0.1022	0.008
K	Ef+	*F. betulina* > *F. betulinaF. pinicola*	0.1056	0.006
K	Ef+	*F. pinicola* > *F. betulinaF. pinicola*	0.1011	0.009
K	Ef+	*G. applanatum* > *F. betulinaF. pinicola*	0.1133	0.003
K	Ef+	*T. versicolor* > *F. betulinaF. pinicola*	0.1033	0.007
K	Ef+	*G. applanatum* > HCTL	0.0822	0.045
K	Ef-	ALL > *F. betulinaF. pinicola*	0.1033	0.02
K	Ef-	*F. betulina* > *F. betulinaF. pinicola*	0.1078	0.014
K	Ef-	*G. applanatum* > *F. betulinaF. pinicola*	0.1156	0.008
Ca	Ef-	*F. betulina* > *F. betulinaF. pinicola*	0.4478	0.022

Ca	Ef-	*F. pinicola* > *F. betulinaF. pinicola*	0.4489	0.021
Ca	Ef-	*G. applanatum* > *F. betulinaF. pinicola*	0.51	0.007
Ca	Ef+	*G. applanatum* > *F. betulinaF. pinicola*	0.4022	0.03
Mg	Ef+	ALL > *F. betulinaF. pinicola*	0.0478	0.012
Mg	Ef+	*F. betulina* > *F. betulinaF. pinicola*	0.0533	0.004
Mg	Ef+	*F. betulina* > HTCL	0.0444	0.021
Mg	Ef+	*G. applanatum* > *F. betulinaF. pinicola*	0.0467	0.014
Mg	Ef-	ALL > *F. betulinaF. pinicola*	0.04	0.022
Mg	Ef-	*F. betulina* > *F. betulinaF. pinicola*	0.0422	0.014
Mg	Ef-	*F. pinicola* > *F. betulinaF. pinicola*	0.0378	0.034
Mg	Ef-	*G. applanatum* > *F. betulinaF. pinicola*	0.0456	0.007
Fe	Ef+	*T. versicolor* > *F. betulinaF. pinicola*	528.8889	0.041
Cu	Ef+	*F. betulina* > *F. betulinaF. pinicola*	8.3333	0.018
Mn	Ef-	*T. versicolorG. applanatum* > ALL	937.7778	0.005
Mn	Ef-	*T. versicolorG. applanatum* > BCTL	876.6667	0.009
Mn	Ef-	*T. versicolorG. applanatum* > *F. betulina*	785.5556	0.023
Mn	Ef-	*F. betulinaF. pinicola* > HCTL	984.4444	0.003
Mn	Ef-	*T. versicolorG. applanatum* > *F. pinicola*	927.7778	0.005
Mn	Ef-	*T. versicolorG. applanatum* > *G. applanatum*	861.1111	0.01
Mn	Ef-	*F. betulinaF. pinicola* > HCTL	984.4444	0.003
Mn	Ef-	*T. versicolorG. applanatum* > HCTL	1267.7778	0
Mn	Ef-	*T. versicolorG. applanatum* > *T. versicolor*	936.6667	0.005
Mn	Ef+	AL > HCTL	978.8889	0.019
Mn	Ef+	BCTL > HCTL	893.3333	0.038
Zn	Ef-	*G. applanatum* > *F. betulinaF. pinicola*	83.6667	0.045
B	Ef+	ALL > *F. betulinaF. pinicola*	10.1111	0.041
B	Ef+	*F. betulina* > *F. betulinaF. pinicola*	11.1111	0.02
B	Ef+	*G. applanatum* > *F. betulinaF. pinicola*	11.6667	0.013
B	Ef+	*T. versicolor* > *F. betulinaF. pinicola*	10.6667	0.028
EC	Ef+	BCTL > ALL	0.4311	0.017
EC	Ef+	*F. betulina* > ALL	0.4967	0.005
EC	Ef+	*F. pinicola* > ALL	0.5133	0.004
EC	Ef+	*G. applanatum* > ALL	0.4087	0.027
EC	Ef+	*T. versicolorG. applanatum* > ALL	0.3922	0.036
EC	Ef+	*T. versicolor* > ALL	0.5078	0.004

5.4 Appendix 2: Identification of cultured species used for experiment.

Ganoderma applanatum strain BL26 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

CTTGGTCATTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGG
AAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATGTGCACG
CCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTATCAGATCGTGAAGCGT
GCTCTTTTACCGGAGCTTGTGAAGCGTGTCTGTGCCTGCGTTTATCACAAACACTATA
AAGTATCAGAATGTGTATTACGATGTAACGCATCTATATACAACTTTCAGCAACGGA
TCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCG
AGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTATAAGCTTTTGTGGTTTGT
AGGCTTGGACTTGGAGGCTTGTCGGCCTTGATCGGTCGGCTCCTCTTAAATGCATTA
GCTTGATTCCTTGCGGATCGGCTCTCGGTGTGATAATATCTACGCCGCGACCGTGAA
GCGTTTGGCGAGCTTCTAACCGTCTCACTTGAGAGACAACTTTATGACCTCTGACCT
CAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Ganoderma applanatum voucher Dai 12757 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

TAAGGGACTGCGGAGGACATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAG
GCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTATCAGA
TCGTGAAGCGTGCTCTTTTACCGGAGCTTGTGAAGCGTGTCTGTGCCTGCGTTTATCA
CAAACACTATAAAGTATCAGAATGTGTATTACGATGTAACGCATCTATATACAACTT
TCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTC
CTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTATAAGC
TTTTGTGGTTTGTAGGCTTGGACTTGGAGGCTTGTCGGCCTTGATTGGTCGGCTCCTC
TTAAATGCATTAGCTTGATTCCTTGCGGATCGGCTCTCGGTGTGATAATATCTACGCC
GCGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCTCACTTGAGACACAACTTTATG
ACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC

Trametes versicolor isolate R47 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GTAGGTGAACCTGCGGAAGGATCATTAACGAGTTTTGAAACGAGTTGTAGCTGGCCT
TCCGAGGCATGTGCACGCTCTGCTCGTCCACTCTACCCCTGTGCACTTACTGTAGGTT
GGCGTGGGCTCCTTAACGGGAGCATTCTGCCGGCCTATGTATACTACAAACACTTTA
AAGTATCAGAATGTAAACGCGTCTAACGCATCTATAATACAACTTTTAGCAACGGAT
CTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGA
GGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTTATAAATCCTTGTGATCTATA
AGCTTGGACTTGGAGGCTTGCTGGCCCTCGTTGGTCGGCTCCTCTTGAATGCATTAG

CTCGATTCCGTACGGATCGGCTCTCAGTGTGATAATTGTCTACGCTGTGACCGTGAA
GTGTTTTGGCGAGCTTCTAACCGTCCATTAGGACAACTTTTAACATCTGA

Trametes versicolor voucher CLZhao 1293 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, region

GCGCTTGTACTCGGAGGACATTAACGAGTTTTGAAACGAGTTGTAGCTGGCCTTCCG
AGGCATGTGCACGCTCTGCTCATCCACTCTACCCCTGTGCACTTACTGTAGGTTGGC
GTGGGCTCCTTAACGGGAGCATTCTGCCGGCCTATGTATACTACAAACACTTTAAAG
TATCAGAATGTAAACGCGTCTAACGCATCTATAATACAACTTTTAGCAACGGATCTC
TTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGG
AGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTTATAAATCCTTGTGATCTATAAG
CTTGGACTTGGAGGCTTGCTGGCCCTTG

Trametes versicolor voucher CLZhao 6616-B internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

CGTGAGGTGACTGCGGAAGGATCATTAACGAGTTTTGAAACGAGTTGTAGCTGGCCT
TCCGAGGCATGTGCACGCTCTGCTCATCCACTCTACCCCTGTGCACTTACTGTAGGTT
GGCGTGGGCTCCTTAACGGGAGCATTCTGCCGGCCTATGTATACTACAAACACTTTA
AAGTATCAGAATGTAAACGCGTCTAACGCATCTATAATACAACTTTTAGCAACGGAT
CTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG
CAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGA
GGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTTATAAATCCTTGTGATCTATA
AGCTTGGACTTGGAGGCTTGCTGGCCCTTGTTGGTCGGCTCCTCTTGAATGCATTAGC
TCGATTCCGTACGGATCGGCTCTCAGTGTGATAATTGTCTACGCTGTGACCGTGAAG
TGTTTTGGCGAGCTTCTAACCGTCCATTAGGACAACTTTTTAACATCTGACCTCAAAT
CAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAAGCGGAGGG

Fomitopsis betulina, formerly *Piptoporus betulinus* voucher FP-125006-T 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

TAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCAT
TAATGAATTATGAAAGGGGTTGTCGCTGGCTGTTAGCAGCATGTGCACGCTCTGATC
ATTATCCATCTTACACACCTGTGCACACACTGTAAGTCGGCTTTTGATGCAAAGTAA
GGGTCTTCATTGACTCTGCTTTAAATTGGGAGCCTGCTTATGTTTTATCACACACTAC
TTCAGTTTAAAGAATGTCAAATCGCGTTTAACGCATTTAAATACAACTTTCAGCAAC
GGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTAT
TCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATCATCAACTCTATTTACTTTTGTGA
ATAGGGCTTGGACTTGGAGGTTTTGCCGGTACTTGTGATCGGCTCCTCTTGAATGCA
TTAGCTCGAACCTTTGTGGATCAGCTTATCGGTGTGATAATTGTCTACGCCGTTACTG
TGAAGCATATATTAAAGGGCTCGGCTTCTAATCGTCCTTCACAGGACAATAACTTTG
ACCTTTGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCAATA

Fomitopsis pinicola isolate AFTOL-ID 770 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 25S ribosomal RNA gene, partial sequence

GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTA
ATGAATTCTGAAAGGGGTTGTAGCTGGCCGTTTCGTTTGAGCGGCATGTGCACGCCC
TGCTCATTATCCATCTCACACACCTGTGCACATACTGTAGGTCGGCTTTTGATTGGAG
TGGGGTCTTCATCGACTCTGCTCTTTAGTTGGGGCCTTCCTATGTTTTATCACACACT
ACTTCAGTTTAAAGAATGTCCTCTTGCGTCTAACGCATTTAAATACAACTTTCAGCA
ACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGT
ATTCCGAGGAGCATGCCTGTTTGAGTGTCATGGAATTCTCAACTCTGTTTACTTTTGT
GAATAGAGCTTGGACTTGGAGGTTTATTGCCGGTACCTGTGATCGGCTCCTCTTGAA
TGCATTAGCTCGAACCTTTTGTGGATCAGCTTATCGGTGTGATAAAAATGTCTACGC
CGTTACTGTGAAGCATATTATTCGGCTTCCAATCGTCCTTCACGGGACAATAACTTTG
ACCTTTGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCA

5.5 Appendix 3: Sequence identification of naturally occurring fungi from PMB and bark

Armillaria mellea isolate G9 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, region

GGNCCCCTGGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCG
AGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTATCA
GATCGTGAAGCGTGCTCTTTTACCGGAGCTTGTGAAGCGTGTCTGTGCCTGCGTTTAT
CACAAACACTATAAAGTATCAGAATGTGTATTACGATGTAACGCATCTATATACAAC
TTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCT
CCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTATAAG
CTTTTGTGGTTTGTAGGCTTGGACTTGGAGGCTTGTCGGCCTTGATCGGTCGGCTCCT
CTTAAATGCATTAGCTTGATTCCTTGCGGATCGGCTCTCGGTGTGATAATATCTACGC
CGCGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCTCACTTGAGAGACAACTTTAT
GACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAA

Clitopilus hobsonii strain CBS 270.36 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAACTTGGTCAA
GCTGTTGCTGGTCCTTCGGGGCATGTGCACGCTTGCCACCAATTTTAACCACCTGTGC
ACCTTTTGTAGACTAGAAACGTTTCTCGAGGCAACTCGGATTGAGAACTGCTGCGCG
AAAGCCAGCTGTTCTTGTGTTTCTCAGTCTATGTTTTTACATACCCCGAATGAATGTA
TCAGAATGTATTGCTTGGCCTTAGTGCCTTTAAATCAAATACAACTTTCAACAACGG
ATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCC
GAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACTATACAAGTTTTTATTAAC

ATGTATAGCTTGGATCATGGGATTTGCGGGCTTTTACAAGTCGGCTATCCTCAAATG
CATTAGCAGAGCTTTTGCCGCTAATCTCTGGTGTGATAATTATCTACGCCATTGAGA
AGTGACATATTGAGGCTTCGCTTCTAATCGTCTTCACGGACAACCTTTGACAATCTG
ACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCAA

Clitopilus passeckerianus isolate P73 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

CCTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAACTTGGTCAAGCTGTT
GCTGGTCCTTCGGGGCATGTGCACGCTTGCCACCAATTTTAACCACCTGTGCACTTTT
TGTAGACTAGAAACGTTTCTCGAGGCAACTCGGATTGAGAACTGCTGCGCGAAAGC
CAGCTATTCTTGTGTTTCTCAGTCTATGTTTTTACATACCCCGAATGAATGTATTAGA
ATGTATTGCTGGGCCTTTGTGCCTTTAAATCAAATACAACTTTCAACAACGGATCTCT
TGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGA
GCATGCCTGTTTGAGTGTCATTAAATTCTCAACTATACAAGTTTTTATTAACCTGTAT
GGCTTGGACCATGGGATTTGCGGGCTTTCTGAGTCGGCTATCCTTAAATGCATTAGC
AGAGCTTTTGCCGCTAATCTCTGGTGTGATAATTATCTACGCCATTGAGAAGTGACA
TGTTGAGGCTTCGCTTCTAATCGTCTTCACAGACAATATTTGACAATCTGACCTCAAA
TCAGGTAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Clitopilus scyphoides isolate T-777 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

AACTGACGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCC
GCACGCGCGCTACACTGACAGAGCCAGCGAGTTCTTTTCCTTGGCCGGAAGGTCTGG
GTAATCTTGTGAAACTCTGTCGTGCTGGGGATAGAGCATTGCAATTATTGCTCTTCA
ACGAGGAATACCTAGTAAGCGTGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCT
TTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGTCTCCGGATT
GGTTTTGGGGAGCCGGCAACGGCACCCTATTACTGAGAAGCTGATCAAACTTGGTCA
TTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTATTGAATAAACTTGGTCAAGCTGTTGCTGGTCCTTCGGGGCATGTGCACGCTTG
CCACCAATTTTAACCACCTGTGCACCTTTTGTAGACTAGAAACGTTTCTCGAGGCAA
CTCGGATTGAGAACTGCTGCGCGAAAGCCAGCTGTTCTTGTGTTTCTCAGTCTATGTT
TTTACATACCCCGAATGAATGTATCAGAATGTATTGCTTGGCCTTAGTGCCTTTAAAT
CAAATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCG
AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCA
ACTATACAAGTTTTTATTAACATGTATAGCTTGGATCATGGGATTTGCGGGCTTTCAC
AAGTCGGCTATCCTCAAATGCATTAGCAGAGCTTTTGCCGCTAATCTCTGGTGTGAT
AATTATCTACGCCATTGAGAAGTGACATATTGAGGCTTCGCTTCTAATCGTCTTCAC
GGACAACTTTTGACAATCTGACCTCAAATCAGGTAGGATTACCCGCTGAACTTAAGC
ATATCAATAAGCGGAGGAAAAGAAACTAACAAGGATTCCCCTAGTAACTGCGAGTG
AAGCGGGAAAAGCTCAAATTTAAAATCTGGTAGTCTTTGGCTGCCCGAGTTGTAATC
TAGAGAAGCGTTATCCGCGCTGGACCGTGTATAAGTCTCCTGGAATGGAGCGTCATA
GAGGGTGAGAATCCCGTCTTTGACACGGACTACCAGGGCTTTGTGATGCGCTCTCAA

AGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGTAAATTCCATCTAAAGCT
AAATATTGGCGAGAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGA
ACTTTGGAAAGAGAGTTAAACAGTACGTGAAATTGTTGAAAGGGAAACGCTTGAAG
TCAGTCGCGTTGGCTAGGAATCAGCCTTGCTTTTGCTTGGTGTACTTCCTGGTTGACG
GGTCAGCATCAATTTTGACCGGTGGAAAAAGATCAGAGGAATGTGGCATCTTCGGA
TGTGTTATAGCTTCTGGTTGTATACATCGGTTGGGATTGAGGAACTCAGCACGCCGC
AAGGTCGGGGTTCGCCCACGTTCGTGCTTAGGATGCTGGCATAATGGCTTTAATCGA
CCCGTCTTGAAACACGGACCAAGGAGTCTAACATGCCTGCGAGTGTTTGGGTGGTAA
ACCCGAGCGCGTAATGAAAGTGAAAGTTGAGATCCCTGTCGTGGGGAGCATCGACG
CCCGGACCAGACCTTCTGTGACGGCTCCGCGGTAGAGCATGTATGTTGGGACCCGA
AAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCT
CGTAGCGATTCTGACGTGCAAATCGATCGTCAAATTTGGGTATAGGGGCGAAAGAC
TAATCGAACCATCTAGTAGCTGGTTCCTGCCGAAGTTTCCCTCAGGAT

Deconica coprophila strain CBS 181.37 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAACCTGATGTG
GTTGTTGCTGGCTCTCTCGAGAGTAATGTGCACGCCCATCATCTTTATATCTCCACCT
GTGCACCTTTTGTAGACCTGGGGATTGAGTAACATCAATCGTTAGGCCTATGTTTTAT
CATATACCCCATAGTATGTATCAGAATGTATCAATGGGCTTCGTGCCTATAAACTAT
ATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACC
TTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAACC
TCTCTAGTTTGTTATGAACTACGTAGATGGCTTGGATGTGGGGGATTATTTTGCAGG
CTTTCACAAGTCAGCTCCCCTGAAATGTATTAGCCGGTGCCCTCTAACCGTCTATTGG
TGTGATAATTATCTACGCCGTGGATGTTTGGAGTTGAAGGTACTGCTTCTAACCGTC
CTTTTTGGACAACTTATGACATTTTGACCTCAAATCAGGTAGGACTACCCGCTGAAC
TTAAGCATATCAATAAGCGGA

Omphalina mutila strain CBS 329.85 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

TCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAACTTGGTCAAGCTGTTGCT
GGTCCTTCGGGGCATGTGCACGCTTGCCACCAATTTTAACCACCTGTGCACCTTTTGT
AGACTAGAAACGTTTCTCGAGGCAACTCGGATTGAGAACTGCTGCGCGAAAGCCAG
CTGTTCTTGTGTTTCTCAGTCTATGTTTTAACATACCCTGAATGAATGTATTAGAATG
TATTGCTGGGCCTTTGTGCCTTTAAATCAAATACAACTTTCAACAACGGATCTCTTGG
CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCA
TGCCTGTTTGAGTGTCATTAAATTCTCAACCATACAAGTTTTTATTAACCTGTATGGC
TTGGATCATGGGATTTGCGGGCTTCCTGAGTCGGCTATCCTTAAATGCATTAGCAGA
GCTTTTGCCGCTATTCTCTGGTGTGATAATTATCTACGCCATTGAGAAGTGACATGTT
GAGGCTTCGCTTCTAATCGTCTTCACAGACAATATTTGACAATCTGACCTCAAATCA
GGTAGGACTACCCGCTGAACTTAAGCATATCAATAA

Psilocybe coprophila strain CBS 417.82 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

GATCATTATTGAATAAACCTGATGTGGTTGTTGCTGGCTCTCTCGAGAGTAATGTGC
ACGCCCGTCATCTTTATATCTCCACCTGTGCACCTTTTGTAGACCTGGGGATTGAGTA
ACATCAATCGTTAGGCCTATGTTTTATCATATACCCCATAGTATGTATCAGAATGTAT
CAATGGGCTTCGTGCCTATAAACTATATACAACTTTCAGCAACGGATCTCTTGGCTC
TCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGC
CTGTTTGAGTGTCATTAAATTCTCAACCTCTCTAGTTTGTTATGAACTACGTAGATGG
CTTGGATGTGGGGGATTATTTTGCAGGCTTTCACAAGTCAGCTCCCCTGAAATGTAT
TAGCCGGTGCCCTCTAACCGTCTATTGGTGTGATAATTATCTACGCCGTGGATGTTTG
GAGTTGAAGGTACTGCTTCTAACCGTCCTTTTTGGACAACTTATGACATTTTGACCTC
AAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAA
ACTAACAAGGATTCCCCTAGTAACTGCGAGTGAAGCGGGAAAAGCTCAAATTTAAA
ATCTGGCGGTCTTTGGCCGTCCGAGTTGTAATCTAGAGAAGTGTTATCCGCGCTGGA
CCGTGTACAAGTCTCCTGGAATGGAGCGTCATAGAGGGTGAGAATCCCGTCTTTGAC
ACGGACTACCAGTGCTTTGTGATACGCTCTCAAAGAGTCGAGTTGTTTGGGAATGCA
GCTCAAAATGGGTGGTAAATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAG
CGAACAAGTACCGTGAGGGAAAGATGAAAAGAACTTTGGAAAGAGAGTTAAACAG
TACGTGAAATTGCTGAAAGGGAAACGCTTGAAGTCAGTCGCGTTGGCCGGGGATCA
ACCTTGCTTTTGCTGGGTGTACTTTCCGGTTGACGGGTCAGCATCAATTTTGACCGTT
GGATAAAGTGTAGGGGAATGTGGCATCTTCGGATGTGTTATAGCCTTTGCTCGTATA
CAACGGTTGGGATTGAGGAACTCAGTACGCCGCAAGGCCGGGAATTTATTCCACGTT
CGTACTTAGGATGCTGGCATAATGGCTTTAATCGACCCGTCTTGAAACACGGACCAA
GGAGTCTAACATGCCTGCGAGTGTTTGGGTGGAAAACCCGAGCGCGTAATGAAAGT
GAAAGTTGAGATCCCTGTCGTGGGGAGCATCGACGCCCGGACCAGACCTTTTGTGAC
GGATCTGCGGTAGAGCATGTATGTTGGGACCCGAAAGATGGTGAACTATGCCTGAA
TAGGGTGAAGCCAGAGGAAACTCTGGTGGAGGCTCGTAGCGATTCTGACGTGCAAA
TCGATCGTCAAATTTGGGTATAGGGGCGAAAGACTAATCGAACCATCTAGTAGCTG
GTTCCTGCCGAAGTTTCCCTCAGGATAGCAGAAACTCATTTCAGATTTATGTGGTAA
AGCGAATGATTAGAGGCCTTGGGGTTGAAACAACCTTAACCTATTCTCAAACTTTAA
ATATGTAAGAACGAGCCGTCTCTTGATTGGACCGCTCGGCGATTGAGAGTTTCTAGT
GGGCCATTTTTGGTAAGCAGAACTGGCGATGCGGGATGAACCGAACGCGAGGTTAA
GGTGCCGGAATTCACGCTCATCAGACACCACAAAAGGTGTTAGTTCATCTAGACAGC
AGGACGGTGGCCATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGC
CGAATGAACTAGCCCTGAAAATGGATGGCGCTTAAGCGTGATACCCATACCTCGCC
GTCAGCGTTGAAGTGACGCGCTGACGAGTAGGCAGGCGT

Psilocybe merdaria strain CBS 371.39 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTATTGAATAAACCTGATGTT
GGTTGTTGCTGGCTCTCTCGAGAGTAATGTGCACGCCCGTCATCTTTATATCTCCACC
TGTGCACCTTTTGTAGACCTGGGGATTGAGTAACATCAATCGTTAGGCCTATGTTTTA
TCATATACCCCATAGTATGTATCAGAATGTATCAATGGGCTTCGTGCCTATAAACTA

TATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
CTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATTAAATTCTCAAC
CTCTCTAGTTTGTTATGAACTACGTAGATGGCTTGGATGTGGGGGATTATTTTGCAG
GCTTTCACAAGTCAGCTCCCCTGAAATGTATTAGCCGGTGCCCTCTAACCGTCTATTG
GTGTGATAATTATCTACGCCGTGGATGTTTGGAGTTGAAGGTACTGCTTCTAACCGT
CCTTTTTGGACAACTTATGACATTGACCTCAAATCAGGTAGGACTACCCGCTGAACT
NAAGCATATCAA