MICROBIAL COMMUNITY AND BIOGEOCHEMICAL CHARACTERISTICS IN RECLAIMED SOILS AT PT BUKIT ASAM COAL MINE, SOUTH SUMATRA, INDONESIA

A thesis presented to the faculty of the Graduate School of Western Carolina University in partial fulfillment of the requirements for the degree of Master of Science in Biology

By

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LIST OF ABBREVIATIONS

AMD: Acid mine drainage
BMP: Best management practice
bp: Base pairs
CNS: Carbon/nitrogen/sulfur
DGGE: Denaturing gradient gel electrophoresis
ICP: Inductively coupled plasma
OM: Organic Matter
PCR: Polymerase chain reaction
PTBA: PT Bukit Asam
RDP: Ribosomal Database Project
USEPA: United States Environmental Protection Agency
WHO: World Health Organization

ABSTRACT

MICROBIAL COMMUNITY AND BIOGEOCHEMICAL CHARACTERISTICS IN RECLAIMED SOILS AT PT BUKIT ASAM COAL MINE, SOUTH SUMATRA, INDONESIA

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Organic soil amendments such as compost, wood chips, and sewage sludge have been reported to influence soil microbial composition, improve soil quality, and increase nutrient concentrations in surface runoff. A potentially negative consequence of adding compost to soils is that it may increase the solubility of metals in surface waters and groundwater. This study investigated the effects of compost age and compost application type on metal (e.g. Al, Fe, Mn, Ni, Pb, Zn) concentrations in subsurface waters at reclaimed areas of the PT Bukit Asam Coal Mine, located near Tanjung Enim, South Sumatra, Indonesia. The mine relies heavily on the use of compost to improve soil productivity of reclaimed and reforested areas. The compost is aged for fourteen-weeks and mainly composed of materials from oil palms (*Elaeis guineensis*) and, to a lesser degree, organic municipal solid waste from the local community. In an effort to determine the most effective method for applying compost to reclaimed soil, compost was applied in a five-centimeter-thick layer spread over the reclaimed soil surface, or mixed with the soil placed in the hole where vegetation was planted. Soil bacterial DNA was extracted on site to characterize the microbial communities within the various aged research plots. All 16S rDNA sequences generated from the DNA using PCR were related to Gram negative-staining bacteria.

RDP Classifier assigned four of the sequences to the phylum Proteobacteria and one to Acidobacteria. These heterotrophic bacteria likely play a role in organic matter turnover and utilize specific major and trace metals for metabolic processes over time, which is consistent with lower values observed in the older plot. In addition, subsurface water metal concentrations, total metal concentrations in soil, grain size distribution, and total percent carbon were compared between study plots of varying ages (3 months to 120 months) and compost treatments. Reclaimed soil without the addition of compost was also examined as a control. Total metal concentrations in soil were all within global background values and below soil contaminant levels. The geochemical data for total metal concentrations in surface and subsurface soil samples showed no sign of metal depletion from the surface or accumulation at depth, which indicates limited metal mobility through the soil profile. Field observations show that minimal metal mobility may have occurred through soil fractures. These data are consistent with the low metal concentrations observed in subsurface waters. Limited metal mobility to shallow subsurface waters is likely to result, in part, from the low permeability of the soils, which was primarily composed of silt and clay. Our results suggest that neither the type of compost application nor age of application had a significant effect on microbial diversity, or metal mobility within the soils, thereby limiting the potential contamination of subsurface waters within the PTBA reforested reclamation areas.

CHAPTER ONE: INTRODUCTION

Approximately 40 percent of global electricity is produced by burning coal (Syarif, 2014). Surface or opencast mining where large parcels of land are disturbed to extract coal, often leads to environmental impacts, including deforestation, decreased biodiversity, degradation of soil health, and decreased water quality (Haigh, 1980; Kilmartin, 1994; Sheoran et al., 2010; Haigh and Kilmartin, 2015; Macdonald et al., 2015). One of the major concerns of surface mining, particularly of coal, is the formation of acid mine drainage (AMD; Geidel et al., 2000). Oxidation of sulfide minerals found in the coal, coal bearing strata, and overburden/interburden material produce hydronium ions (H^+) and releases metal cations into the environment (Geidel et al., 2000; RoyChowdhury et al., 2015), resulting in ecosystem degradation (Kundu and Ghose, 1997; RoyChowdhury et al., 2015). To reduce the potential of such environmental impacts, effective reclamation is crucial.

There are a number of different methods and technologies for successful reclamation following mining; however, these treatments are typically site specific and should be chosen based on the physical, chemical, and biological characteristics present (Kuter, 2013). Nonetheless, a common approach to surface mining reclamation is the reconstruction of surface topography, followed by revegetation of the reclaimed area. Such mine reclamation techniques, require an adequate supply of essential nutrients in “spoil material” that allows for species establishment, vegetation growth, and microbial processes to take place (Singh et al., 2002, Lone et al., 2008; Kavamura and Esposito, 2010). Furthermore, revegetation of reclaimed sites can be challenging due to severely compacted soils caused by heavy machinery, resulting in rapid storm runoff, low soil water storage, and a shortage in crucial nutrients required for plant growth (Sheoran et al., 2010; Haigh and Kilmartin, 2015).

Soil is often referred to as the foundation of a natural system because it is the medium for plant growth (Zipper et al., 2013), and is where many biogeochemical processes occur. Therefore, it is necessary to improve degraded soils affected by mining practices, following landscape reconstruction. The use of soil amendments for improving soil quality, vegetation growth, and microbial activity is a common practice in contaminated sediments such as mine tailings. A wide range of amendments (e.g. fly ash, sewage sludge, and lime) have been applied to reclaimed soils; one of the most common is compost (decomposed organic material). Compost, including municipal solid waste compost (MSWC), increases organic matter (OM) within soil, aiding in soil quality (He et al., 1995; Soumare et al., 2003).

Organic matter in soil exists in various stages of decomposition (Canada Department of Agriculture, 1972). It is a major source of nitrogen, phosphorus, potassium, sulfur, and organic carbon, all of which are essential plant nutrients (Schnitzer and Khan, 1978; Miller and Donahue, 1990; Ellert and Bettany, 1995) and microbial activity (Sheoran et al., 2010). Soil substitutes used in mine reclamation and made up of overburden are capable of forming soil-like properties (e.g., OM incorporation) once they are revegetated, however these spoil materials are initially OM deficient, providing a paucity of bioavailable nutrients (Roberts et al., 1988; Zipper et al., 2013). The addition of sewage sludge, sawdust, hay, bark, wood chips, and MSWC have been shown to raise OM content (Sydnor and Redente, 2002; Munshower, 1994; Hall, 1985), and increase bacterial growth (Lindemann et al., 1984).

Nutrient cycling, and carbon cycling in particular, is important for microbial activity because nutrients serve as an energy source that helps fuel metabolic processes that produce necessary nutrients for plant growth (Sheoran et al., 2010). While the volume of organic carbon present within soil may influence microbial activity (Williamson and Johnson, 1991). Microbial activity may also influence metal toxicity in reclaimed coal mine soils (Haigh and Kilmartin, 2015). Moreover, metal mobilization is made possible through methylation, autotrophic, and heterotrophic leaching processes associated with microbial activity (Gadd, 2004). Reduction and oxidation processes caused by microbial metabolism may also influence the solubility of metal compounds (Gadd, 1993; Gharieb et al., 1999; Lovley, 2000). It is not uncommon for MSWC to contain metals (Baldwin and Shelton, 1999), and as microorganisms play an essential role in the decomposition of OM, as they can influence metal mobility. Restoring nutrient cycling and microbial populations in disturbed soils is also vital for successful ecosystem restoration projects (Sheoran et al., 2010).

In addition to metal mobility caused by microbial activity, Singh and Kalamdhad (2012) found the addition of OM can increase metal solubility through complexation. Farrell et al., (2010) found that metal concentrations increased in soil solutions following the application of MSWC. This increase in metal solubility may allow metals to migrate downward to groundwater (Blake et al., 2007), raising water quality concerns. Furthermore, several studies found that metal concentrations such as nickel, lead, and zinc may increase significantly in soils and water as a result of complexation associated with the addition of organic amendments (Planquart, 1999; Jordao et al., 2006; Smith, 2009).

It appears that the degree to which metal mobility is altered by the addition of compost (OM) depends not only on the nature of the compost, but on site conditions. More specifically, the amount of rainfall/infiltration, microbial OM turnover, chelation processes, and soil composition. The latter influences metal sorption-desorption, pH, and cation exchange capacity.

Thus, metal mobility in contaminated areas is controlled by a wide range of factors (Lucas and Knezek, 1972; Bailey et al., 1999; Planquart, 1999; Hudson-Edwards et al., 1999; Jordao et al., 2006; Smith, 2009; Sheoran et al., 2010; Singh and Kalamdhad, 2012). This raises questions on whether the addition of compost to contaminated sites that is of benefit to reforestation poses a risk to water quality by releasing metals into waters located on site.

Major and trace element analyses have been conducted in contaminated soils of Indonesia to better understand water quality, geological chemistry, and the effects of soil amendments on metal mobility. However, the effects of different applications of one compost on metal mobility in subsurface waters, and reclaimed soil characteristics (i.e. OM, total metal concentrations, grain size, and microbiology) as a function of time have not been studied. For example, major and trace element analyses to document water quality and poor soils typically focus on the use of different soil amendment types rather than the method of application (Beesley et al., 2014). Therefore, examining the best method for applying compost to reclaimed soils aids in development of best management practices for improving water and soil quality in mining reclamation areas with limited access to various types of soil amendments.

The coal mine where my study was located is approximately 165 km west of Palembang in South Sumatra, Indonesia and is owned by PT Bukit Asam (PTBA), an Indonesian state-owned coal company (Figure 1) (Susilawati and Ward, 2006). Various mining reclamation and rehabilitation efforts have been implemented by PTBA. The company conducts routine revegetation programs in reclaimed areas that use soil amendments in the form of compost derived from palm plants (fruit) and MSWC from the local community to enhance soil fertility. In addition, pioneer and cover crops of economic value are planted and profits are given to the local community following project completion. In order to improve reclamation management practices on soil and water quality in reclaimed areas, PTBA has collaborated with multiple universities. This study is part of a larger project focusing on improving forest production, soil health, and in this case, water quality. Inherent in the study is an analysis of soil microbial communities, and the potential impacts of different compost application methods on metal mobility. Specific questions included:

1. What is the microbial diversity of reclaimed soils within PTBA reclamation areas, and could they play a role in OM decomposition and metal mobility?
2. Are metal concentrations in subsurface water influenced by different compost treatments?
3. Are metal concentrations in subsurface water influenced by the amount of time since compost was applied to reclaimed soils when focusing on one type of compost treatment?
4. Are there differences between percent soil organic carbon as a result of compost treatment and the amount of time since compost was applied to reclaimed soils?
5. What are the total metal concentrations for Al, Fe, Mn, Ni, Pb, and Zn in the reclaimed soils, and are they above the World Health Organization maximum permissible limits for metals in soil?

CHAPTER TWO: METHODS AND MATERIALS

2.1: Study Area

The region PTBA Coal Mine is located in exhibits a humid, tropical climate characterized by semi-stable annual air temperatures (Gautama, 1994). Daily temperatures range from 20° to 30° C; annual precipitation ranges from 2820 mm to 3832 mm (Gautama, 1994). The climate exhibits a distinct dry and rainy season; rainfall is highest from November to March and driest from May to October.

The coalfields of PTBA formed in a Tertiary sedimentary basin of South Sumatra (Gautama, 1994). Magmatic intrusions aided in the formation of semi-anthracite to sub-bituminous rank coal deposits within the basin. The high-grade coal partly explains why these coalfields have been in operation since 1919. The lithology of the overburden and interburden are primarily made up of sandstone, siltstones, claystones, and shale (Amijaya, and Littke, 2006; de Coster, 1974; Gautama, 1994).

In order to accomplish the project goals, three study plots were selected for analysis, each corresponding to a different reclamation plot, which vary in age. Within each plot, major and trace metal concentrations were measured within subsurface water as a function of compost treatment approach and time since compost application. The three study plots are referred to as Agrotourism Tupak, Agroforestry Tupak – Blok 1, and Banko Barat 3. The Agrotourism Tupak reclamation site was constructed three months prior to data/sample collection. Individual plots were constructed by the PTBA reclamation management team to determine the influence of selected compost application methods on soil health and plant growth. The first technique was used as a control and consisted of vegetation planted in the reclaimed soil without the addition of compost. The second treatment involved the placement of compost into a hole where it was mixed into the reclaimed soil and vegetation was planted soon after. The compost used in our research plots was derived from the fruit of oil palm trees (*Elaeis guineensis*) and municipal solid waste compost (MSWC) from the local community that was aged for fourteen-weeks at the PT Bumi Sawindo Parai (PTBSP) composting facility located off site. The third way compost was applied was, a five-centimeter-thick layer of compost over the soil surface after vegetation was planted into the soil. Two older plots were also chosen for study; to our knowledge both plots had a five-centimeter-thick layer of compost aged for 14 weeks applied over the soil surface. Agroforestry Tupak – Blok 1 was started in 2016 and was approximately 12-months-old at the start of our study, and Banko Barat 3 was created around 2005 and, thus, was roughly 120-months-old.

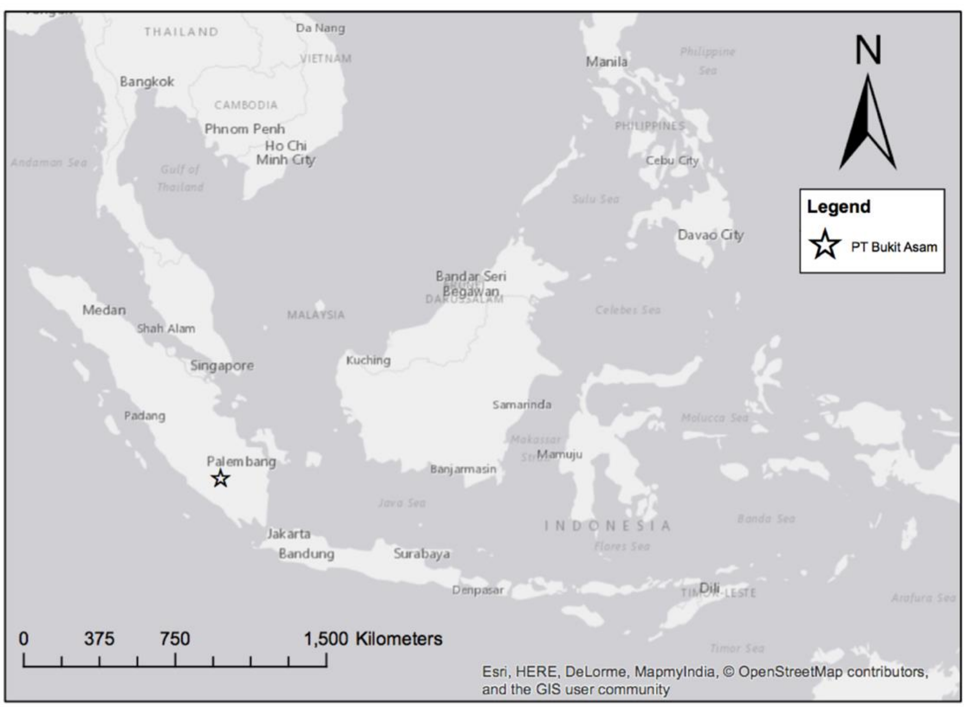

Figure 1. PT Bukit Asam coal mine is located in South Sumatra, Indonesia.

2.2: Sample Collection and Well Installation

A total of 15 monitoring wells (Figure 2) were installed in holes augured to a depth of ~60 cm at a ~45° angle (Table1) Angled installation was used to increase the intersection of the well screen with vertical soil fractures observed during soil profile characterization. These vertical fractures are likely to enhance the migration of major and trace metals through the soils by macropore flow (Dowdy and Volk, 1983; Sherene, 2010). Following well installation, soil microbial samples were taken at each shallow monitoring well for DNA extraction and microbial community characterization. Samples were taken in triplicate at ~5 cm depths within each site for a total of 45 microbial soil samples. An additional set of sediment samples were collected at a depth of ~5 cm in order to determine major and trace metal concentrations, soil carbon content, and grain size distribution. Soil samples collected for trace metal, carbon, and grain size analysis were shipped to the U.S. where they were analyzed at Western Carolina University (WCU).

Several soil pits were dug to ~60 cm depths in order to build a soil profile conceptual model based on field observations (Figure 3). In pits where a layer of compost was spread over the soil, there was a visible 2 cm thick layer of partially decomposed compost on the soil surface that had a munsell color of 7.5 YR 2.5/1. These reclaimed soils were not true soils because they were backfilled following mining activity. Backfill material was sourced from the original topsoil that was stripped off the land surface and stockpiled during mining activity. Typical South Sumatra soil orders are Inceptisols, Entisols, Ultisols, Oxisols, Histosols, Mollisols, and Andisols (Shofiyati et al., 2010). In addition, rock materials were primarily made of sandstones, siltstones, claystones, and shale (Amijaya and Littke, 2006; de Coster, 1974; Gautama, 1994), which were also present in the stockpile sediment.

It is important to note the heterogeneity of the backfill materials. The material is mixed, and therefore not stratified, therefore soil horizons did not exist within the soil profile. The soil consisted of a sticky clay material that had a coarse blocky structure with smooth surfaces along the entire profile. In addition, visible mottels and iron precipitates from oxidation/reduction reactions were present on the surface of clay peds with a color of 10YR 4/4. These iron precipitates suggested that there may be flow through observed soil fractures towards the bottom of the soil profile. There were two types of fractures observed within soil pits, (1) fractures around peds that were 2.5 cm, and vertical fractures that were ~20 cm, which may be more important for potential metal mobility to subsurface waters. In addition, distinct organic and/or manganese coatings were also present on the smooth surface of clay peds with a color of 10YR 2/1 that appeared to mobilize downward by macropore flow in visible soil fractures within the soil profile.

The shallow monitoring wells were used to collect subsurface water from approximately 5 precipitation events during the rainy season. In addition, sediment samples were collected at the bottom of auger holes. A total of 30 sediment samples collected from the surface and at depths of ~60 cm were analyzed for major (Al, Fe, Mn) and trace elements (Ni, Pb, Zn) at WCU. These elements were selected based on results available from Aberystwyth University which showed the exhibited high concentrations in mine waters (Moore, 2016).

Figure 2. Photograph depicting monitoring wells that were installed in the Agrotourism Tupak reclamation area and their relative locations to each other. Note: The land surface is actually level and there is not a slope to the topography as this photograph may suggest.

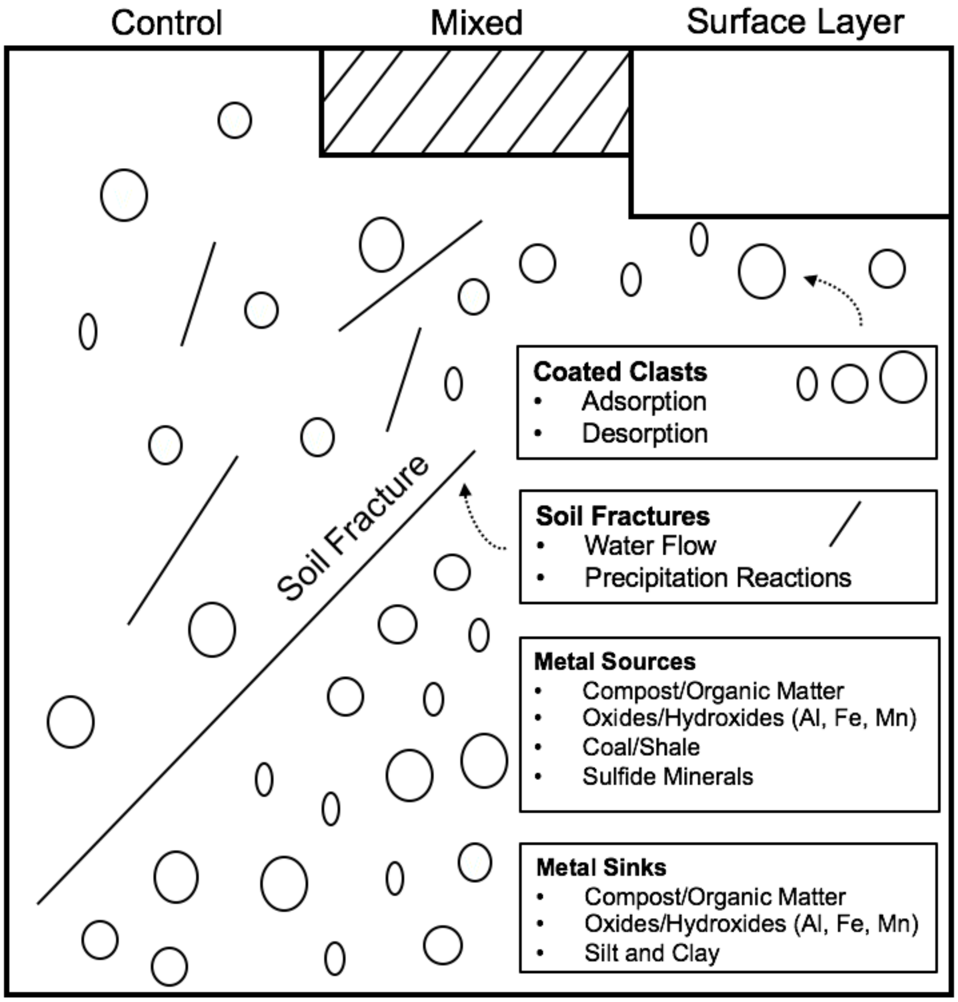

Figure 3. Conceptual model based on soil profiles observed in the field in reforested reclamation areas. Observations were based on the different compost application techniques which include the control – no compost treatment, compost mixed into reclaimed soil, and compost spread as a 5-cm thick layer over reclaimed soil surface. This conceptual model indicates potential geochemical processes in relation to observed soil characteristics.

Table 1. Naming scheme and locations of monitoring wells and samples collected at PTBA. The first two letters of the site identification column refer to the study plot, (AT) Agrotourism Tupak, (AF) Agroforestry Tupak – Blok 1, (BB) Banko Barat 3, followed by the well number, and treatments used (T0) no compost treatment "control", (T1) compost was mixed and placed into a hole and mixed with sediment before planting vegetation, (T2) a layer of compost was spread over the sediment, and the presence of (C) indicates compost that was matured for 14 weeks was used

Site Identification	Site Age (Months)	Compost Treatment	UTM Zone	Easting (X)	Northing (Y)
AT1T0	3	Control	48M	0364973	9590892
AT2T0	3	Control	48M	0364983	9590896
AT3T0	3	Control	48M	0364982	9590897
AT4T1C	3	Mixed	48M	0365051	9590875
AT5T1C	3	Mixed	48M	0365017	9590890
AT6T1C	3	Mixed	48M	0365033	9590885
AT7T2C	3	Surface layer	48M	0365021	9590889
AT8T2C	3	Surface layer	48M	0365048	9590888
AT9T2C	3	Surface layer	48M	0365057	9590873
AF10T2C	12	Surface layer	48M	0364889	9590838
AF11T2C	12	Surface layer	48M	0364856	9590851
AF12T2C	12	Surface layer	48M	0364848	9590833
BB13T2C	120	Surface layer	48M	0370430	9583342
BB14T2C	120	Surface layer	48M	0370413	9583340
BB15T2C	120	Surface layer	48M	0370403	9583337

2.3: Soil Microbial Analysis

2.3.1: DNA Extraction

A subset of 250 mg of soil was taken from each of the 45 samples collected for DNA extraction using the SurePrep™ Soil DNA Isolation Kit (Fisher BioReagents, Fair Lawn, NJ, USA). The extraction procedure followed the manufacturer's instructions. DNA was not detected in any of my extracted samples using agarose gel electrophoresis. Therefore, we I analyzed a subset of samples using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) to determine if there was quantifiable DNA present, the lower detection limit for the instrument is 2 ng/uL; my results did not go above 10 ng/uL. In addition, we observed sediment samples under a microscope and discovered microbes were present, which suggested that the DNA we were able to obtain came from intact cells.

2.3.2: Polymerase Chain Reaction (PCR)

The initial polymerase chain reaction (PCR) analysis was conducted on a Mastercycler Personal Thermal Cycler (Eppendorf, Westbury, NY). Nested PCR was conducted to amplify either ~800 base pair (bp) gene fragments of the fungal 18S rRNA internal transcribed spacer (ITS) region or ~1500 bp partial gene fragments of the bacterial 16S rRNA locus region. Fungal analysis used primers EF4 and ITS4R (Anderson et al., 2003), whereas bacterial PCR used 27F and 1492R (Corinaldes et al., 2005) for the first round of amplification. The second round for the nested PCR reaction was conducted to amplify ~300 bp of the fungal 18S rRNA region using primers ITS1F and ITS2R (Anderson et al., 2003), and ~550 bp of the 16S rRNA genes were amplified using primers 341F and 907R (Casamayor et al., 2000). The PCR reactions for both fungal and bacterial analysis contained 23 µL Nuclease Free Buffer Mix H_2O, 25 µL 2X Promega MasterMix (Mg^{2+}, Taq, dNTP's; Promega, Madison, WI), and 0.25 µM of the forward and reverse primers, for a total volume of 50 µL. Thermal cycler conditions for all fungal and the first round of bacterial PCR were as follows: initial denaturation at 94°C for 3 minutes, followed by 30 cycles for denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 2 minutes, and final elongation at 72°C for 10 minutes. The PCR samples were held at 4°C until they could be analyzed using 1% agarose gel electrophoresis to determine the quantity and integrity of the DNA. The PCR reactions for fungal analysis showed signs of contamination in negative controls and were not further examined.

2.3.3: DGGE and 16S rDNA Sequencing

In order to assess microbial community variations among different compost treatments within our study plots, denaturing gradient gel electrophoresis (DGGE) was used. Approximately 550 bp partial gene fragments of the 16S rRNA region were amplified (chemistry as above) from bacterial DNA extracted from sediment samples using universal bacterial primers 341F with a GC clamp and 907R (Operon, Inc., Huntsville, AL; Casamayor et al., 2000). A "touchdown" PCR was used for amplification of the first round PCR products from bacteria. The thermal cycler conditions included initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, initial annealing at 65°C for 1 minute, elongation at 72°C for 3 minutes, and final elongation at 72°C for 7 minutes. The annealing temperature was decreased by 1°C from 65°C to 55°C every cycle in the first 12 cycles and followed by a constant temperature of 55°C (Casamayor et al., 2000). The DGGE analyses were conducted using a BioRad DCode apparatus (BioRad, Hercules, CA). A 15 cm by 20 cm polyacrylamide gel, with denaturing gradients ranging from 20 to 60% urea/formamide, facilitated DNA fragment separation into distinct bands in the gel. Samples (~20 µL PCR products and 4 µL 6X loading buffer) were loaded into the gel vertically and DGGE was run in a 1X TAE buffer at 60°C for a total of 4 hours at 200 V (Muyzer et al., 1993). Distinct bands of DNA were cut from DGGE gels for sequencing to identify particular bacterial species present within treatment plots. The closest matches to each sequence were determined by using the Ribosomal Database Project (RDP) website tools Classifier and SeqMatch (https://rdp.cme.msu.edu).

2.4: Water Analysis

Samples were filtered into pre-cleaned sample bottles using 0.45-micron membrane filters, followed by freezing and shipment to Aberystwyth University for analysis of major (Al, Fe, Mn) and trace metals concentrations (Ni, Pb, Zn). The analyses were carried out using an Agilent 7700 Series inductively coupled plasma mass spectrometer (ICP-MS; Agilent, Santa Clara, CA).

2.5: Soil Analysis

2.5.1: CNS Analysis

Preparation for total carbon analysis was conducted by drying sediment samples at 100°C for ~24 hours. Sediment samples were ground to a powder, then homogenized using a mortar and pestle. Following homogenization, ~25 mg of powdered sediment was combined with ~20 to 30 mg of WO_3 (tungsten trioxide). Samples were analyzed for total carbon content using the Vario EL III Elemental Analyzer (Elementar, Mt. Laurel, NJ) at WCU. It is important to note that CNS analysis is unable to differentiate between carbon in shale, coal, or compost that may be present in the soil samples.

2.5.2: Acid Digestion and ICP Analysis

Sediment samples were analyzed for major and trace metals (i.e., Al, Fe, Mn, Ni, Pb, and Zn) using the Optima 4100DV inductively coupled plasma optical emission spectrometer (ICP-OES; Perkin Elmer, Waltham, MA). Analyses were performed using a procedure modified from that used at the Nevada Bureau of Mines and Geology in Reno, Nevada. Sediment samples were air dried for a 24 to 48-hour period or until completely dry. Between 0.2 to 0.5 g of sediment was subsampled for acid digestion. A mortar and pestle was used to break up aggregates before placing the sample in a nitric acid-washed 125 mL Nalgene bottle. Subsequently, 3 mL of ~37% hydrochloric acid and 1 mL of ~68% trace metal grade nitric acid was added to the samples, after which the lids were placed on the bottles. The bottles were then placed in a hot water bath at a temperature of 90°C to 95°C for one hour before being allowed to cool. After digestion, the sample solution was transferred to a nitric acid-washed 100 mL volumetric flask and brought to volume using ultra-pure water. The final step required the sample to be filtered into 50 mL conical tubes using a 0.45-micron filter, and then analyzed by ICP-OES. Accuracy and precision were generally within +/- 5 %.

2.5.3: Grain Size Analysis

The grain size distribution of the <2 mm sediment samples was measured using a Mastersizer 2000 particle size analyzer (Malvern Instruments, Malvern, UK). The percent silt and clay in the sample was determined and assumed to represent the chemically active sediment within the samples. Prior to analysis, ~5 g of sediment was combined in a 50 mL beaker with 5 mL of pyrophosphate and ~30 to 40 mL of deionized water. The mixture was stirred and left to sit overnight.

2.6: Statistical Analysis

Principal components analysis (PCA) was used to visualize correlations on site characteristics (metals, carbon, soil particles) and microbial communities identified following DGGE analysis. A correlation value of ≥ 0.70 or ≤ -0.70 was used to characterize variables as influential within PCA. A Kruskal-Wallis analysis was used to determine how plot ages and compost treatment types correlated with (1) major and trace metal concentrations in subsurface water samples, (2) percent carbon in the soil, and (3) percent silt and clay in the soil. Pair-wise correlation analysis was conducted for determining the strength and relationship between metal concentrations analyzed from subsurface water samples. Due to metal concentrations not being normally distributed, the Spearman method was used (Zamani, A. A., Yaftian, M. R., and Parizanganeh, A., 2012) in addition to pair-wise comparisons.

CHAPTER THREE: RESULTS

3.1: Soil Microbial Analysis

3.1.1: Polymerase Chain Reaction (PCR)

Following PCR reactions for fungal 18S rRNA gene fragments, my negative control showed amplification. Amplification within my negative control suggests contamination occurred within samples or that reagents and lab artefacts contained trace fungal DNA that could be detected using our methods and and was of similar intensity in banding patterns as the samples. Therefore, further fungal analyses were not conducted. Bacterial PCR reactions for 16S rRNA gene fragments resulted in amplification of 16 soil samples out of 45 total.

3.1.2: DGGE and 16S rDNA Sequencing

A total of 17 species (estimated by banding patterns) were detected via DGGE from soil samples collected from our study area (Figure 4). The Agrotourism Tupak study plot had a variable number of species in relation to the method of composting used. Only 1 to 2 species were observed within control sites lacking compost with an average band number of 1.5. In plots where compost was mixed into the soil, I only detected 1 species. I found 3 to 6 species with an average of 5.0 species in sites where a layer of compost was spread over the surface. Agroforestry Tupak – Blok 1 had a range of 3 to 7 species with an average of 4.5 species. Samples from Banko Barat 3 exhibited 3 to 5 species, with an average of 4.2.

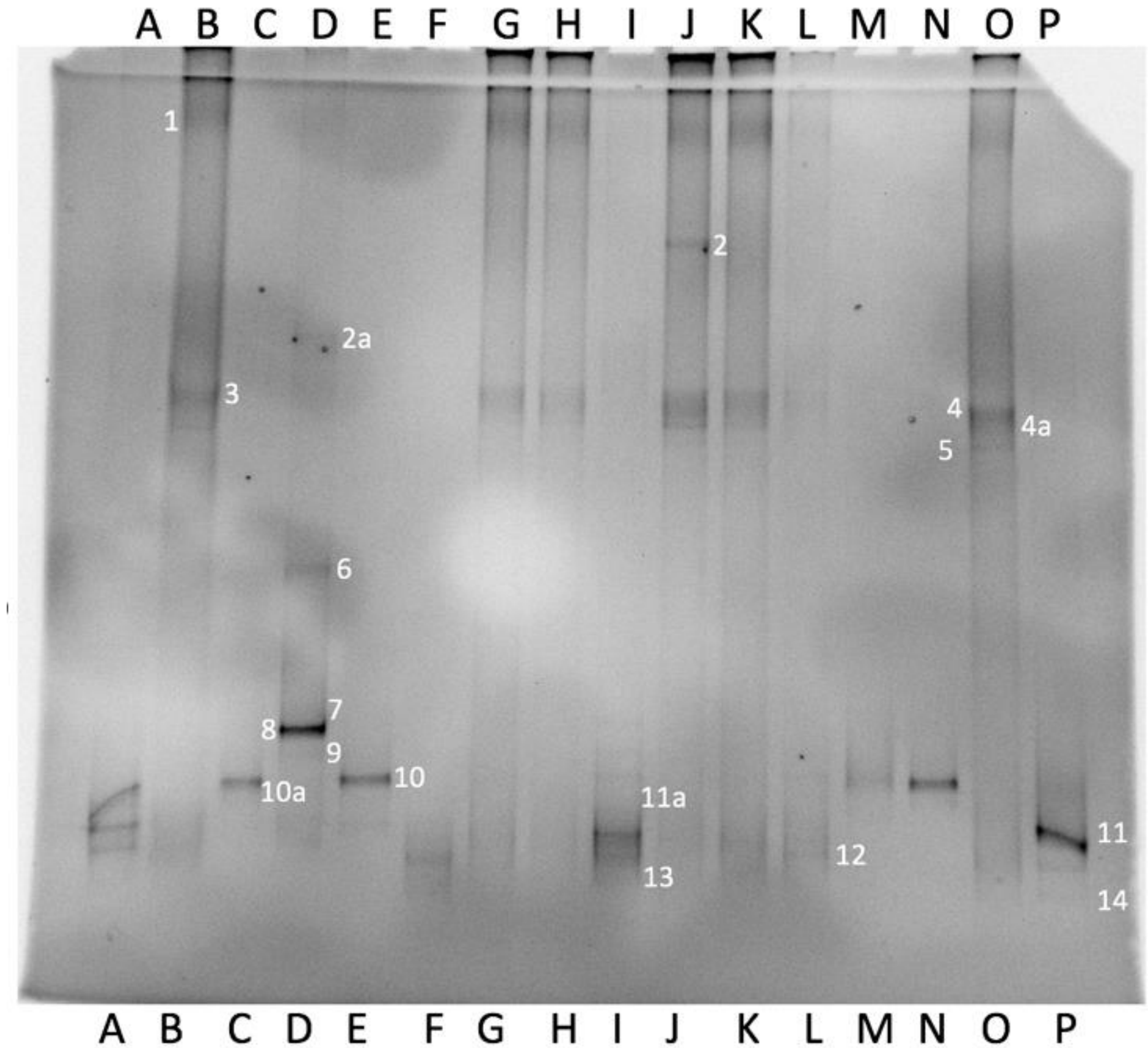

Figure 4. Denaturing gradient gel electrophoresis (DGGE) of bacterial community banding patterns from reclaimed soil. Letters A through P represent the microbial sample analyzed. Refer to Table 1 for naming scheme details and site location information, (A) AF11T2C, (B) BB13T2C, (C) AT1T0, (D) BB14T2C, (E) BB14T2C, (F) BB14T2C, (G) AF12T2C, (H) AF12T2C, (I) AF12T2C, (J) AT7T2C, (K) AT7T2C, (L) AT7T2C, (M) AT6T1C, (N) AT3T0, (O) BB13T2C, (P) AT9T2C. The white numbers represent the bands used in PCA, excluding 11a.

Sequenced bacterial 16S rRNA gene fragments from soil samples were related to Gram-negative bacteria including *Massilia aurea*, *Stenotrophomonas maltophilia*, *Delftia* spp., Acidobacteria spp., *and Ramlibacter* spp. Sequence matches were all above 89% based on the

RDP (Tables 2 and 3) which assigns sequences derived from bacterial 16S rRNA gene fragments to the corresponding taxonomy model used in RDP.

Table 2. Sequence matches for 16S rRNA gene fragments from DNA collected from reclaimed soil. The Ribosomal Database Project (RDP) software program Classifier was used and produced confidence values (in percentages) that denote the closest match to each taxonomic rank

PHYLUM	CLASS	ORDER	FAMILY	GENUS
Proteobacteria (100%)	Betaproteobacteria (100%)	Burkholderiales (100%)	Oxalobacteraceae (100%)	*Massilia (100%)*
Proteobacteria (100%)	Betaproteobacteria (100%)	Burkholderiales (100%)	Comamonadaceae (100%)	*Delftia (97%)*
Proteobacteria (100%)	Betaproteobacteria (100%)	Burkholderiales (100%)	Comamonadaceae (100%)	*Ramlibacter (52%)*
Proteobacteria (100%)	Gammaproteobacteria (100%)	Xanthomonadales (100%)	Xanthomonadaceae (100%)	*Stenotrophomonas (100%)*
Acidobacteria (100%)	Acidobacteria Gp_1 (98%)	Unknown	Unknown	*Candidatus Koribacter (52%)*

Table 3. Sequence matches for 16S rRNA gene fragments from DNA collected from reclaimed soil. The Ribosomal Database Project (RDP) software program SeqMatch was used to find the closest match to known cultured and uncultured bacteria contained in the RDP database (percent similarity and accession numbers for each match are in parentheses)

Band	Best Match Cultured Organism	Best Match Uncultured Organism
8	*Massilia aurea (97.1%; S000650723)*	uncultured Oxalobacteraceae bacterium (100.0%; S002476753)
10	*Delftia spp. (98.8%; S000146281; S001169258)*	*uncultured Delftia sp. (100.0%; S000976715)*
11a	*Ramlibacter spp. (89.0%; S000388105; S000394160)*	*uncultured Ramlibacter sp. (89.0%; multiple records)*
11	*Stenotrophomonas maltophilia (95.5%; S000009493)*	*uncultured Stenotrophomonas sp. (98.9%; S001178092)*
12	*Terriglobus roseus (63.2%; S000712490)*	uncultured Acidobacteria bacterium (80.3%; multiple records)

3.1.3: Principal Components Analysis (PCA)

Patterns in site characteristics in the different research plots were determined by means of PCA (Figure 3). Physical and chemical parameters included in the analysis were median metal concentration in subsurface waters, metal concentrations for topsoil and subsoil samples. Bacterial banding patterns were excluded from this analysis.

The PCA resulted in a separation between the different-aged plots (Figure 5). Dimension 1 accounted 32.3 % of the variance and dimension 2 accounted for a variance of 24.4 %. In dimension 1 almost all median subsurface water metal concentration, excluding Fe, resulted in highly positive component loadings (Table 4). Furthermore, percent carbon (0.89) within subsoil resulted in a positive component loading among sites within dimension 1. Dimension 2 revealed high correlations among sediment concentrations of manganese and zinc. Topsoil manganese concentrations correlated at 0.70, whereas subsoil samples had a value of 0.72. Zinc had similar component loading values to manganese with topsoil; loading of 0.78. Subsoil displayed a component loading of 0.86. Dimension 3 did not show any correlation values above the arbitrary 0.70 threshold.

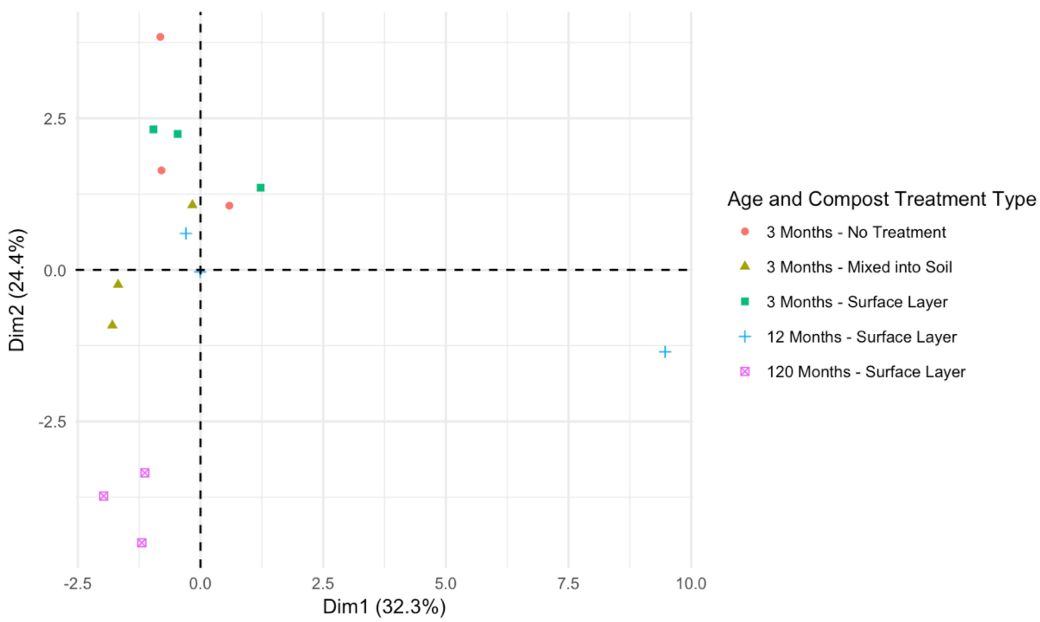

Figure 5. Principal components analysis (PCA) of sediment samples and median metal concentration in subsurface water. The DGGE banding patterns of bacteria were excluded to determine physical and chemical characteristics present within research plots.

Table 4. Principal components analysis (PCA) results of sediment samples and median metal concentration in subsurface water. Bold values indicate variables that correlated at a high level in the PCA analysis

Table 4. Principal components analysis (PCA) correlation results of sediment samples and median metal concentrations in subsurface waters. Bold values indicate variables that correlated at a high level in PCA analysis

Full Site Characteristics 3 Months - 120 Months	Dimension 1	Dimension 2	Dimension 3
Median Al (ng/mL)	**0.92**	-0.27	0.03
Median Mn (ng/mL)	**0.91**	-0.15	0.03
Median Fe (ng/mL)	0.44	-0.65	0.01
Median Ni (ng/mL)	**0.95**	-0.24	-0.07
Median Zn (ng/mL)	**0.92**	-0.32	0.02
Median Pb (ng/mL)	**0.93**	-0.26	0.02
% Carbon - Topsoil	0.47	0.24	-0.57
% Carbon - Subsoil	**0.89**	0.12	-0.16
% Silt & Clay - Topsoil	-0.27	0.26	-0.50
% Silt & Clay - Subsoil	0.32	0.50	-0.06
Al - Topsoil (mg/Kg)	0.05	-0.41	0.20
Al - Subsoil (mg/Kg)	0.30	-0.42	0.74
Fe - Topsoil (mg/Kg)	0.23	-0.02	0.58
Fe - Subsoil (mg/Kg)	-0.10	-0.16	0.93
Mn - Topsoil (mg/Kg)	0.58	**0.70**	-0.15
Mn - Subsoil (mg/Kg)	0.38	**0.72**	0.13
Ni - Topsoil (mg/Kg)	0.64	0.63	0.03
Ni - Subsoil (mg/Kg)	-0.06	**0.80**	0.46
Pb - Topsoil (mg/Kg)	0.48	0.45	0.43
Pb - Subsoil (mg/Kg)	-0.26	0.50	0.49
Zn - Topsoil (mg/Kg)	0.25	**0.78**	-0.12
Zn - Subsoil (mg/Kg)	-0.06	**0.86**	0.20

A PCA was also conducted that included DGGE results. (Band 11a was excised and observed only once. It was located adjacent to band 11. Band 11a could not be included in the PCA because it was potentially obscured by band 11). Physical, chemical, and microbial parameters included in the analysis were the presence or absence of particular species banding patterns, % C, grain size, and total metal concentrations in topsoil. The PCA of all surface samples collected from all research plots and species (Figure 6A) resulted in a separation between the different-aged plots. Dimension 1 accounted for 30.2 % of the variance and dimension 2 accounted for 18.6 % of the variance. There was a distinct separation in dimension 2 between the younger reclaimed plots, Agrotourism Tupak, and Agroforestry Tupak – Blok 1 when compared to the older site Banko Barat 3 (Figure 6A). The results of PCA among all treatments and aged plots in Figure 6A showed that dimension 1 was primarily driven by species 1, 2a, 3, 4a, 5, and 12 (Table 5). Zinc was also found to have a highly correlated component loading of 0.81 in dimension 2 (Table 5).

The PCA of all surface samples collected from the different-aged plots using the compost treatment where a layer of compost was spread over the soil was conducted to determine if age since compost application may correlate with microbial diversity (Figure 6B). There was a distinct age separation between plots that varied in age since compost was applied. Component loadings (Table 6) were high in dimension 1 for total metal concentrations in topsoil for Mn (-0.71) and Ni (-0.82). The only total metal concentration with a high component loading in dimension 2 was zinc which had a value of 0.81. Species that had the highest component loadings were 4a, 4, 6, 9 12, and 13 which were found to be most influential in dimensions 1, 2 and 3.

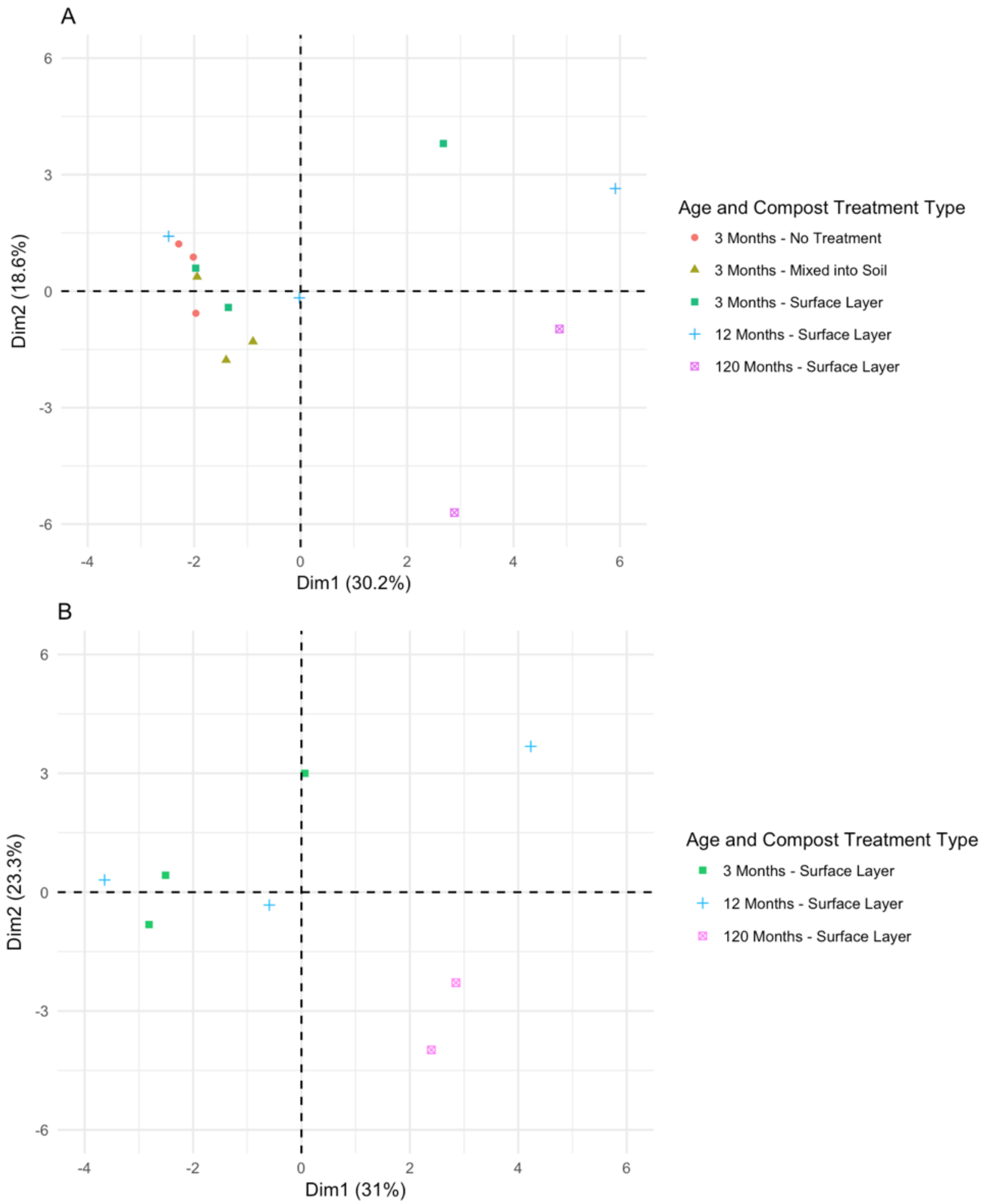

Figure 6. (A) Principal components analysis (PCA) of all surface samples and denaturing gradient gel electrophoresis (DGGE) banding patterns of bacteria. (B) Principal components analysis (PCA) of surface samples and denaturing gradient gel electrophoresis (DGGE) banding patterns of bacteria (excluding species 10a) collected from the different-aged plots but only surface layer treatments were used in PCA.

Table 5. Principal components analysis (PCA) correlation results from Figure 6A of total metal concentrations in soils, % carbon, % silt + clay, and denaturing gradient gel electrophoresis (DGGE) banding patterns of bacteria. Bold values indicate variables that correlated at a high level in PCA analysis

Surface Characteristics 3 Months - 120 Months	**Dimension 1**	**Dimension 2**	**Dimension 3**
% Carbon - Topsoil	0.17	0.68	0.39
% Silt & Clay - Topsoil	0.35	0.05	0.52
Al - Topsoil (mg/Kg)	0.69	0.05	-0.20
Fe - Topsoil (mg/Kg)	-0.05	0.07	-0.52
Mn - Topsoil (mg/Kg)	-0.50	0.68	0.18
Ni - Topsoil (mg/Kg)	-0.54	0.63	-0.02
Pb - Topsoil (mg/Kg)	-0.27	0.50	-0.36
Zn - Topsoil (mg/Kg)	-0.18	**0.81**	0.23
Species 1	**0.85**	0.44	-0.22
Species 2	0.27	0.49	-0.10
Species 2a	**0.73**	0.06	0.45
Species 3	**0.85**	0.44	-0.22
Species 4	0.49	-0.13	**-0.78**
Species 4a	**0.80**	0.16	-0.19
Species 5	**0.85**	0.44	-0.22
Species 6	0.58	-0.63	-0.36
Species 7	0.60	0.34	0.52
Species 8	0.29	**-0.73**	0.29
Species 9	0.65	-0.29	0.60
Species 10	0.33	0.09	0.55
Species 10a	-0.23	0.16	0.11
Species 11	0.55	0.01	0.35
Species 12	**0.89**	-0.03	0.00
Species 13	0.39	0.19	-0.63
Species 14	0.11	-0.58	0.13

Table 6. Principal components analysis (PCA) correlation results from Figure 6B of total metal concentrations in soils, % carbon, and % silt + clay collected from surface layer treatments within the different-aged plots and denaturing gradient gel electrophoresis (DGGE) banding patterns of bacteria (excluding species 10a). Bold values indicate variables that correlated at a high level in PCA analysis

Surface Characteristics 3 Months - 120 Months	**Dimension 1**	**Dimension 2**	**Dimension 3**
% Carbon - Topsoil	0.00	**0.85**	-0.10
% Silt & Clay - Topsoil	0.63	0.30	-0.40
Al - Topsoil (mg/Kg)	0.69	-0.13	0.21
Fe - Topsoil (mg/Kg)	-0.08	-0.56	0.75
Mn - Topsoil (mg/Kg)	**-0.71**	0.58	-0.13
Ni - Topsoil (mg/Kg)	**-0.82**	0.49	0.04
Pb - Topsoil (mg/Kg)	-0.67	0.25	0.41
Zn - Topsoil (mg/Kg)	-0.23	**0.93**	0.00
Species 1	0.68	0.48	0.52
Species 2	0.01	0.48	0.24
Species 2a	0.63	0.29	-0.40
Species 3	0.68	0.48	0.52
Species 4	0.40	-0.37	**0.81**
Species 4a	**0.75**	0.17	0.40
Species 5	0.68	0.48	0.52
Species 6	0.56	**-0.77**	0.21
Species 7	0.59	0.59	-0.29
Species 8	0.33	-0.64	-0.54
Species 9	**0.70**	-0.04	-0.63
Species 10	0.56	0.25	-0.49
Species 11	0.31	0.17	-0.52
Species 12	**0.85**	0.01	0.05
Species 13	0.01	-0.01	**0.71**
Species 14	-0.04	-0.59	-0.43

The PCA of younger surface samples and banding patterns of bacteria collected at Agrotourism Tupak exhibited a strong separation based on the compost treatment used (Figure 7). The majority of variance observed was described by dimension 1 which accounted for 42.4 % of the variance; dimension 2 accounted for 17.0 % of the variance. Component loadings below 0.70 indicated poor correlation among sediment characteristics in dimension 1 (Table 5).

Presence of several species' (e.g., 1, 2, 2a, 5, and 12) banding patterns was very highly correlated with the first principal component (loading = 0.96). Furthermore, species 11 and 13 showed high component loadings of 0.76. There were high component loadings within dimension 2 among sediment concentrations of iron (0.76) manganese (0.70), and nickel (0.72).

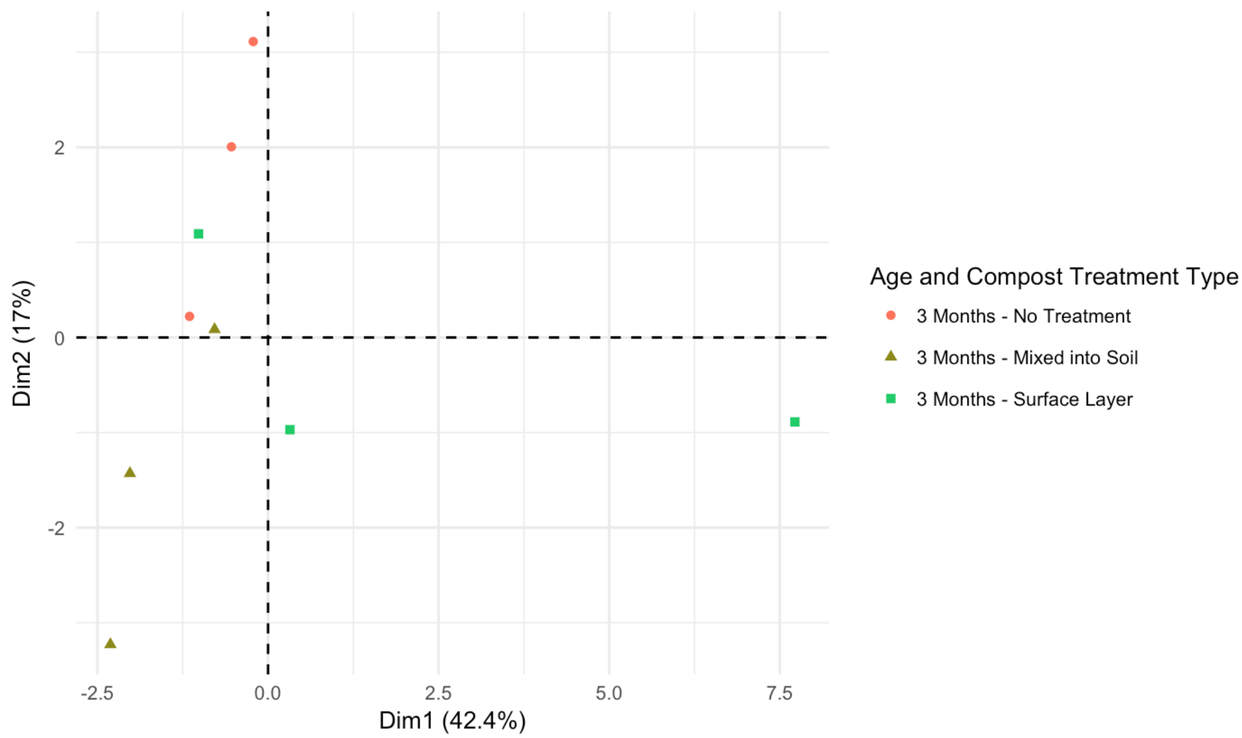

Figure 7. Principal components analysis (PCA) of total metal concentrations in soils, % carbon, % silt + clay, and denaturing gradient gel electrophoresis (DGGE) banding patterns of bacteria collected from reclaimed soil at the Agrotourism Tupak research plot.

Table 7. Principal components analysis (PCA) correlation results from Figure 7 of total metal concentrations in soils, % carbon, % silt + clay, and denaturing gradient gel electrophoresis (DGGE) banding patterns of bacteria collected from reclaimed soil at the Agrotourism Tupak research plot. Bold values indicate variables that correlated at 0.70 or greater in PCA

Surface Characteristics 3 Months	**Dimension 1**	**Dimension 2**	**Dimension 3**
% Carbon - Topsoil	0.20	0.04	-0.59
% Silt & Clay - Topsoil	-0.18	0.52	-0.34
Al - Topsoil (mg/Kg)	0.30	0.45	-0.39
Fe - Topsoil (mg/Kg)	0.21	**0.76**	0.36
Mn - Topsoil (mg/Kg)	0.19	**0.70**	-0.09
Ni - Topsoil (mg/Kg)	0.55	**0.72**	0.34
Pb - Topsoil (mg/Kg)	0.60	0.51	0.42
Zn - Topsoil (mg/Kg)	0.54	0.52	0.11
Species 1	**0.96**	-0.17	-0.19
Species 2	**0.96**	-0.17	-0.19
Species 2a	**0.96**	-0.17	-0.19
Species 3	**0.96**	-0.17	-0.19
Species 5	**0.96**	-0.17	-0.19
Species 10	0.39	0.35	-0.31
Species 10a	-0.07	0.39	0.03
Species 11	**0.76**	-0.28	0.53
Species 12	**0.96**	-0.17	-0.19
Species 13	**0.76**	-0.28	0.53
Species 14	0.04	-0.19	0.90

3.2: Water Analysis

Several major and trace metals were significantly correlated (Table 8). Manganese had strong correlations with nickel ($r = 0.75$), but was not correlated with iron. All other measures of elements were weak to moderately correlated with each other (Table 8).

Table 8. Correlation matrix for metal concentrations in subsurface water samples. Bold values indicate a significance level 0.05 followed by (*) very weak correlation (**) weak correlation (***) moderate correlation and (****) strong correlation

	Al (ng/mL)	Mn (ng/mL)	Fe (ng/mL)	Ni (ng/mL)	Zn (ng/mL)	Pb (ng/mL)
Al (ng/mL)	1.00					
Mn (ng/mL)	**0.27***	1.00				
Fe (ng/mL)	**0.65*****	0.20	1.00			
Ni (ng/mL)	**0.42****	**0.75*******	**0.43****	1.00		
Zn (ng/mL)	**0.58*****	**0.37****	**0.64*****	**0.46****	1.00	
Pb (ng/mL)	**0.44****	**0.30****	**0.59*****	**0.45****	**0.69*****	1.00

Neither compost treatment nor age were found to consistently differ among metal concentrations in subsurface waters (Figure 8A-8F). Manganese and zinc showed no significant differences among different-aged plots or the control sites when compared to compost treatments (8C and 8F). Although, there were water samples that had manganese concentrations above Indonesia drinking water standards (400 ng/mL), the median concentration were below these limits. Median values ranged from 26.83 ng/mL to 210.20 ng/mL among sites (Table 9). Zinc had lower concentrations in subsurface waters in comparison to manganese with median values that ranged from 0.27 ng/mL to 4.80 ng/mL among sites (Table 9).

In contrast, aluminum concentrations in subsurface waters showed a significant difference between the Agrotourism Tupak control sites and Banko Barat 3 (Figure 8A); however, this is not consistent with the other metals measured (Figures 8B-8F). In addition, one water sample had an aluminum concentration above the USEPA aquatic life criteria standards. However, median concentration of aluminum grouped by compost treatment and age combinations ranged from 1.14 ng/mL to 4.50 ng/mL (Table 9) and were below drinking water standards for the USEPA and Indonesia (Figure 8A).

Iron concentrations showed significant differences between Agrotourism Tupak control sites when compared to Agroforestry Tupak – Blok 1 and Banko Barat 3. Median concentration ranged from 2.18 ng/mL to 4.05 ng/mL for the different compost treatments and aged plots (Table 9). In addition, iron concentrations were below Indonesia drinking water standards (300 ng/mL), maximum concentration of 67.94 ng/mL which was in water samples collected from Agroforestry Tupak – Blok 1 (Figure 8B).

Nickel concentrations in subsurface waters were below USEPA aquatic life standards (52 ng/mL) and had median concentration that ranged from 1.09 ng/mL to 7.91 ng/mL among sites (Table 9). Agroforestry Tupak – Blok 1 had the highest median Ni concentration of 7.91 ng/mL (Table 9) and a maximum concentration of 36.0 ng/mL (Figure 8D). Significant differences were observed between Agrotourism Tupak control sites and surface layer compost treatments within Agrotourism Tupak and Banko Barat 3 research plots (Figure 8D).

Lead concentrations among the different compost treatments and age groups were more variable in comparison to the other metals analyzed (Figure 8E). Subsurface water samples analyzed for Pb showed median concentration that ranged from below instrument detection limits ($4.50e^{-08}$ ng/mL) to 1.26 ng/mL (Table 9). Agrotourism Tupak control sites differed significantly from all the surface layer compost treatments among the three different-aged plots (Figure 8E). In addition, there were several water samples that had Pb concentrations above USEPA and Indonesia water quality standards, and a maximum concentration of 106.6 ng/mL was observed in Agroforestry Tupak – Blok 1 (Figure 8E).

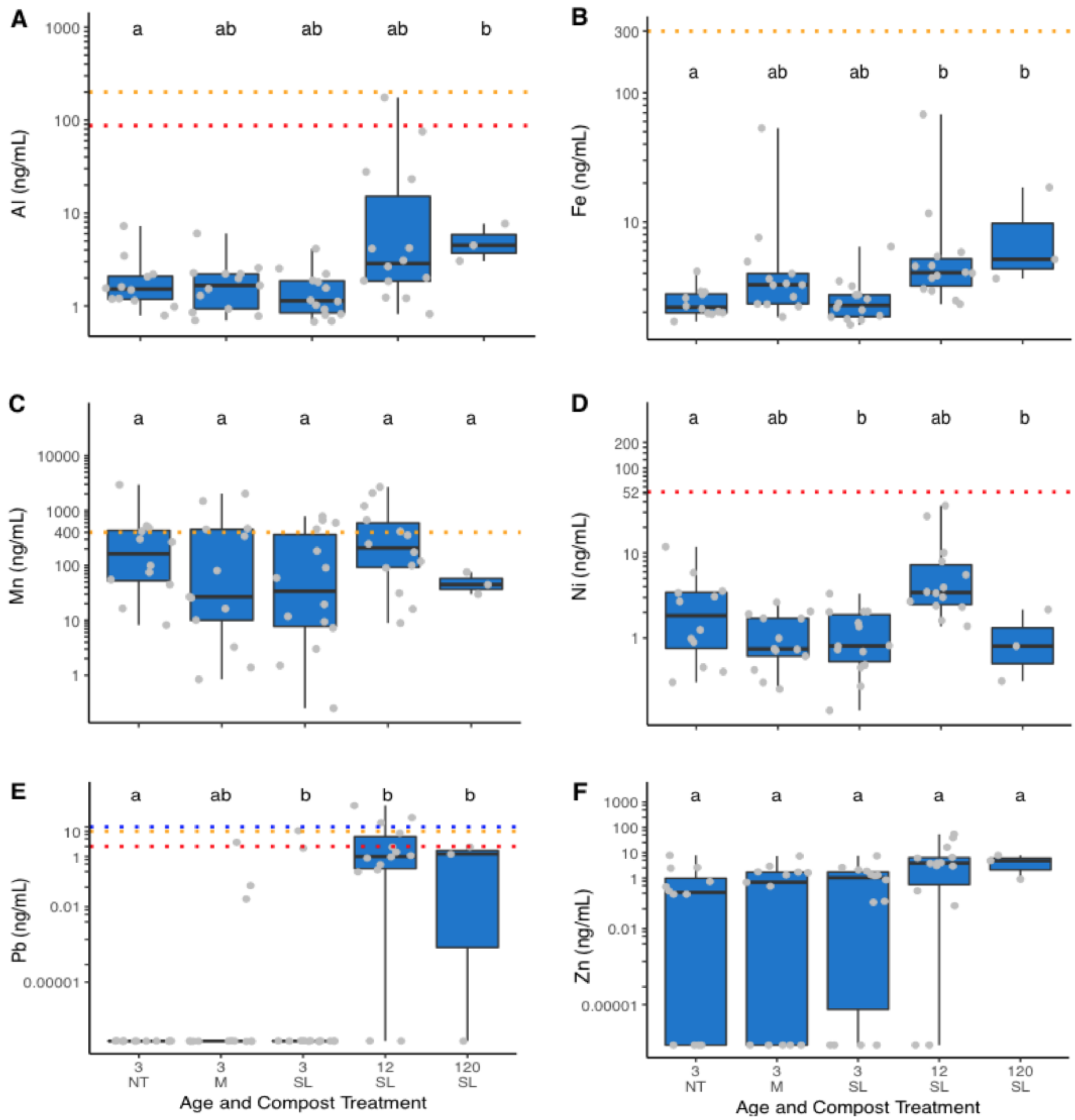

Figure 8. Concentrations of (A) aluminum (ng/mL), (B) iron (ng/mL), (C) manganese (ng/mL), (D) nickel (ng/mL), (E) lead (ng/mL), and (F) zinc (ng/mL) in subsurface waters collected in the 3-month old Agrotourism Tupak plot, 12-month old Agroforestry Tupak - Blok 1 plot, and 120-month old Banko Barat 3 plot. Concentrations are plotted using a $\log_{10}$ scale. Compost treatments were classified as (NT) no treatment, or control, (M) compost was mixed in with reclaimed soil, and (SL) a 5-cm thick layer of compost applied over reclaimed soil. Orange dotted lines indicate the Indonesian drinking water standards for major and trace metals, red dotted lines indicate USEPA freshwater aquatic life chronic criteria standards of major and trace metals; and blue dotted lines indicate USEPA drinking water standards for major and trace metals. Treatments sharing the same letter were not significantly different in pair-wise comparisons among different aged plots and compost treatments.

Table 9. Metal concentrations (medians, means [standard deviations]) in subsurface water samples grouped by compost treatment and age combinations, (AT) Agrotourism Tupak, (AF) Agroforestry Tupak – Blok 1, (BB) Banko Barat 3

Research Plot	Site Age (Months)	Compost Treatment	Al (ng/mL)	Fe (ng/mL)	Mn (ng/mL)	Ni (ng/mL)	Pb (ng/mL)	Zn (ng/mL)
AT	3	Control	1.52, 2.08 [1.78]	2.18, 2.43 [0.67]	183.06, 436.40 [813.05]	1.97, 2.88 [3.27]	NA, NA [NA]	0.27, 1.23 [2.26]
AT	3	Mixed	1.66, 1.92 [1.39]	3.26, 7.27 [13.90]	26.83, 379.93 [644.39]	0.74, 1.13 [0.77]	NA, 0.29 [1.01]	0.67, 1.36 [2.02]
AT	3	Surface layer	1.14, 1.52 [0.95]	2.27, 2.61 [1.24]	39.35, 206.55 [287.41]	0.81, 1.19 [0.90]	NA, 0.89 [2.80]	1.04, 1.35 [1.97]
AF	12	Surface layer	2.88, 23.15 [48.09]	4.05, 9.03 [17.11]	210.20, 585.39 [834.75]	3.42, 7.91 [10.44]	0.99, 12.84 [28.90]	3.82, 9.93 [16.11]
BB	120	Surface layer	4.50, 5.07 [2.36]	5.14, 9.10 [8.19]	44.69, 49.98 [23.12]	0.80, 1.09 [0.95]	1.26, 1.20 [1.17]	4.80, 4.51 [3.47]

3.3: Soil Analysis

3.3.1: Total Carbon

There was a significant difference in the percent carbon (% C) within topsoil between the Agroforestry Tupak – Blok 1 and Banko Barat 3 sites (Figure 7A, Table 10). However, subsoil samples (Figure 7B, Table 10) did not show a statistical difference among compost application treatments or plot age groups. Agrotourism Tupak topsoil samples collected in control sites had a mean of 2.62% C, and subsoil samples had a mean of 1.95% C. Sites that had compost mixed into the soil had a mean of 4.23% C within topsoil samples, and a mean of 2.45% C in subsoil samples. Sites containing a surface layer of compost had a mean of 4.27% C within the topsoil, and a mean of 3.88% C in subsoil samples. Agroforestry Tupak – Blok 1 had slightly higher carbon percentages with a mean of 6.34% C in topsoil, and a mean of 6.31% C in subsoil. Banko Barat 3 had, in general, fairly low carbon percentages with a mean of 1.77% C in topsoil samples, and a mean of 0.62% C within subsoil samples.

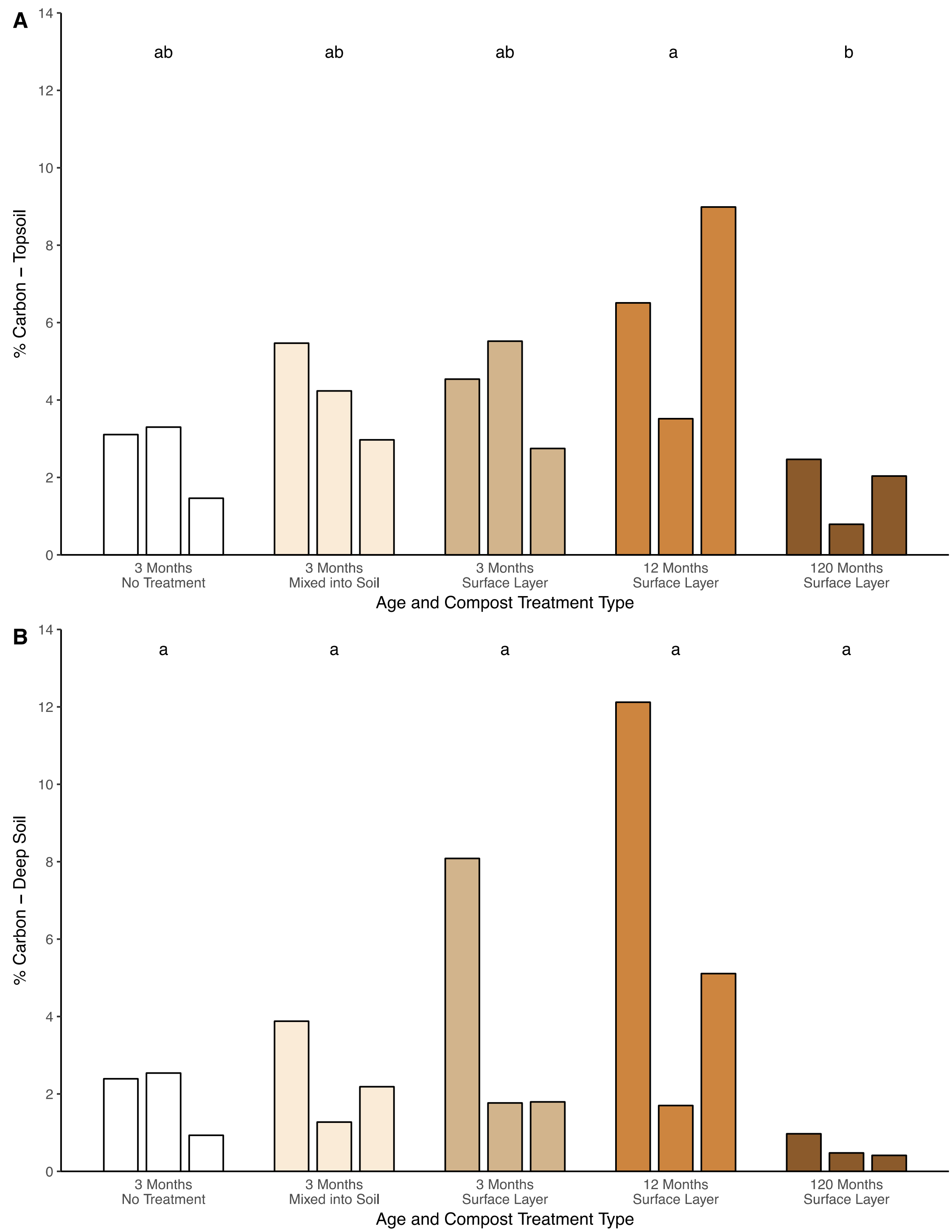

Figure 9. Percent carbon in sediment collected from (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas. Treatments sharing the same letter were not significantly different in pair-wise comparisons among different aged plots and compost treatments.

Table 10. Percent carbon (medians, means [standard deviations]) in topsoil and subsoil samples that are grouped by compost treatment and age combinations, (AT) Agrotourism Tupak, (AF) Agroforestry Tupak – Blok 1, (BB) Banko Barat 3

Research Plot	Site Age (Months)	Compost Treatment	% Carbon Topsoil	% Carbon Subsoil
AT	3	Control	3.11, 2.62 [1.01]	2.39, 1.95 [0.89]
AT	3	Mixed	4.23, 4.23 [1.25]	2.19, 2.45 [1.32]
AT	3	Surface layer	4.54, 4.27 [1.41]	1.80, 3.88 [3.64]
AF	12	Surface layer	6.51, 6.34 [2.74]	5.11, 6.31 [5.31]
BB	120	Surface layer	2.04, 1.77 [0.87]	0.48, 0.62 [0.31]

3.3.2: Total Metal Concentrations in Soil

The geochemical results show no significant differences in metal concentrations among compost treatments and age combinations within topsoil and subsoil samples collected at PTBA (Figures 10-15, Table 11). In addition, all the metals analyzed within this study were within range of global background soils, and below the World Health Organization's maximum permissible metal limits for soils, consensus-based threshold effect, and probable effect guidelines.

Table 11. Metal concentrations (medians, means [standard deviations]) for (A) topsoil and (B) subsoil grouped by compost treatment and age combinations, (AT) Agrotourism Tupak, (AF) Agroforestry Tupak – Blok 1, (BB) Banko Barat 3

A. Topsoil

Research Plot	Site Age (Months)	Compost Treatment	Al (mg/Kg)	Fe (mg/Kg)	Mn (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
AT	3	Control	3540.27, 3268.71 [513.39]	26994.64, 28656.71 [6480.61]	741.02, 702.72 [125.78]	9.93, 11.51 [3.13]	19.53, 19.43 [0.72]	93.54, 103.78 [40.13]
AT	3	Mixed	2668.46, 3042.44 [801.09]	13927.95, 13213.19 [2469.72]	421.58, 494.73 [169.90]	5.77, 6.14 [2.71]	12.57, 14.69 [4.84]	56.41, 66.60 [18.06]
AT	3	Surface layer	3630.06, 3288.95 [702.20]	21386.94, 19964.32 [2989.66]	696.68, 815.51 [257.15]	11.64, 11.31 [1.57]	20.49, 19.85 [2.34]	86.82, 94.28 [23.97]
AF	12	Surface layer	9903.46, 8580.10 [2793.67]	20467.52, 19642.04 [5323.04]	684.54, 792.73 [206.11]	8.76, 10.01 [4.87]	15.87, 17.03 [3.21]	84.45, 91.35 [12.77]
BB	120	Surface layer	NA, NA [NA]	NA, NA [NA]	155.32, 168.64 [32.99]	2.61, 2.77 [0.37]	15.35, 15.74 [0.98]	41.33, 38.27 [10.19]

B. Subsoil

Research Plot	Site Age (Months)	Compost Treatment	Al (mg/Kg)	Fe (mg/Kg)	Mn (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
AT	3	Control	3207.49, 3702.58 [1244.30]	22590.20, 21583.42 [2277.08]	748.47, 847.79 [311.33]	14.74, 13.54 [2.11]	20.87, 19.92 [5.09]	81.61, 91.76 [36.48]
AT	3	Mixed	3628.20, 3607.58 [463.93]	21380.88, 19631.37 [3044.66]	828.50, 762.53 [204.45]	10.18, 9.94 [2.62]	19.64, 18.34 [2.37]	84.74, 79.21 [10.01]
AT	3	Surface layer	3265.38, 3773.56 [996.40]	22195.10, 21231.68 [1906.14]	792.21, 774.88 [38.67]	NA, NA [NA]	NA, NA [NA]	NA, NA [NA]
AF	12	Surface layer	3666.19, 3906.61 [1771.30]	20910.67, 19811.70 [5392.38]	866.98, 837.39 [204.67]	6.90, 8.76 [3.35]	18.33, 18.04 [2.85]	67.95, 72.77 [14.18]
BB	120	Surface layer	NA, NA [NA]	NA, NA [NA]	85.68, 148.36 [152.26]	0.58, 4.35 [6.56]	18.54, 17.02 [5.91]	52.98, 45.26 [17.36]

Topsoil and subsoil metal concentrations for aluminum were variable among sites. Topsoil samples had median concentration that ranged from 2669 mg/Kg to 9904 mg/Kg among the Agrotourism Tupak and Agroforestry Tupak – Blok 1 sites. Banko Barat 3 only had one sample available; its aluminum concentration was 1158 mg/Kg (Figure 10A). In subsoil samples, median concentration ranged from 3207 mg/Kg to 3666 mg/Kg among the two younger plots; the concentration within the one sample at Banko Barat 3 was 7712 mg/Kg (Figure 10B).

Iron had the highest median concentrations of all the metals analyzed within topsoil and subsoil samples. Topsoils exhibited a median that ranged from 13,928 mg/Kg to 21,387 mg/Kg. Subsoil medians ranged from 20,911 mg/Kg to 22,590 mg/Kg in the younger research plots. Banko Barat 3 only had one sediment sample available in topsoil with a concentration of 29,748 mg/Kg (Figure 11A); the sites, subsoil concentration of 31,828 mg/Kg (Figure 11A).

Although there were no significant differences in metal concentrations among compost treatments and age combinations, the lowest concentrations of Mn were observed within Banko Barat 3 (Figure 12) where the median concentration was 155 mg/Kg for topsoil samples and

85.68 mg/Kg for subsoil. The median concentration for Agrotourism Tupak and Agroforestry Tupak - Blok 1 plots ranged from 422 mg/Kg to 741 mg/Kg in topsoil samples, and 792 mg/Kg to 8667 mg/Kg in subsoil samples.

Minor variations in metal concentrations for nickel, lead, and zinc among topsoil and subsoil samples were present; however, differences between compost treatment and age were not significant (Figures 13-15). Median concentration for nickel ranged from 2.61 mg/Kg to 9.93 mg/Kg among compost treatments and age in topsoils, and 0.58 mg/Kg to 14.74 mg/Kg in subsoils. Lead median concentration ranged from 12.57 mg/Kg to 20.49 mg/Kg in topsoils and 18.33 mg/Kg to 20.87 mg/Kg in subsoils. Zinc had a topsoil median concentration that ranged between the sites from 41.33 mg/Kg to 93.54 mg/Kg, and 52.98 mg/Kg to 84.74 mg/Kg in subsoils.

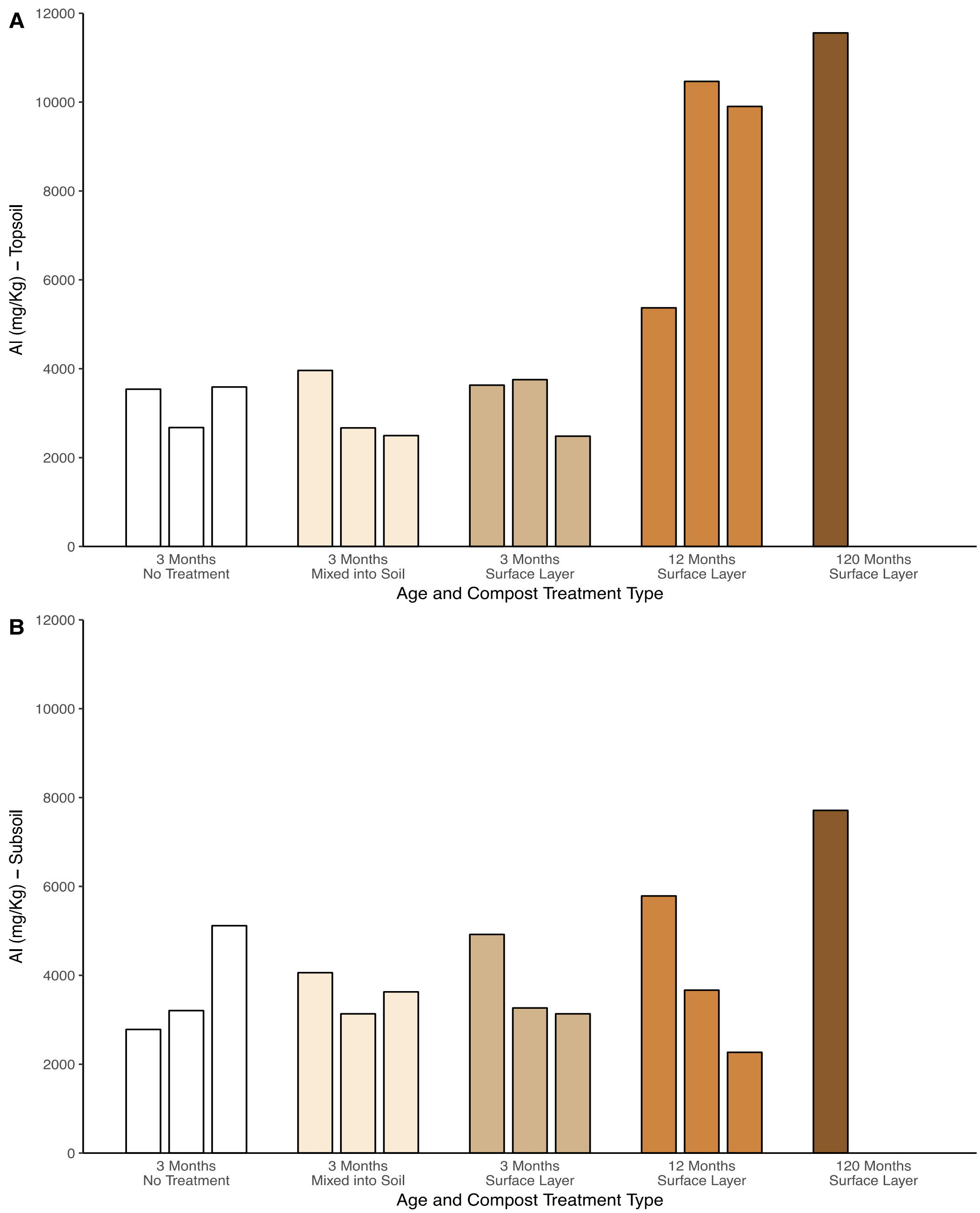

Figure 10. Concentrations of Al (mg/Kg) in sediment collected from the (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

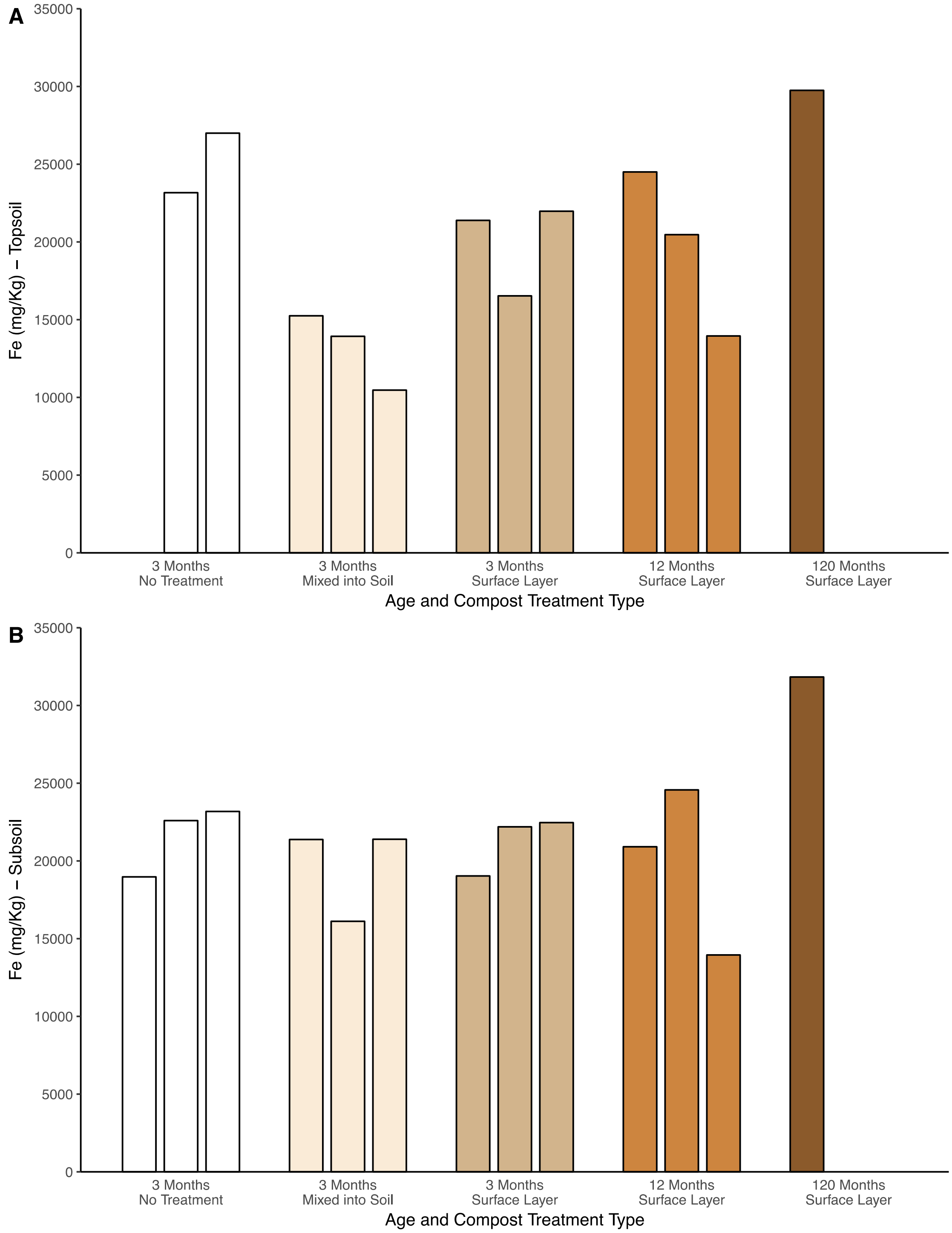

Figure 11. Concentrations of Fe (mg/Kg) in sediment collected from (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

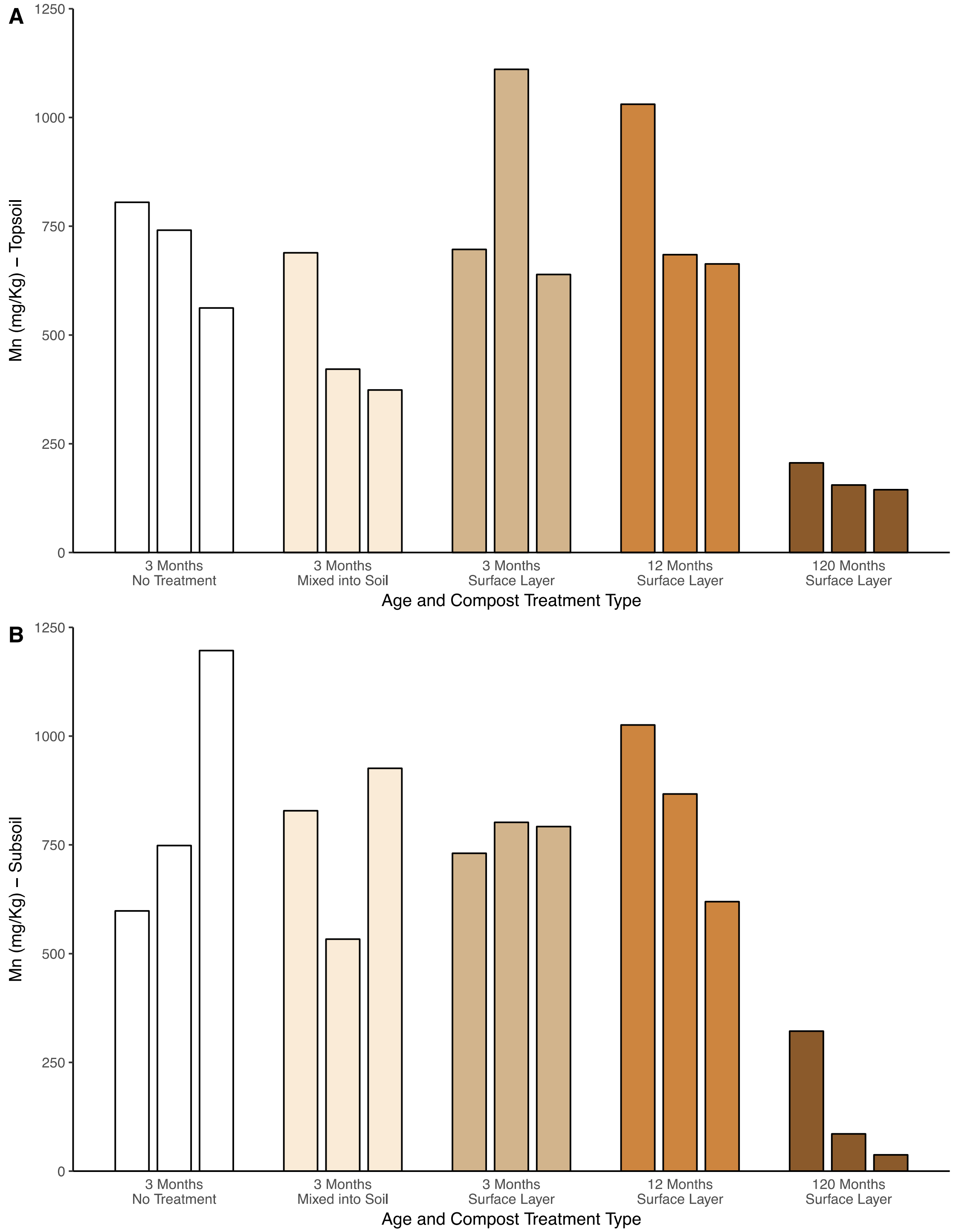

Figure 12. Concentrations of Mn (mg/Kg) in sediment collected from the (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

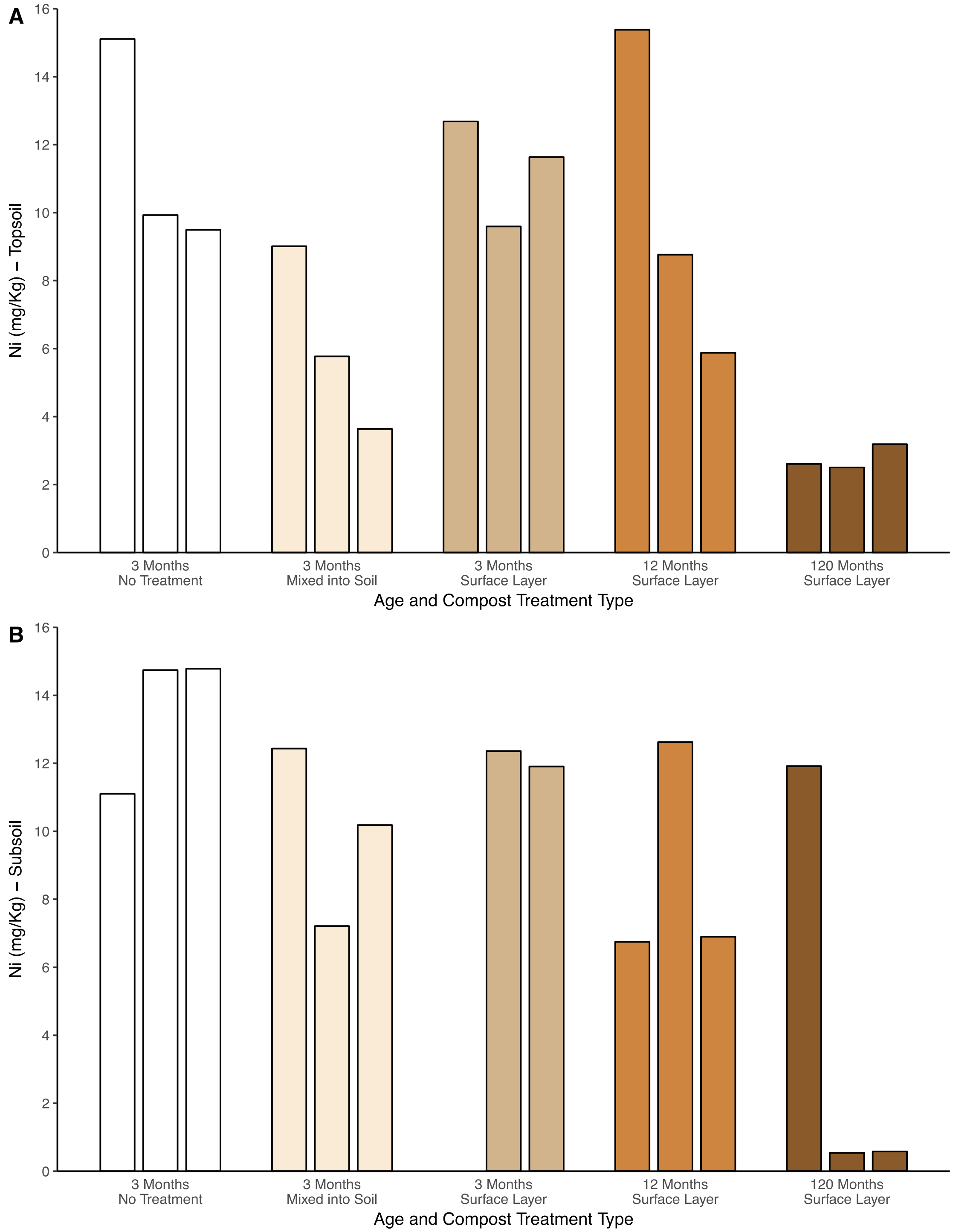

Figure 13. Concentrations of Ni (mg/Kg) in sediment collected from the (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

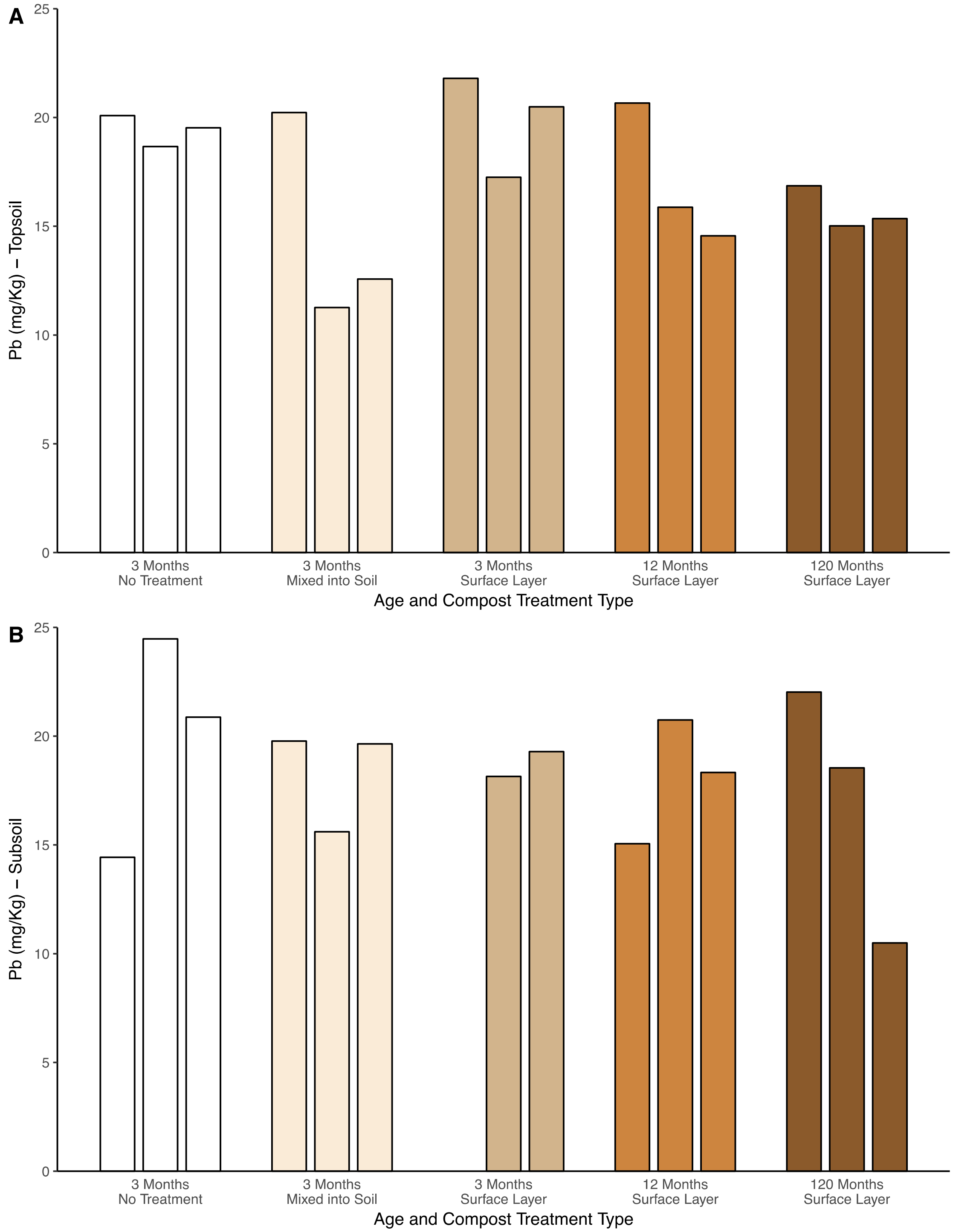

Figure 14. Concentrations of Pb (mg/Kg) in sediment collected from (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

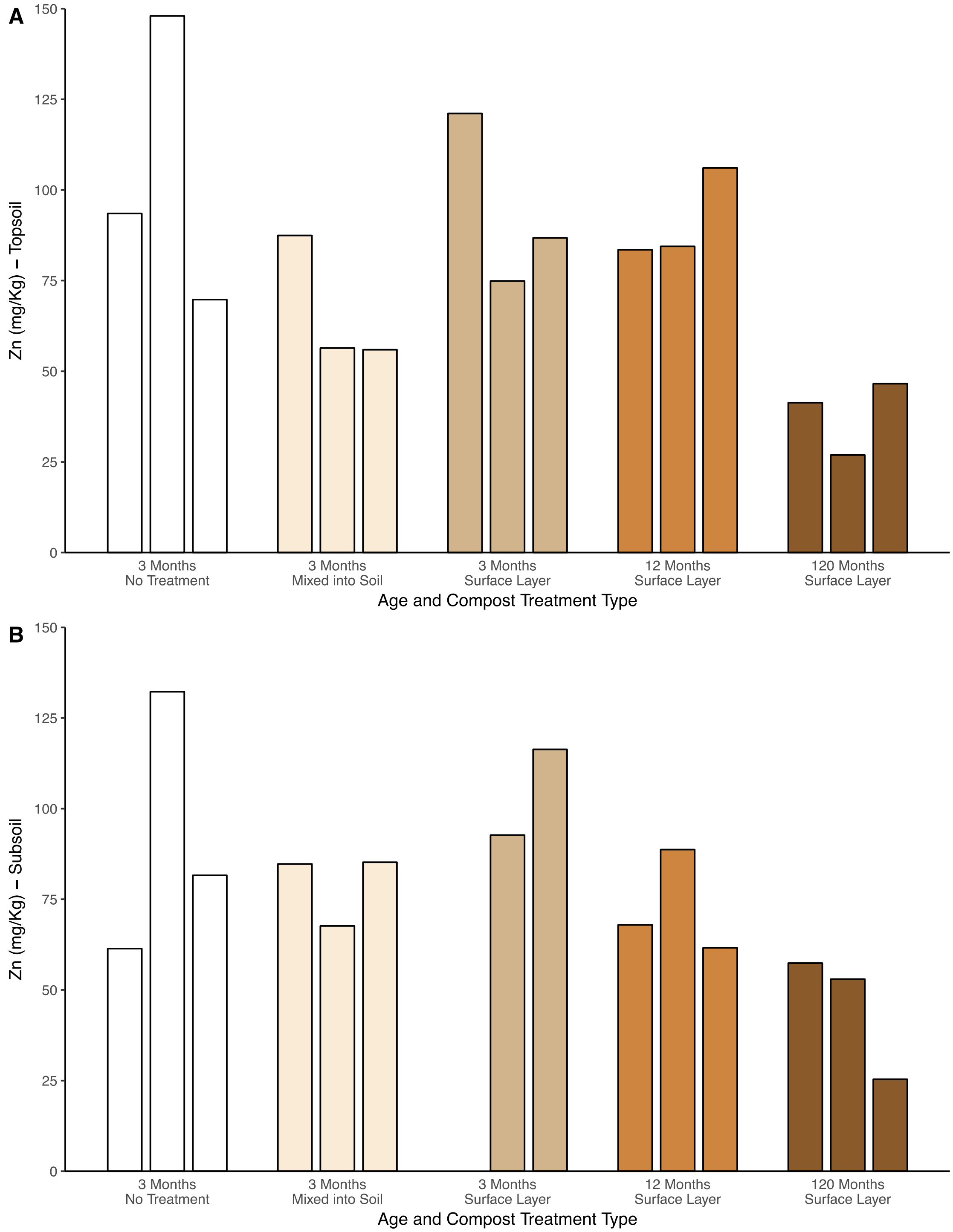

Figure 15. Concentrations of Zn (mg/Kg) in sediment collected from the (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

3.3.3: Grain Size Distribution

We found no significant differences in the amount of fine (silt plus clay) particles in samples when compared to compost treatments and age combinations (Figure 16, Table 12). Banko Barat 3 had the lowest percentage of silt and clay (the mean concentration was 71.94 % in topsoil and 70.85 % in subsoil). Agrotourism Tupak had the highest percentage of fine-grained material (the mean in topsoil was 81.75 %, the mean in subsoil samples was 88.64 % within control sites). Agrotourism Tupak sites that had compost mixed into the reclaimed soil exhibited a mean of 77.49 % fine particles in topsoil and a mean of 78.58 % in subsurface soils. Treatments within the three different-aged plots that had a 5-cm thick layer of compost spread over the reclaimed soil surface had means that ranged from 71.94% to 78.54% in topsoil, and 70.85% to 88.28% in subsoil samples.

Table 12. Percent silt plus clay (medians, means [standard deviations]) in topsoil and subsoil samples that are grouped by compost treatment and age combinations, (AT) Agrotourism Tupak, (AF) Agroforestry Tupak – Blok 1, (BB) Banko Barat 3.

Research Plot	Site Age (Months)	Compost Treatment	% Silt/Clay Topsoil	% Silt/Clay Subsoil
AT	3	Control	79.76, 81.75 [8.10]	90.33, 88.64 [5.28]
AT	3	Mixed	71.87, 77.49 [13.48]	85.63, 78.58 [13.90]
AT	3	Surface layer	74.48, 78.54 [7.40]	83.93, 85.07 [11.27]
AF	12	Surface layer	69.82, 77.67 [19.40]	86.90, 88.28 [6.23]
BB	120	Surface layer	75.84, 71.94 [13.63]	72.43, 70.85 [4.52]

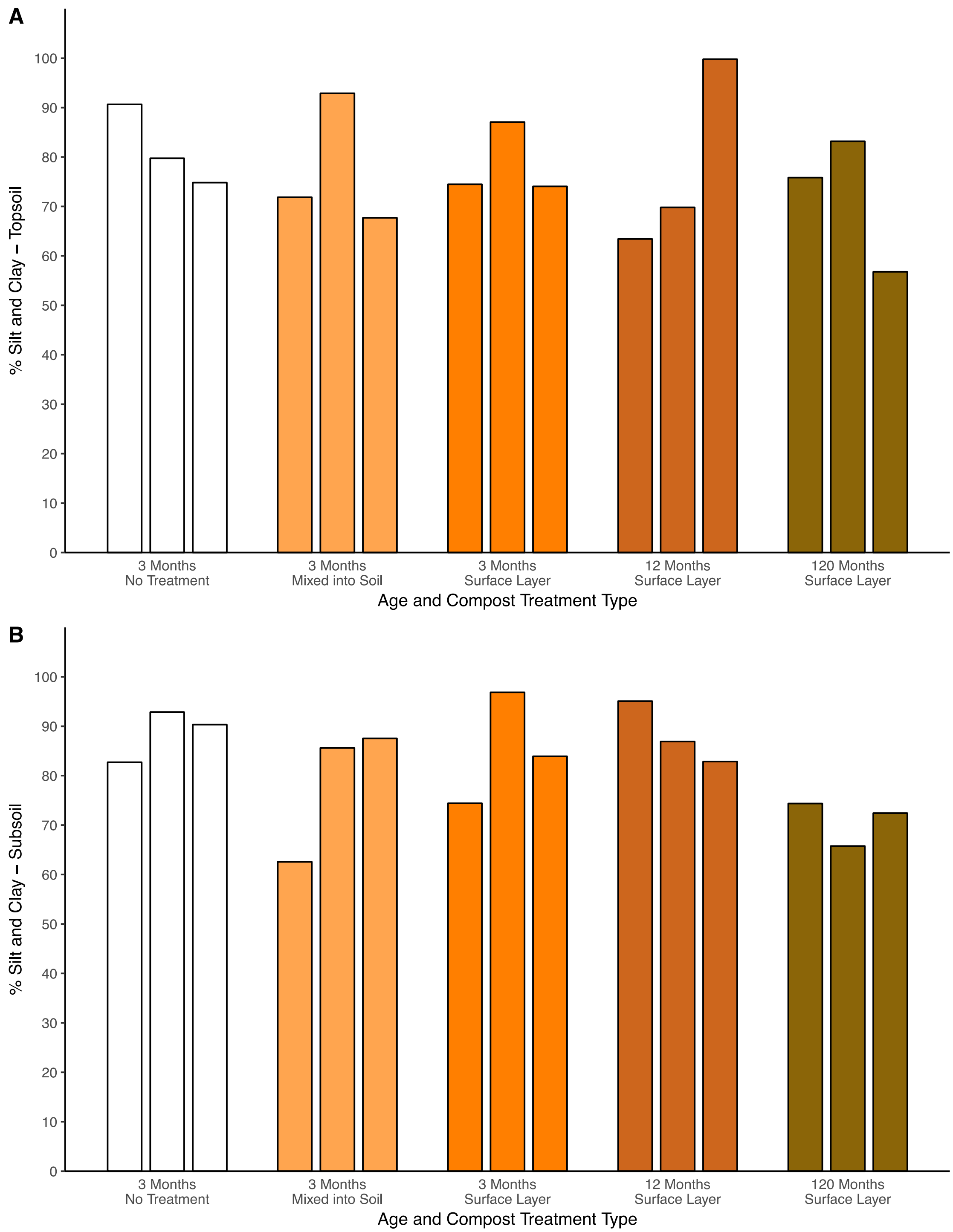

Figure 16. Percent silt and clay in sediment collected from (A) topsoil and (B) subsoil located in areas where three different compost treatments were used at Agrotourism Tupak (3 months), one compost treatment used at Agroforestry Tupak – Blok 1 (12 months), and one compost treatment used at Banko Barat 3 (120 months) reclamation areas.

Several soil physical and biogeochemical characteristics were significantly correlated (Table 13). Manganese was correlated with total carbon (r = 0.42) and percent fine sediment (<63 µm) (r = 0.39), in addition to nickel (r = 0.73), lead (0.49), and zinc (0.66). Aluminum was not correlated with any of the variables (e.g., total carbon, % silt and clay, major, and trace metals) included within correlation analysis. Iron is correlated with nickel (r = 0.60) and lead (r = 0.63). In addition, nickel tends to be correlated with lead (r = 0.78) and zinc (r = 0.70); zinc is correlated with lead (r = 0.57).

Table 13. Correlation matrix for metal concentrations in soil samples. Bold values indicate a significant correlation at the 0.05 level

	Carbon %	**Silt/Clay %**	**Al (mg/Kg)**	**Fe (mg/Kg)**	**Mn (mg/Kg)**	**Ni (mg/Kg)**	**Pb (mg/Kg)**	**Zn (mg/Kg)**
% Carbon	1.00							
% Silt/Clay	0.08	1.00						
Al (mg/Kg)	0.19	-0.09	1.00					
Fe (mg/Kg)	-0.49	-0.02	0.28	1.00				
Mn (mg/Kg)	**0.42**	**0.39**	0.23	0.23	1.00			
Ni (mg/Kg)	0.14	0.18	0.05	**0.60**	**0.73**	1.00		
Pb (mg/Kg)	-0.02	-0.06	0.17	**0.63**	**0.49**	**0.78**	1.00	
Zn (mg/Kg)	0.37	0.34	-0.03	0.23	**0.66**	**0.70**	**0.57**	1.00

CHAPTER FOUR: DISCUSSION

4.1: 16S rDNA Sequence Descriptions

Soil bacteria utilize carbon, major, and trace metals such as iron, manganese, nickel, and zinc for metabolic processes (Madigan et al., 2017). They also play an essential role in OM decomposition, which may increase metal mobilization over time (Williamson and Johnson, 1991; Gadd, 1993; Gharieb et al., 1999; Lovley, 2000; Gadd, 2004; Sheoran et al., 2010; Madigan, et al., 2017). Soil samples collected for this study contained the phyla Proteobacteria and Acidobacteria (Table A1). These heterotrophic bacteria are commonly found within soil samples and can significantly influence soil forming processes via direct/indirect breakdown of OM (e.g., nutrient cycling) (Sheoran et al., 2010; Li et al., 2014). Therefore, carbon turnover is likely linked with heterotrophic bacteria identified within this study.

Typical characteristics of the genus *Massilia* (phylum Proteobacteria) include aerobic metabolism and nonsporeforming, motile, and rod-shaped cells (George, M. G., et al., 2005) that produce yellow-pigmented colonies and belong to the family Oxalobacteraceae, class Betaproteobacteria (La Scola et al., 1998; Gallego et al., 2006). *Massilia* was first discovered in the blood of an immunocompromised patient (La Scola et al., 1998) but has recently been found in a variety of environments. Isolates have been collected from drinking water (Gallego et al., 2006), sediment samples (Wery et al., 2003; Ferrari et al., 2005; Nagy et al., 2005), and areas with metal contamination (Cai et al., 2009; Abou-Shanab et al., 2010; Kuffner et al., 2010; White et al., 2011) such as mine tailings (Du et al., 2012; Feng et al., 2016). Furthermore, *Massilia* is commonly found in early succession and decomposition stages of soils amended with plant residues (Pascault et al., 2010). The sample in this study was collected from the Banko

Barat 3 research plot which is 120 months old (Table A1). In recent years *Massilia* was found to be a rhizosphere and root-colonizing bacteria within soil that can also rapidly attach to mycorrhizal fungi hyphae (Scheublin et al., 2010). They have been found to be associated with plant growth by production of the phytohormone known as indole-3-acetic acid (IAA) (Kuffner et al., 2010), and siderophores (Hrynkiewicz et al., 2010). Rhizosphere bacteria that produce siderophores may promote plant growth by inhibiting colonization of harmful pathogens and bacteria on the roots and increasing Fe availability to the plant (Alexander and Zuberer, 1991).

Species from the family Comamonadaceae (phylum Proteobacteria, class Betaproteobacteria) have an aerobic metabolism and cells are commonly straight or slightly curved rods. They are motile by a single polar flagellum or via polar tufts of up to five flagella; they lack endospores, and in the presence of CO or H_2 they can be chemoorganotrophic or facultatively chemolithotrophic, or able to use inorganics as an energy source (Willems et al., 1991). Furthermore, the genus *Delftia* which also belong to the family Comamonadaceae, has been isolated from a variety of environmental and clinical samples including freshwaters, soils, rhizospheres, activated sludge, and immunocompromised individuals (Wen et al., 1999; Bilgin et al., 2015). Recently it has been discovered that *Delftia* species can show resistance to Zn (II) and Pb (II) contaminated soils, and are capable of detoxifying Cr-contaminated soils (Morel et al., 2011; Ubalde et al., 2012). In addition, they encourage plant growth activity by phosphate solubilization (Chen, et al., 2006), nitrogen fixation (Ahemad, 2015), production of siderophores, IAA, and aid in increased root development (Morel et al., 2015) which enhances nutrient absorption (Ahemad, 2015) in a number of plant species known for hyperaccumulation of metals (Morel et al., 2011; Ubalde et al., 2012; Morel et al., 2015). Many members of Comamonadaceae are commonly isolated during ecological studies due to their widespread geographic distribution (Willems et al., 1991).

The genus *Stenotrophomonas* (family Xanthomonadaceae, class Gammaproteobacteria, phylum Proteobacteria) are heterotrophic bacteria well-adapted to environments that are nutrient deficient (Ryan et al., 2009). They have an aerobic metabolism with oxygen as the electron acceptor and cells are typically straight or curved rods and are motile by two or more polar flagella (Palleroni and Bradbury, 1993). *Stenotrophomonas* is frequently isolated from environmental samples associated with the rhizosphere (Juhnke et al., 1989; Berg et al., 1996) and vascular systems of plants (Ryan et al., 2009). *Stenotrophomonas* may aid other microorganisms in plant colonization and plant productivity through extracellular enzyme and IAA production, nitrogen fixation, and making sulfate available for plants through sulfur oxidation. In addition, they may prevent fungal pathogens from harming plants by creating competition for iron which is conducted by capturing siderophores produced from other microorganisms.

Acidobacteria, a large and diverse phylum, contain species that contain rod-shaped, nonphotosynthetic, and nonsporeforming cells that typically occur as single cells, in pairs, or in short chains of cells. They are strictly aerobic, mobile by peritrichous flagella, and many have acidophilic growth patterns at pH ranges of 3.0 to 6.0 (Thrash and Coates, 2010). Species are commonly found in samples originating from acid mine drainage and soils (Kishimoto et al., 1991). The species detected within PTBA reclaimed soil samples are related to those commonly found to be associated in environmental samples such as soils, freshwaters, and mine spoil. It is therefore not surprising that this species was sequenced from our reclaimed soil samples among different compost applications and/or sites of varying age.

The identified bacteria were all heterotrophic and commonly found in environmental samples similar to the site conditions observed within this study and may play a role in OM turnover. Previous studies show that the bacteria *Massilia*, *Delftia*, and *Stenotrophomonas* are commonly associated with vegetation (e.g., rhizospheres, roots, stems, etc.) and likely play a role in enhancing vegetation growth, which is beneficial to reclamation (e.g., increasing OM, improving microbial activity, etc.). In addition, *Massilia* has been commonly early succession and decomposition stages of soils amended with plant residues (Pascault et al., 2010) which is consistent with the reclamation stage of the reforested areas at PTBA. These heterotrophic bacteria likely play a significant role in ecological functions such as OM turnover, and enhancing vegetation growth (e.g., nitrogen fixation, siderophore, and IAA production) that may ultimately improve site conditions.

4.2: Total Major and Trace Metal Concentrations in Soils

The total concentrations of manganese, nickel, lead, and zinc in samples collected from reclaimed soils at PTBA are within range of global soil background concentrations (Table 11, Figures 12-15) (Buonicore, 1996). Thus, the metal concentrations are all below the World Health Organization (WHO) maximum permissible levels of metals in soils (Table 11, Figure 11-15) (Chiroma et al., 2014). These results suggest that the contaminant concentrations in reclaimed soils in the reforested reclamation areas at PTBA are below levels of concern. It is worth noting that the concentration of the soil particulate-associated metals are also below the consensus-based threshold effect and probable effect guidelines cited in MacDonald et al.

(2000) (Table 11, Figure 13-15). Topsoils, then, are unlikely to pose a significant threat to local aquatic biota if they were eroded and transported to an adjacent water body.

Many studies have found that fill materials composed of overburden/interburden are rich in sulfide bearing minerals that, upon oxidation, release trace metals locked in their crystalline grain structures (Amijaya and Littke, 2006; Miller and Mackin, 2013; Moore, 2016). Based on the results reported herein, the major and trace metals within reclaimed soils of the study plots do not appear to be of major concern. There are two potential explanations for the observed concentrations. First, the backfill material was composed of material that did not contain significant amounts of coal, black shale, or sulfide minerals. The stockpiling of surface materials to be used for backfill was presumably designed to limit the incorporation of these materials during reclamation of the reforested areas. Second, low soil metal concentrations may be related to the utilized acid digestion method, which relied on 3:1 ratio of hydrochloric to nitric acid. This procedure which does not fully dissolve the mineral grains. Thus, it is possible, although unlikely, that unoxidized sulfide minerals containing trace metals exist with the soil, but were not measured by the analytical method used here. If this is the case, the bioavailability of the metals would be low as they remain locked in the mineral grains. Therefore, even if these metals are present, they are unlikely to negatively impact biota.

Total aluminum concentrations in the soil did not show a correlation with any of the other major and trace metals analyzed, nor did it correlate with % C, or % silt and clay (Table 13). Aluminum is often associated with aluminum oxides and kaolinite $Al_2Si_2O_5(OH)_4$ (Keller, 1964), both of which may exist as white coatings or grains within the soil. Within the study plots, there were no visible white coatings on ped faces or fractures within the soil profile. The lack of these features suggests, in combination with the correlation analysis (Table 13), that aluminum does not play a significant role in sorption/desorption of the trace metals (e.g., nickel, lead, and zinc) (Table 13).

Table 13 shows that % C and % silt and clay are both associated with manganese which may indicate that manganese is bound to organic carbon and/or clay minerals within the soil. In addition, it has been well established that manganese and iron oxides/hydroxides are excellent scavengers of trace metals (Jenne, 1968), and exist as coatings on soil particles (an observation made in our field area as well) (Figure 3). Total trace metal concentrations for nickel, lead, and zinc are all correlated with manganese; nickel and lead are also correlated with iron (Table 13). These relationships suggest that the analyzed trace metals (e.g., nickel, lead, and zinc) are potentially sorbed to the manganese and iron oxides/hydroxides within soil, and which were observed on ped surfaces and along linear soil fractures (Figure 3). This hypothesis could be tested in future studies by using sequential extraction methods that determine the soil phases with which metals are associated.

The total major and trace metal data above clearly demonstrate that the reclaimed soils at PTBA are not contaminated; rather, concentrations are within range of global background soils, indicating that toxic trace metals are unlikely to be a significant issue within the reforested areas. This is supported by the lack of observed acid mine drainage in the area. However, this study only examined shallow soils (~60 cm in depth) and does not preclude potential issues (e.g., acid mine drainage, metal contamination, etc.) that may originate from deeper sulfide bearing fill materials if it were to be come exposed to oxygenated waters.

4.3: Potential Age Effect(s) on Soil Characteristics and Microbiology

The data above shows that metal concentrations in reclaimed soils are below contaminant levels. Although our results exhibit variations in concentrations, there is no statistical difference among the analyzed metals and plots that varied in age since compost was applied (Figure 10-15). However, total metal concentrations for manganese, nickel, and zinc do appear to be lower in the 120-month old Banko Barat 3 site (Table 11, Figure 12, 13, 15). In contrast % C in topsoil showed a significant difference between the Agroforestry Tupak – Blok 1 and Banko Barat 3 research plots. The noted differences in carbon between the sites is consistent with field observations. Undecomposed and/or partially decomposed organic matter was widespread in the topsoils at Agroforestry Tupak – Blok 1, but was not observed in the reddish brown soils at Banko Barat 3. The reduction in organic carbon may be related to decomposition of the compost over time paired with the comparably lower OM input from vegetation at the site.

It has been established that organic carbon, major and trace metals, particularly iron, manganese, nickel, and zinc are essential macro/micronutrients, and are utilized by heterotrophic bacteria (Table 2, 3, A1) (Marshall et al., 1980; Lindemann et al., 1984; Mullen et al., 1989; Walker et al., 1989; Williamson and Johnson, 1991; Madigan et al., 2017). The phyla Proteobacteria and Acidobacteria (Table 2) were detected in soil samples and may use some metals, as mentioned above, for biological functions such as metabolic processes, activating enzymes, countering oxygen toxicity, and DNA/RNA polymerases (Madigan et al., 2017).

There were negative component loadings (Table 6) for manganese and nickel in the younger plots which suggest the higher values may have a relationship with microbial diversity in different aged plots with one type of compost application. It is important to note that although manganese and nickel are essential micronutrients to bacteria, they can be harmful in excess amounts. However, PCA conducted on the different compost applications and age groups showed there were positive component loadings (Table 5) for topsoil % C, manganese, nickel, and zinc (component loadings ranged from 0.63 to 0.81). This indicates a potential relationship between the geochemical data and microbial diversity in the sites. These component loadings are consistent with correlation analyses described in Table 13 where manganese is correlated and likely bound to carbon and/or clay particles. In addition, nickel, zinc, and lead were also correlated with manganese and likely sorbed to manganese oxides/hydroxides (Table 13).

Previous studies have found that microbes are typically associated with clay sized fractions and/or OM within the soil, where they may play a role in metal sorption due to their large surface area (Marshall et al., 1980; Mullen et al., 1989; Walker et al., 1989), and/or precipitation of dissolved metals (Tebo et al., 1984; Schultze-Lam et al., 1996). This suggests that manganese is bound to the organic and/or clay fractions observed in soil fractures (Figure 3); bacteria that are present can potentially biosorb essential nutrients such as organic carbon, manganese, nickel and zinc. Microbial biosorption of carbon, major, and trace metals over time would likely result in lower values in Banko Barat 3, which is consistent with field observations and the presented geochemical data (Table 11, Figure 9, 12, 13, 15). Although, lead is potentially adsorbed to manganese and iron oxides/hydroxides as well, it has been shown that there is no level of lead exposure that is beneficial to living organisms (Flora et al., 2012), which may explain why lead concentrations remained constant among the different aged plots (Figure 14). In addition, lead was found to correlate with iron and may be bound to the iron oxides/hydroxides (Table 13), which also did not appear to be affected by age (Figure 11).

According to the correlation analyses, total aluminum concentrations in shallow soil do not appear to be associated with any of the other soil characteristics we analyzed (e.g., % C, % silt and clay, total metal concentrations). As mentioned above, aluminum may be associated with kaolinite, and is therefore bound within its crystalline structure. Although there is no statistical difference between aluminum concentrations in soils, there does appear to be a slight increase in aluminum concentrations over time, indicating a potential age effect (Figure 10). The increase of aluminum concentrations through time may be related to the release of aluminum ions from the kaolinite crystalline structure as a result of increased soil acidity and/or weathering of mineral grains (Zołotajkin et al., 2011; Miller and Mackin, 2013).

The data presented above show that there is a distinct age separation between microbial diversity and geochemical data among the younger research plots (Agrotourism Tupak and Agroforestry Tupak – Blok 1) and the older research plot, Banko Barat 3. More specifically, % C, manganese, nickel, and zinc are correlated to one another and are essential nutrients to heterotrophic bacteria. Thus, these microbes likely play a role on carbon turnover, and utilized the metals for necessary biological functions, thereby producing the slightly lower values at the Banko Barat 3 site. In addition, correlation analyses show that lead may primarily be associated with iron oxides/hydroxides and, since lead has no necessary benefit to organisms, may explain why lead concentrations remained constant in the different aged research plots. Furthermore, total aluminum concentrations in shallow soils appeared to increase over time, which may be a result of weathering processes that release aluminum ions from mineral grains.

4.4: Major and Trace Metal Mobility in Soils and Subsurface Waters

Major and trace metal concentrations for aluminum, iron, manganese, nickel, lead, and zinc in subsurface waters collected from PTBA reforested reclamation areas were low (Figure 8A-8F) in comparison to previous analyses on waters in active mining pits (see, for example, Moore, 2016). In addition, metal concentrations for iron and nickel were below USEPA aquatic life chronic criteria, and USEPA and Indonesia drinking water standards (Figure 8B, 8D). Several water samples did exhibit aluminum, manganese, and lead concentrations that exceeded either the USEPA aquatic life chronic criteria and/or USEPA and Indonesia drinking water standards. However, median concentrations were all below water quality limits. Moreover, concentrations for aluminum and manganese were typically much lower than the geochemical data provided by Moore (2016), who found aluminum concentrations varied from ~50 ng/mL to ~11,000 ng/mL and manganese concentrations from 2000 ng/mL to ~21,400 ng/mL.

Significant variations were observed in metal concentrations in subsurface water samples of the different aged plots. Field observations did not show any evidence of acid mine drainage within the selected reclamation areas, indicating that sulfide minerals are unlikely to have played a significant role in the observed variations in dissolved metal concentrations within the subsurface waters. The variations in metal concentrations may be related to minor differences in the concentration of organic matter as well as iron and manganese oxides and hydroxides in the soils. The differences may also be related to variations in the quantity of shale and coal fragments observed within the fill materials (which are potential sources of these metals).

Metal mobility can be increased by leachate from organic matter (OM) which forms organo-metallic complexes that then move downwards through the soil profile (Schnitzer, 1969; Schnitzer and Khan, 1978; Soumare et al., 2003; Schwab et al., 2007). In order to test for metal mobility through the soil profile, and potentially into shallow subsurface waters, soil samples were collected from the land surface (~5 cm) and ~60 cm depths at each well location within the Agrotourism Tupak research plot. These samples were taken in order to investigate potential metal mobility among the different compost applications. The results show that particulate metal concentrations for all major and trace metals analyzed within this study were not depleted in the surface materials, nor did the concentrations increase at depth (Figure 10-15). This is consistent with other studies that suggest organic amendments can, in some instances, sorb metals and reduce their risk of leaching to groundwaters (Aziz and Smith, 1992; USEPA, 1992; Brown et al., 2003; Brown et al., 2004; van Herwijnen et al., 2007; Nwachukwu and Pulford, 2008; Nwachukwu and Pulford, 2009). As suggested earlier, these geochemical data imply that metal mobility is low among the different compost application methods used within the Agrotourism Tupak research plots. Thus, leaching to subsurface waters is unlikely (USEPA, 1992) unless a site has a shallow groundwater table or macropore flow (Sherene, 2010) is providing a connection to groundwater.

Field observations showed some mobilization of organics, manganese, and/or iron oxides/hydroxides (Figure 3) as coatings along vertically oriented linear fractures. It is possible that metals reach the underlying subsurface water via these soil fractures. However, the magnitude of mobilization is presumably limited given low metal concentrations measured in subsurface waters (Figure 8A-8F).

Soil samples collected from the surface (~5 cm) and at ~ 60 cm depths at each well location among the different research plots were primarily composed of silt and clay (Figure 16). Silt and clay rich soils are typically characterized by a low intrinsic permeability, which is function of pore size (Fetter, 2001). Finer grain fractions have more surface area, which allows for a larger area of water contact, resulting in an increase to frictional resistance to flow (Fetter, 2001). Thus, it is unlikely that significant water movement occurs through the soil matrix due to its low permeability (10^{-6}-10^{-1}) and hydraulic conductivity (10^{-9}-10^{-4} cm/s) (Fetter, 2001), reducing the potential for groundwater contamination. However, based on field observations and grain size distribution data (Figure 16), there may be localized lateral flow through the surface layer of compost, leading to throughflow and/or surface runoff, which was evident based on eroded ditches observed in the field.

CHAPTER FIVE: CONCLUSIONS

Multiple soil samples were collected from the PTBA reforested reclamation areas and used to extract 16S rRNA gene fragments for sequencing. Sequencing was conducted in order to identify specific bacteria that likely have an effect on OM turnover, metal sorption/desorption processes, and enhance vegetation growth among various compost application techniques and time since compost application (age) occurred. The bacteria detected from soil samples were all heterotrophic bacteria commonly found in environmental samples collected from soils, rhizospheres, vegetation root systems, and stems. Some of these bacteria (e.g., *Massilia*) have been commonly found in early succession and decomposition stages of soils amended with plant residues which is consistent with our recently reclaimed study areas. The heterotrophic bacteria detected within these soil samples among the different study plots likely have a significant impact on ecological processes such as nitrogen fixation, OM turnover, metal sorption/desorption, and plant growth. Therefore, these bacteria are expected to have a very important impact within the reforested reclamation areas at PTBA.

Numerous environmental samples were collected and analyzed from the PTBA reforested reclamation areas to determine how compost application techniques and time since compost application (age) affect soil and water quality. Total metal concentrations in soils were all within the range of global soil background concentrations and below the WHO maximum permissible levels of metals in soils. The concentrations were also lower than consensus-based threshold effect and probable effect guidelines for aquatic environments, suggesting that the erosion of the soils, and its transport to adjacent surface waters is unlikely to affect aquatic biota. Correlation analyses were conducted to provide insights into the sources and sinks of metals within the soil.

The analysis showed that manganese was correlated with, and likely bound to, organic carbon, which was consistent with field observations. Manganese, nickel, and zinc as well as % C were generally lower in the older Banko Barat 3 study plot than in younger plots (Agrotourism Tupak and Agroforestry Tupak – Blok 1). These results are consistent with PCA which showed a distinct age separation between Banko Barat 3 and the two younger plots. These differences are likely related to the identified heterotrophic bacteria utilizing OM, iron, manganese, nickel, and zinc over time, all of which are essential nutrients used for metabolic processes.

Total metal concentrations analyzed from soil samples taken from the surface (~5 cm) and at ~60 cm depths indicate there was neither a depletion in surface metal concentrations or an accumulation of subsurface metals at any of the sites. Therefore, metal mobility to subsurface waters is unlikely, a conclusion that is consistent with low dissolved metal concentrations in subsurface waters. Although some mobility of organic material was observed along soil fractures, the low permeability of these soils make it is unlikely for water to easily move through the soil matrix. Based on field observations it is more likely that significant surface runoff is generated during the rainy season, which has the potential to erode the soils.

This study therefore suggests that neither type of compost application nor age of application had a significant effect on microbial diversity, metal contamination of the soils, or dissolved metal concentrations in subsurface waters within the PTBA reforested reclamation areas. Therefore, reclamation managers at the PTBA coal mine should focus on the compost application method that best enhances vegetation growth and may increase microbial diversity over time.

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APPENDIX

Table A1. DNA sequence matches for partial 16S rRNA gene fragments for isolates collected from from reclaimed soil at PT Bukit Asam Coal Mine, South Sumatra, Indonesia which had various compost treatments and/or age. The Ribosomal Database Project (RDP) software program SeqMatch was used including percentages and accession numbers that designate the closest match to a known isolate described in the RDP program.

Site Identification	Site Age (Months)	Compost Treatment	DGGE Band	Best Match Cultured Organism	Best Match Uncultured Organism
AF12T2C	12	Surface Layer	11a	*Ramlibacter sp. (89.0%; S000388105; S000394160)*	*Ramlibacter spp. (89.0%)*
AT7T2C	3	Surface Layer	12	Acidobacteria (100.0%)	Acidobacteria bacterium (80.3%)
AF11T2C	12	Surface Layer	12	Acidobacteria (100.0%)	Acidobacteria bacterium (80.3%)
AF12T2C	12	Surface Layer	12	Acidobacteria (100.0%)	Acidobacteria bacterium (80.3%)
BB13T2C	120	Surface Layer	12	Acidobacteria (100.0%)	Acidobacteria bacterium (80.3%)
BB14T2C	120	Surface Layer	12	Acidobacteria (100.0%)	Acidobacteria bacterium (80.3%)
AT1T0	3	Control	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
AT3T0	3	Control	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
AT6T1C	3	Mixed	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
AT7T2C	3	Surface Layer	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
AF11T2C	12	Surface Layer	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
AF12T2C	12	Surface Layer	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
BB14T2C	120	Surface Layer	10	*Delftia sp. (98.8%; S000146281; S001169258)*	*Delftia spp. (100.0%; S000976715)*
BB14T2C	120	Surface Layer	8	*Massilia aurea (97.1%; S000650723)*	Oxalobacteraceae bacterium (100.0%; S002476753)
AT7T2C	3	Surface Layer	11	*Stenotrophomonas maltophilia (95.5%; S000009493)*	*Stenotrophomonas spp. (98.9%; S001178092)*
AT9T2C	3	Surface Layer	11	*Stenotrophomonas maltophilia (95.5%; S000009493)*	*Stenotrophomonas spp. (98.9%; S001178092)*
AF11T2C	12	Surface Layer	11	*Stenotrophomonas maltophilia (95.5%; S000009493)*	*Stenotrophomonas spp. (98.9%; S001178092)*
AF12T2C	12	Surface Layer	11	*Stenotrophomonas maltophilia (95.5%; S000009493)*	*Stenotrophomonas spp. (98.9%; S001178092)*
BB14T2C	120	Surface Layer	11	*Stenotrophomonas maltophilia (95.5%; S000009493)*	*Stenotrophomonas spp. (98.9%; S001178092)*