Impact of stockpiling on soil microbial communities and their functions

by

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Abstract

Soil stockpiling before mining activities is a by-law-mandated procedure that has adverse effects on soil health, which raises concerns about the suitability of stockpiled soils as a reclamation substrate. Relatively few studies have addressed the effects of stockpiling on soil biology, and particularly the impact of this disturbance on soil microbial communities is not clear. Since microbes have a fundamental role in nutrient cycling and can respond rapidly to changing soil conditions, the impact of soil stockpiling on microbial communities can be used as an indicator of soil quality, to shed light on the usefulness of the stored topsoil in the restoration of disturbed ecosystems. Using marker gene sequencing, I analyzed the structure and composition of microbial communities in a chronosequence of 0.5-28-year-old stockpiled soils at increasing depths (0-300 cm), in two oil-extraction locations in northern Alberta, Canada.

In Chapter 2, I analyze the effect of stockpiling on soil prokaryotic communities. My results indicate that stockpiling shifts the microbial community composition outside the range of natural variability. Furthermore, while microbial communities in younger and older stockpiles were dissimilar to the reference soils, the communities of intermediate-age stockpiles were more similar to those in the reference soils, which may indicate that initial disturbance leads to a shift in the microbial community, which then recovers following several years of storage, but eventually, long-term storage leads to a secondary divergence from the range of natural variability. Additionally, the bacterial diversity decreased significantly with increasing stockpile depth, which could be attributed to the harsh conditions of the deeper stockpile layers and the scarcity of nutrients.

In Chapter 3, I examine the impact of soil stockpiling on fungal communities and their functions. I found that fungal communities of the stockpiles differ from the communities in the nearby undisturbed reference soils. Also, similar to previous studies, there was a decrease in fungal richness and overall diversity with increasing stockpile depth. Furthermore, soil stockpiling generated a shift in the inferred function in the form of putative fungal guilds and trophic modes. Ectomycorrhizal fungi decreased and saprotrophic fungi increased in the stockpiles relative to the reference soils. These findings indicate that stockpiling may have important implications for ecosystem functions and services associated with fungal communities, such as litter decomposition and plant growth promotion when these stockpiles are used to reclaim ecosystems.

In Chapter 4, I assess the predictors associated with the variability in microbial communities of stockpiled soils. I apply the null model operational framework proposed by Stegen et al. (2013), to shed light on the assembly processes influencing the β-diversity in the microbial communities of stockpiled soils. There was a significant correlation between specific microbial taxa and the conditions found in the disturbed soils. However, less than 20% of the variability in the microbial communities was explained by the predictors assessed by the study. Regarding the assembly processes shaping the communities, stochastic factors like drift and dispersal exerted the most important influence in all microbial groups (Bacteria, Archaea, and fungi). Therefore, the disturbance generated by the soil mechanized handling and management seems to be more important in the assembly of microbial communities of the stockpiles than the commonly attributed harsh physicochemical conditions created by stockpile depth and storage time.

Taken together, the results of my thesis provide important insights into the impact of soil stockpiling on microbial communities and their functions and shed light on the effects of stockpile depth and storage time on soil microbial diversity and community composition. Similarly, the

results reveal that selective pressures promoted by environmental filters or legacy effects, may not be as important as usually described in the literature for the structuring and variability of the microbial communities in the stockpiled soils. These findings are useful for reclamation specialists/agencies to determine optimal conditions for the storage of topsoil and for stockpiled topsoil to be used effectively in post-mining reclamation operations.

Preface

This thesis is an original work done by Julian Ariel Cabrera Hernandez at the Department of Biological Sciences, University of Alberta, under the supervision of Dr. Brian D. Lanoil. The thesis was written according to the guidelines provided by the Faculty of Graduate Studies and Research, University of Alberta.

Chapter 1 of the work corresponds to a literature review to introduce the concepts and background relevant to the understanding of the research.

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Chapter 5 of this thesis presents the conclusions derived from this thesis, delineates the contributions of the study to the field, and proposes future avenues of research.

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CHAPTER 1
Literature review

1.1. The Athabasca Oil Sand Region

This thesis research takes place in two oil extraction sites within the Athabasca Oil Sands Region (AOSR), one of the world's largest oil reserves and an important component of the Canadian economy (Government of Alberta, 2022). The AOSR area occupies around 142,200 km^2 (US Geological Service, 2021), and is located in the Canadian boreal forest. The area exhibits a mid-boreal and subhumid climate, with annual mean temperatures of -2 to +1 °C, and precipitation rates of 300 to 600 mm (Johnson & Miyanishi, 2008). The relief of the area is characterized by vast lowland wetlands interspersed with undulating and level till as well as lacustrine plains (Downing & Pettapiece, 2006). The high diversity of habitats and wildlife in the region has been attributed to the interaction of climate, topography, and vegetation (Alberta Parks, 2014).

The region was covered by the Laurentide ice sheet until 10,000-12,000 years ago (Johnson & Miyanishi, 2008). The glacial sediments left behind or transported by the glacial sheets are the materials that have formed most of the Canadian soils, and the mixture of these sediments and crushed rocks is called glacial till (Krzic et al., 2021). Glacial till in the AOSR, mostly of local origin, is the most common sediment deposit in the region, while the material of recent origin includes colluvial, aeolian, fluvial, and organic deposits (Fung & Macyk, 2000)

1.1.1 Soils in the AOSR

Like most soils in Canada, soils in the AOSR are young when compared with soils globally, since they were formed when ice sheets retreated, leaving behind glacial sediments, which were

eventually transformed into soils by physical, chemical, and biological factors (Krzic et al., 2021). The most common soil types found in the AOSR are luvisols, brunisols, and organic soils (Dimitriu & Grayston, 2010). Luvisols are typical of well-drained forests, like those in the uplands (Downing & Pettapiece, 2006; Dimitriu & Grayston, 2010), and are characterized by a lack of humus incorporation into the mineral layer and thick forest litter layers (Krzic et al., 2021). Luvisols are characterized by eluvial (Ae) and textural (Bt) horizons with silicate clays accumulating (Fung & Macyk, 2000). In well-drained aeolian or fluvial materials, brunisols are commonly found (Downing & Pettapiece, 2006). Brunisols are characterized by a lack of textural Bt or sesquioxide (Bf) horizons, due to insufficient clay accumulation (Fung & Macyk, 2000), and since they do not exhibit the well-developed horizons that characterize other soil types (e.g., podzols or luvisols), these soils are commonly considered immature or in-transition (Earle, 2019).

In lowland positions, organic soils are dominant, varying between 40 cm and 3 m thick, overlying the glacial deposits throughout the region. The organic matter (OM) accumulates since the lack of oxygen in water-saturated systems reduces the rate of heterotrophic OM mineralization (Krzic et al., 2021). The types of OM found in the AOSR region are (1) fen peat, which originates from sedges and grasses, (2) forest peat, made up mainly of trees, shrubs remnants, and mosses and (3) bog peat, derived from *Sphagnum* mosses (Fung & Macyk, 2000). These boreal peatlands are responsible for the long-term sequestration of carbon dioxide and, therefore, are important players in climate stability (Sun et al., 2014). Mesisols, made up of peat that is in a medium state of decomposition are the prevalent organic soils; in the poorly drained soil of the AOSR, like fens and bogs (Fung & Macyk, 2000; Downing & Pettapiece, 2006)

1.1.2 The Alberta Boreal Forest

The AOSR lies within the boreal forest or taiga, which is located ~ 50 and 70º N latitude (Johnson & Miyanishi, 2012), forming a circumpolar belt that extends through North America and northern Eurasia (Apps et al., 1993), including regions of Russia, Sweden, Finland, Canada, and the American state of Alaska (Mery, 2010). Boreal ecosystems represent around 25% of the world's total closed forested zone (Dimitriu & Grayston, 2010; Boonstra et al., 2016), and are one of the largest and most important ecosystems worldwide (Mery, 2010; Gauthier et al., 2015). They are the most significant pool of living biomass on the earth's surface (DeAngelis, 2008), and the largest source of soil organic matter (Fath, 2018), most of which is peat (Mery, 2010). Additionally, this ecosystem has been demonstrated to sequester ~20% of the carbon consumed by forests worldwide (Pan et al., 2011). Consequently, disturbance impacting the productivity of these ecosystems may seriously affect world climate (Chapin III et al., 2000; Dimitriu & Grayston, 2010). Around 28% of the world's boreal zones are located in Canada and extend from Newfoundland and Labrador to the Yukon and northern British Columbia (Government of Canada, 2020). However, the ecosystem dynamics and structure of the boreal forests vary geographically and therefore boreal forests in North America differ from their European counterparts (Boonstra et al., 2016).

The boreal forest natural region covers 58% of Alberta, occupying most of the northern portion of the province (Alberta Parks, 2014). The varying relief of the region has given rise to upland forests interwoven by lowland wetlands (Beckingham & Archibald, 1996), and watercourses that constitute the habitat of wildlife species and productive aquatic communities (Alberta Wilderness Association, 2022). Therefore, the region is known to be biologically, topographically, and climatically diverse, and provides a broad spectrum of services like the supply of goods, climate regulation, and support of primary production (Brandt et al., 2013; Gauthier et al., 2015). Based

on the climate, soil, topography, and vegetation, eight sub-regions have been recognized in the boreal forest of Alberta, these regions are Dry Mixedwood, Central Mixedwood, Lower Boreal Highlands, Upper Boreal Highlands, Athabasca Plain, Peace-Athabasca Delta, Northern Mixedwood and Boreal Subarctic (Downing & Pettapiece, 2006).

1.1.3 Vegetation of the boreal forest in Alberta

Boreal forests harbor a low tree diversity (Mery, 2010), but are almost totally covered by a continuous belt of trees (Downing & Pettapiece, 2006), which are adapted to the long winters and short summers of the zone (Mery, 2010). The vegetation that characterizes boreal forests is the coniferous, deciduous, and mixed wood forests (Downing & Pettapiece, 2006). This vegetation is characterized by a temporal and spatial mosaic of plant species that generates an array of diverse ecosystems (Macdonald et al., 2012). Among the dominant conifers found in the region are the jack pine (*Pinus banksiana*), white spruce (*Picea glauca*), and black spruce (*Picea mariana*), whereas aspen (*Populus tremuloides*) and balsam poplar (*Populus balsamifera*) are dominant deciduous species (Alberta Parks, 2014). The wetlands on the other hand are dominated by black spruce (*Picea mariana*) and mosses, such as sphagnum (*Sphagnum* spp.) (Downing & Pettapiece, 2006). The plant community of the region is known to influence the soil processes and formation, for instance in the uplands, the needles of the conifer that dominates the region, accumulate on the forest floor generating acidic conditions that reduce the rate of organic matter decomposition (Mery, 2010).

1.1.4 The microbial communities of the boreal forests

The boreal forests host diverse fungal communities that play essential roles in the organic matter decomposition and establish complex associations with the native vegetation (Sterkenburg et al., 2015; Pec et al., 2017). Sukdeo et al. (2018), suggest that the fungal groups *Cortinariu*s,

Amphinema, *Russula,* and *Piloderma,* are native to boreal ecosystems, where they are found establishing ectomycorrhizal associations with roots of trees of the genera *Picea* and *Pinus* (Soop, 1993; Iwański & Rudawska, 2007; Geml et al., 2010; Scott et al., 2019), and are known to play important functions in soil carbon stabilization (Tedersoo & Nara, 2010; Averill et al., 2014). This is consistent with the dominance of the fungal genera *Piloderma, Dioszegia,* and *Macrolepiota*, classified under the phyla Basidiomycota, in the boreal forests of Alberta (Dimitriu et al., 2010). Similarly, Stefani et al. (2018), determined that *Agaricomycetes* (Basidiomycota) is the dominant fungal group in the undisturbed soils of the AOSR, followed by the paraphyletic group *Zygomycetes* and *Dothideomycetes* (Ascomycota). All these reports align with the body of knowledge that attributes the dominance of ectomycorrhizal fungus of the phyla Basidiomycota in the boreal forests (Read, 1991; Tedersoo & Nara, 2010).

Scarce reports have documented the composition of the prokaryotic microbial communities that are native to the boreal forests of Alberta. One study found that the dominant groups in the region were in the order *Rhizobiales,* of the class *Alphaproteobacteria*, followed by Group 4 of Acidobacteria, the *Xanthomonadales* of *Gammaproteobacteria*, and the *Planctomycetales* of the phylum Planctomycetes (Masse et al., 2017). Likewise, the results of more recent studies indicate that Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, and Verrucomicrobia are dominant in the AOSR (Stefani et al., 2018; Santana Martinez, 2021). These studies are consistent with reports indicating that Actinobacteria, Acidobacteria, Proteobacteria, and Bacteroidetes are the dominant phyla in boreal ecosystems (Roesch et al., 2007; Sun et al., 2014), and non-boreal ecosystems (Janssen, 2006; Roesch et al., 2007; Lladó et al., 2017) globally. The dominance of some of these bacterial groups in soils worldwide, and the variation in their relative abundances,

has been linked to the amount of nutrients present in the soil (Fierer et al., 2007) or the soil pH (Lauber et al., 2009; Chu et al., 2010).

1.2 Oil sands extraction in Alberta

Oil sands are a mixture of clay, sand, water, and extra-heavy crude oil known as bitumen (Alberta Energy Regulator, 2022). It has been estimated that the Canadian oil sands originated when crude oil flowed from the Rocky Mountains toward the sand deposits in Alberta 50-100 million years ago (Gray et al., 2009). Then microbial activity removed the low molecular weight components and left the more complex and viscous fraction (i.e., bitumen)(Chandler et al., 2016).

Oil sands reserves of ~ 165 billion barrels make the Athabasca Oil Sands Region (AOSR) the most important single oil reserve in the world (Dimitriu & Grayston, 2010), and is a fundamental component of the Canadian economy (Alberta Government, 2022), generating around 85% of the oil produced in the country (Alberta Energy Regulator, 2022). The estimated bitumen reserves in the AOSR are enough to supply the Canadian domestic crude oil demand for ~250 years (Czarnecki et al., 2005).

The main oil sand deposits in the region are the Peace River oil sand deposit, Cold Lake oil sand deposit, and Athabasca oil sands deposit (Johnson & Miyanishi, 2008), most of these areas are inhabited by First Nation people and were not disturbed by Europeans until the start of the recent oil and forest developments (Johnson & Miyanishi, 2008). Even though the presence of oil sands was known to exist in Alberta since the end 18th century (Berkowitzt & Speightt, 1975), commercial exploitation of oil sands in Alberta commenced by 1967, with the incursion of the Great Canadian Oil Sands (CAPP, 2022).

In the AOSR region, bitumen is extracted by applying *in-situ* and open-pit mining, depending on the depth of the deposits (Government of Canada, 2016). Thus, the near-surface oil sand deposits are exploited using open-pit mining (Gray et al., 2009; MacKenzie, 2013), where the oil sands are scooped up with trucks and shovels (Alberta Energy Regulator, 2022), and then hot water is used to separate the bitumen from other oil sands components in separation vessels (Czarnecki et al., 2005). However, when the oil sands deposits are located in the deeper soil layers (>75 meters underground), oil sands are extracted by employing *in-situ* drilling, in which high-temperature steam is injected to decrease the viscosity and increase the fluidity of the bitumen so that it can be pumped out with extraction wells (Johnson & Miyanishi, 2008). In the AOSR ~80% of the bitumen is currently extracted using in situ thermal extraction methods (Alberta Government, 2022). Some *in-situ* oil sand extraction methods that have been used in the AOSR and nearby mining zones are steam-assisted gravity drainage (SAGD) and cyclic steam stimulation (CSS). SAGD can recover 60% of the bitumen in place, but their use is recommended in thick rich bitumen (Gray et al., 2009). The SAGD technology has been applied by major Canadian oil companies in the AOSR. The CSS, on the other hand, can extract ~20% of bitumen in place and has been used by Canadian Natural Resources at Wolf Lake and Primrose extraction sites (Sunshine Oilsands, 2022).

1.2.1 Environmental impact associated with oil sands mining

The present extinction rates and decrease of species populations caused by habitat loss exceed what was projected from the fossil record (Barnosky et al., 2011). Ecosystem disturbances generated by anthropogenic activities have been linked with ecosystem shifts to alternative compositional and functional states (Scheffer et al., 2001; Falk et al., 2006), which may have serious consequences for biodiversity (Liao et al., 2018), land use and water quality (Ibarra & de las Heras, 2005; Timoney & Lee, 2009).

The evidence seems to indicate that the impact of human activity in the North American boreal forests has occurred since the retreat of the glaciers 8,000–12,000 years ago (Johnson & Miyanishi, 2012), and by the year 2020, the human footprint generated in the AOSR represented 9%, from which 4.5% corresponds to the forestry footprint followed by 2.3% of the energy footprint (Alberta Biodiversity Monitoring Institute, 2020). This is consistent with reports indicating that the rate of disturbances in the Canadian boreal forests is one of the fastest worldwide (Hansen et al., 2010; Komers & Stanojevic, 2013).

Mining has been regarded as one of the anthropogenic operations that most seriously impact the environment (Ibarra & de las Heras, 2005; Stracher, 2019). In the oil sand extraction activities developed in the AOSR, surface mining, *in-situ* extraction, and bitumen upgrading generate most of the environmental concerns (Timoney & Lee, 2009; Kurek et al., 2013). This is evidenced by reports recognizing that the environmental impact associated with oil-mining activities is substantial and has generated vast zones of disturbed land and destroyed or fragmented ecosystems (Rowland et al., 2009). Even more concerning is the fact that the projections indicate that more than 4,000 km^{2} of northern Alberta will eventually be mined (Rooney et al., 2012). Furthermore, it is expected that the impact of the disturbance generated by oil sand extraction will be intensified by climate change in the near future (Johnson & Miyanishi, 2008).

Another factor that has generated concern about the magnitude and perspectives of the oil sand industry in Alberta, has been the contribution of oil sand mining to the emissions of greenhouse gasses (Rooney et al., 2012; He et al., 2024), since the oil sand extraction and process applied to convert bitumen into crude oil, releases volatile organics, SO_2, and NOx (Johnson & Miyanishi, 2008), some of which are known to impact global warming and accelerate climate change. Indeed, a report by the Carnegie Endowment for International Peace (2013), has estimated that oil sand

mining and upgrading emits more greenhouse gasses than the generated by the conventional production of more accessible or lighter crude oil. However, it is worth noting that this assertion is still debated since other reports indicate that the emission of greenhouse gasses by oil sand mining and processing is within the same range as that generated in the processing of other crude oils (Government of Canada, 2013).

1.2.2 Impact of open pit mining

Around 4,750 km^2 of the oil sand deposits in the boreal region of Alberta are accessible by open pit mining, and ~ 99% of that amount has already been leased (Energy Alberta, 2015). The footprint generated by open pit mining activities on the landscape is evidenced by the modification of the landscape, generation of discard dumps (Mushia et al., 2016), and loss of boreal coniferous and deciduous forests (Timoney & Lee, 2009). As an example, in the year 2016, the ecosystem disturbance generated by oil-sand open-pit mining activities in the AOSR expanded to more than 900 km^2 (Alberta Environment and Parks, 2021). The relatively high impact of open pit mining is associated with the removal of forest cover and a considerable proportion of the overburden (~15-50 meters), to gain access to the oil sands (Rowland et al., 2009). The subsequent removal of oil sands leaves behind kilometers-wide pits in which oil extraction residues may accumulate (Rowland et al., 2009; MacKenzie, 2013). These residues may percolate to nearby water bodies (Routson et al., 1979) and affect wildlife and human health. Similarly, the emission of pollutants like polycyclic aromatic hydrocarbons and heavy metals from open-pit mining activities in the AOSR, may affect human health (Kelly et al., 2009; Timoney & Lee, 2009; Kurek et al., 2013).

1.2.3 Impact of *in-situ* oil sand extraction

Most of the bitumen in the AOSR is extracted by applying *in-situ* operations (Alberta Government, 2022). There is a general conception that the impact of open pit mining is greater than that of *in*

situ extraction (Flint, 2004). However, the works of Jordaan et al. (2009) challenge this view asserting that the disturbance generated by *in-situ* extraction on land per unit of production is lower than surface mining, but *in-situ* extraction generates a more dispersed spatial footprint, which increases landscape fragmentation (Jordaan et al., 2009). Landscape fragmentation has been defined as "a large expanse of habitat that is transformed into a number of smaller patches of smaller total area, isolated from each other by a matrix of habitats unlike the original" (Wilcove, 1987). Habitat fragmentation causes disturbances in the fluxes of wind, radiation, nutrients, and water in the ecosystem (Saunders et al., 1991), and has been linked to a reduction in diversity (Fahrig, 2003; Didham, 2010; Liu et al., 2019), to a decrease dispersal potential of dispersal-limited taxa (Cote et al., 2017) and the ease of colonization by invasive plant species (Zambrano et al., 2019).

1.2.4 Reclamation and regulations

Due to the magnitude and characteristics of the disturbance produced by oil sand extraction in the AOSR, bioremediation treatments (i.e. the use of living organisms to remove or stabilize pollutants from soils) are insufficient to rehabilitate the impacted ecosystems in the region (Johnson & Miyanishi, 2008). Consequently, reclamation, which involves the physical reconstruction of heavily degraded soils, is a better option to restore post-mining sites, both in the AOSR and other mining sites around the world (Johnson & Miyanishi, 2008).

The goal of land reclamation is to return mined lands to their pre-disturbance conditions (Bohrer et al., 2017), which includes the rehabilitation of the ecosystem function, services, and land use (Ezeokoli et al., 2019a). Reclamation practices and technologies have been developed and widely used in the last 20 years around the world (Ibarra & de las Heras, 2005). Generally, the following stages are considered in land reclamation: (1) collection of information about the site to be

disturbed, (2) tree clearing (also part of the mining activities), (3) soil removal and storage to be applied in reclamation operations, (4) salvaged soil placement, (5) revegetation of the placed reclamation material, and (6) monitoring and maintenance (Naeth et al., 2013a).

1.2.5 Assessing the success of land reclamation

Early Alberta legislation and norms regarding post-mining land rehabilitation assessed the land condition (e.g., soil erosion and weed infestation), land agricultural productivity (i.e. crops yielded in a determined area, in a defined period), or human safety, as an indication of good restoration practices (Chandler et al., 2016). The development of industrial activities in Alberta raised concerns about the accelerated ecosystem destruction and prompted the need for broader and more stringent regulations to maintain ecosystem health and integrity (Chandler et al., 2016), thus originating the first land reclamation policies in the province in 1963 (Powter et al., 2012).

By the mid-1980s, the objective of reclamation was to achieve an equivalent (or better) land productivity compared to the condition of the pre-disturbed ecosystem (Chandler et al., 2016), but due to the complexities and challenges involved with assessing the similarities in productivity of undisturbed and reclaimed soils, the goal shifted to achieve "equivalent land capability" with the Reclamation Regulations of 1993 (Chandler et al., 2016). Consequently, oil sands mining companies in Alberta are legally obligated to reclaim disturbed land to "equivalent land capability", which is defined as "the ability of the land to support various land uses" and its function is similar to the one that existed before the disturbance (Environmental Protection and Enhancement Act, 1993).

To determine the success of the reclamation of a disturbed site, a common approach consists of the selection of a *reference ecosystem* whose conditions are similar to those that existed before the

disturbance generated by the mining activities and a *target ecosystem* that exhibits the conditions that are expected after the reclamation is completed (Harris, 2003; Zahraei, 2015). The characteristics of reference and target ecosystems represent the range of natural variability, defined as the spatial and temporal variability in the ecosystem processes, community composition, and dynamics that existed before the major modifications generated by settlers (Swanson et al., 1994; Wong & Iverson, 2004). Since it has been widely accepted that more sustainable and healthier ecosystems are those in which the natural disturbances fall within the range of natural variability (Gayton, 2001), the conditions of both the target and reference ecosystems are used as a guide to assess the restoration of native populations, ecosystem diversity, and to determine the success of reclamation.

However, returning the disturbed land to a pristine state has been considered idealistic under the argument that ecosystems are not static (Johnson & Miyanishi, 2008) and that ecosystems change as a result of fluctuations in environmental conditions (Williams et al., 2007). Similarly, it has been asserted that the "pristine site" used as a reference may not represent the initial conditions of the disturbed site or may have already been impacted directly or indirectly by anthropogenic activities (e.g. climate change, invasive species, or habitat fragmentation), (Falk et al., 2006), which makes the goal difficult to attain or even impractical.

Likewise, the current reclamation norms have been criticized since the restoration of important ecosystem components such as the native tree cover composition is not explicitly mandated by the regulations. As a consequence, some of the largest mining companies in the AOSR have failed to describe the plant communities that existed before the start of the mining activities, which complicates the comparison between the reclaimed sites with the pre-disturbed ones (Rooney et al., 2012), and the settlement of accurate restoration goals. This lack of precision and ambition in

the reclamation guidelines has been evidenced by the reconstruction of well-drained hills instead of even terrain, using salvaged soils, which has caused former peatlands to be reclaimed into upland forests (Rooney et al., 2012). Since peatlands have played a crucial role in carbon sequestration since the deglaciation of the Laurentide ice sheet (Harden et al., 1992), the transformation of lowland to upland forest ecosystems will result in a drastic decrease in carbon sequestration and storage (Rooney et al., 2012). Similarly, due to the leniency of the reclamation guidelines regarding the functional and compositional characteristics of the soil community to be restored, it has been a common practice to use peat in the restoration of upland boreal forests (Dioumaeva et al., 2002), which is believed to increase soil microbial heterotrophic respiration rates in the reclaimed sites, disrupting the carbon exchange rates between plant, soil, and the atmosphere (Dietrich & MacKenzie, 2018), such an increase in microbial organic carbon degradation is assumed to increase with global warming, thus generating more CO_2 that goes to the atmosphere (Hicks Pries et al., 2017).

1.2.6 Topsoil salvage

Because soils provide pivotal ecosystem services and functions (Weil & Brady, 2017), the first step toward the goal of generating a healthy and self-sustaining ecosystem consists of rehabilitating the functions of the soil (Macdonald et al., 2012). However, the reclamation of post-mining sites is a complex multistep procedure of soil engineering and wide-scale landscaping (Rowland et al., 2009; Macdonald et al., 2012). Therefore, the procedure needed to restore post-mining sites to functional, resilient, and self-sustaining ecosystems, like those of the AOSR, may go beyond the boundaries of ecological restoration.

As part of the mining activities, surface soils (~10-15 cm) and overburden are removed to allow access for drilling and other mining operations (Kundu & Ghose, 1997; Bohrer et al., 2017;

Muñoz-Rojas, 2018). The removed surface soils are considered a valuable resource for land reclamation (Kundu & Ghose, 1997) since they are generally rich in nutrients, native plant seeds, and complex microbial communities (Golos et al., 2016, Mushia et al, 2016; Buss et al., 2019). Since 1983 salvaging surface soil has been a legislated requirement for several industrial activities in Alberta, including oil sands mining (Government of Alberta, 2022). The salvaged topsoil is used in the reclamation of disturbed ecosystems, especially at sites where mining has concluded (Paterson et al., 2019).

The direct placement of the removed topsoil (i.e. use of fresh topsoil in the reclamation of disturbed ecosystems) can lead to the successful reclamation of post-mining sites (Anderson et al., 2008; Birnbaum et al., 2017b; Dhar et al., 2019; Ngugi et al., 2020), and the re-establishment of a diverse plant community (Macdonald et al., 2012). Moreover, physicochemical and microbial characteristics of the soil are improved when soil vertical structure is minimally altered and is directly applied in the reclamation of a post-mining site (Bulot et al., 2017). Therefore, reports have considered it a good practice to use fresh soil in the reclamation of disturbed ecosystems (Naeth et al., 2013a). In one case, successful reclamation using fresh soil restored 70% of the native forest understory species richness of a post-mining site (Koch, 2007). Similarly, with the direct application of topsoil, the establishment of a self-sustaining forest (i.e. self-renewing without the addition of extra inputs or human intervention) in a post-mining site in Spain was achieved (Ward, 2000). However, direct placement of topsoil in a reclamation site is not always a reasonable option since there may be no nearby sites ready to be reclaimed (Strohmayer, 1999; Gorzelak et al., 2020) or because the topsoil generated may not be well-suited to reclaim a particular post-mining site, i.e. reclaiming lowland ecosystems with upland soil may affect the establishment of native plant propagules (Mackenzie & Naeth, 2010). Under these circumstances, the removed

topsoil is stockpiled and may remain stored for long periods, until a post-mining site is available to be reclaimed (Johnson et al., 1991a; Kundu & Ghose, 1997; Strohmayer, 1999). Currently, there is a considerable proportion of sites disturbed by mining activities in the AOSR that will need to be reclaimed using stockpiled soils (Buss et al., 2020).

1.2.7 Salvaged soil amendments and treatments

Two main types of salvaged soil amendments are used in the reclamation of post-mining sites of the AOSR. One of them consists of peat extracted from 1-2 m depth from lowlands (Zahraei, 2015), and the other is LFH, an organic horizon composed of identifiable litter (L), fragmented and fermenting litter (F), and humus (H) (Paré et al., 1993), from upland forest soils generated by the accumulation of twigs, leaves, and mosses (MacKenzie, 2013). Peat and LFH are salvaged before the start of the mining activities and can be used in the reclamation of post-mining sites, alone or mixed with the underlying mineral soil, forming peat mineral mix (PMM) or forest floor mineral mix (FFM) respectively, (Fung & Macyk, 2000).

Compared to PMM, FFM has a greater amount of woody organic matter, which aids in the establishment of mesofauna, microbial communities, and plant diversity, (Brown, 2010). FFM also provides native plant propagules in the reclamation of boreal upland forest ecosystems (Fung & Macyk, 2000; Mackenzie & Naeth, 2010), and accelerates the restoration of forest ecosystems, as demonstrated by a study on the impact of reclamation with PMM and FFM on microbial communities (Zahraei, 2015), according to which the microbial biomass and microbial genes involved in the nitrogen cycle (i.e., NirS, and NifH) increased in the site reclaimed using FFM relative to that reclaimed with PMM (Zahraei, 2015).

Soil salvaging and application in reclamation in Alberta historically did not discriminate between upland and lowland material (Naeth et al., 2013). However, most of the area being disturbed by the mining activities lies in the wetlands, therefore peat and PMM are more abundant than FFM (Naeth et al., 2013), and are widely used in mine reclamation in the AOSR (MacKenzie & Naeth, 2007; Naeth et al., 2013). LFH in the upland forests of Alberta is variable but is not higher than 20 cm in depth (Naeth et al., 2013). Therefore, the limited amount of the LFH in the AOSR makes it impossible to exclusively use this material in all reclamation projects (Naeth et al., 2013).

It has been common practice to apply fertilizers to fresh or stockpiled soils or to combine the salvaged soil with overburden, peat, or mine residues to enrich the nutritional properties of the substrate and to create a soil-like material able to become a functional ecosystem that supports plant and microbial communities (Williamson & Johnson, 1990; Rowland et al., 2009). Likewise, by-products of oil sand extraction and upgrading like tailing sands and coke to PMM have been applied to improve plant growth (Wolter, 2012). For example, the amendment of PMM with biochar increases the availability of several nutrients and seed germination (Dietrich & MacKenzie, 2018). Since biochar also reduces microbial peat decomposition and stabilizes soil organic carbon, the use of PMM amended with biochar has been proposed for the reclamation of upland ecosystems.

The soil that is finally constructed using salvaged soil material and amendments lacks the general characteristics found in naturally occurring soils. Therefore, the engineered substrate cannot be classified in any of the orders currently accepted by the Canadian System of Soil Classification (CSSC). The anthroposolic order has been proposed by Naeth et al. (2012), to group soils that have been constructed by humans, like those generated from reclamation using peat mineral mix or mine residues. The proposed category will also include soils that have been highly modified by

anthropogenic activity, like those in which one or more of the natural horizons have been removed or replaced (Naeth et al., 2012). However, based on the relatively few mentions found in the literature, it can be said that this proposal has not found significant support or diffusion across the soil science community.

1.3 Impact of stockpiling on soil attributes

1.3.1 Effect of topsoil stockpiling on soil physical and chemical components

Soil stockpiling can lead to a severe disturbance of soil edaphic properties and soil structure (Izquierdo et al., 2005; Anderson et al., 2008), due to the mixing of soil horizons (Ghose & Kundu, 2004) that occurs as part of the mechanized process applied during stripping and stockpiling (Abdul-Kareem & McRae, 1984; MacKenzie, 2013). The impact of mechanized handling and heap forming have been considered to be more responsible for the loss of some soil-desirable properties than that generated by the topsoil storage time (Ghose & Kundu, 2004). Interestingly, these disturbances in the soil structure seem to persist even after the soil has been applied to reclamation (Fowler et al., 2015).

Further, stockpiling has been known to generate a reduction in soil aggregates, especially micro aggregates (Wick et al., 2009; Bach et al., 2010; Block et al., 2020). Due to the pivotal role of soil aggregates in protecting soil carbon and their hydraulic properties (Chen et al., 2014; Weil & Brady, 2017), the effect of the disturbance generated by stockpiling on soil aggregates may have serious consequences on soil structure such as the increase in soil bulk density (Shrestha & Lal, 2011) and a decrease in the water holding capacity (Ghose & Kundu, 2004; Shrestha & Lal, 2011). However, even when soil aggregates are significantly affected by stripping and stockpiling practices (Abdul-Kareem & McRae, 1984; Six et al., 1998), soil aggregates have been shown to

re-form with time since disturbance (Wick et al., 2009), or in the course of restoration (Schäffer et al., 2007).

Similarly, topsoil stockpiling has been associated with a loss of trace metals like Fe, Cu, Zn, and Mn (Ghose & Kundu, 2004), cations such as Ca, Mg, Na, and K (Ghose, 2001), and essential nutrients like nitrogen and carbon pools (Ghose & Kundu, 2004; Sheoran et al., 2010; Shrestha & Lal, 2011). Soil organic carbon is highly influenced by the proportion and turnover of soil aggregates (Six et al., 1998), and therefore the loss of soil aggregates can result in the loss of a considerable proportion of soil organic matter (Wick et al., 2008). This may explain the increase in the intra-aggregate carbon and a decrease in the outside-aggregate carbon in stockpiled soils (Wick et al., 2009), and may indicate that microbes can generally mineralize the organic carbon that is outside soil aggregates, but not the organic carbon inside soil aggregates, which is made available when soil structure is disrupted due to the stripping and stockpiling process. This could also explain the ephemeral increases in soil organic carbon that have been reported in relatively young stockpiled soils (Stratechuk, 2020). Additionally, the decrease in the organic carbon in the stockpile has also been attributed to a dilution of the organic carbon in the topsoil with the mineral subsoil during soil stripping and mound formation (Visser et al., 1984). The combination of these factors may have dramatic effects on soil biology and compromise stockpiled soil quality and its usefulness as a reclamation substrate (Harris et al., 1989; Johnson et al., 1991a; Block et al., 2020).

1.3.2 Effect of soil stockpiling on plant communities

The lack of commercially available native plant seeds to be used in restoration practices is a limitation to restoring disturbed sites to their pre-disturbance conditions (MacKenzie, 2013). Therefore, plant-viable seeds in the salvaged topsoil are essential in the rehabilitation of native plant communities of disturbed ecosystems (Munshower, 2017). However, there is a growing body

of literature addressing the negative impact of the disturbance generated by soil stockpiling on plant propagules and seed banks (Strohmayer, 1999; Navie et al., 2007; Valliere et al., 2021), and the evidence has shown that vegetation coverage, diversity, and richness is significantly higher in sites reclaimed using directly placed soil contrasted with those sites reclaimed using stockpiled soils (Dhar et al., 2019).

Salvaged topsoil depth may be a major factor influencing the decrease of salvaged soil's native plant propagules (Navie et al., 2007). For example, Buss & Pinno, (2019), revealed that seed emergence was significantly affected by stockpile depth. Likewise, Mackenzie & Naeth (2019) found that most of the seeds and rhizomes below one soil meter did not germinate and that the impact of stockpiling was more important for plant seeds that lack dormant stages or hard seed coat (Mackenzie & Naeth, 2019). Similarly, Golos et al. (2016), compared seed emergence between fresh and stockpiled soils and found stockpile age as a fundamental factor affecting seed emergence and community composition. Therefore, stockpile depth or age may be the determining factors affecting plant seed banks and seed emergence.

1.3.3 Effect of stockpiling on soil invertebrates

The re-establishment of soil mesofauna in post-mining sites usually requires the restoration of a vegetative plant cover (Witt, 1997). Also, the time soil remains stockpiled is very important for soil fauna, since according to Viert (1989), the life of soil fauna is limited to a few months in stockpiled soils. A study showed that the mesofauna present in the reclaimed sites of the AOSR was significantly lower than that in the undisturbed soils (Battigelli & Leskiw, 2006). This may indicate that the disturbance generated to the soil still impacts the mesofauna community, even after reclamation treatment has been applied.

1.3.4 Effect of stockpiling on soil microbial communities

Relatively little attention has been paid to the impact of soil stockpiling on microbial communities or to their potential as bioindicators of stockpiled soils' health. Microbial communities may increase in diversity and composition at the early stages of the stockpiling, probably in response to the availability of nutrients that were made accessible by the disturbance, but then after some time, a general decline or stabilization in the microbial community occurs (Johnson et al., 1991; Ngugi et al., 2020). Such a decline seems to happen almost concomitantly with a significant reduction in soil organic carbon and nitrogen in the stockpiles (Amir et al., 2022). This could indicate that the initial flush in microbial communities consists of the proliferation of copiotrophic microorganisms (Fierer et al., 2007).

Age since stockpiling is one of the main factors affecting soil microbial communities (Ghose, 2001; Ghose & Kundu, 2004). Multiple reports have suggested that topsoil has a "shelf life", indicating that some of the soil health attributes, including microbial diversity, may deteriorate with increasing storage time, and that eventually, the removed topsoil becomes biologically sterile and therefore unsuitable to be used in land reclamation (Gould & Liberta, 1981; Kundu & Ghose, 1997; Ghose, 2001). Consistent with this idea, Ghose (2001) and Ghose and Kundu (2004), reported a drastic decrease in the bacterial and fungal communities of the stockpiled soil, and that such a decrease was more significant with increasing stockpile age.

After a relatively short period of soil storage, the microbial community composition recovers to resemble the communities in the reference soils (Gorzelak et al., 2020). Arbuscular mycorrhizal fungi (AMF) populations decreased in response to the initial disturbance, but after a period of soil storage, the proportion of AMF in stockpiled soils reached the level found in the reference ecosystems (Birnbaum et al., 2017b). These results are consistent with other studies reporting the

restoration of the AMF propagules in removed topsoil after 5 years of revegetation (Jasper et al., 1987). The restoration of the microbial community structure or biodiversity in the stockpiled soils may have been correlated with the carbon inputs of the plant community, and since plant communities are more developed in older stockpiles, then AMF will populate them preferentially (Birnbaum et al. 2017b). However, the fact that microbial communities or functional groups may recover with time since disturbance challenges the idea of a "shelf life" for stockpiled soil.

Due to limited space, topsoil tends to be stored in large piles (Strohmayer, 1999). The height of the topsoil heaps is another factor that has historically generated concerns when referring to the impact of stockpiling on soil microbial communities (Block et al., 2020). The reduced productivity and low biomass in the deep regions of the stockpiles may be the cause of the loss in microbial diversity (Paterson et al., 2019). Likewise, there is a decrease in nitrogen proportion in the deeper layers of the stockpiled soil (Fischer et al., 2022) and this decrease is linked to the reduction in microbial diversity. Severe conditions have been attributed to the deep layers of the stockpiles, which are likely generated by the reduced levels of oxygen (Mackenzie & Naeth, 2010; Naeth et al., 2013). The scarcity of oxygen in the stockpiled soil's lower layers generates anaerobic zones that promote the dominance of anaerobes and facultative aerobe microbial populations, (Abdul-Kareem & McRae, 1984; Harris et al., 1989; Johnson et al., 1991a: Anderson et al., 2008). Together the loss of nutrients and oxygen may influence the structure and dynamics of microbial communities in the stockpiled soils.

The literature registers multiple works referring to the impact of stockpile depth on microbial communities. The works of Block et al. (2020), on the effect of stockpile depth on microbial communities, found that both bacterial and fungal communities were significantly reduced by stockpile depth. These results align with the findings of Johnson et al. (1991), who reported a

decrease in the fungal and bacterial aerobic populations with increasing stockpile depth within the top 2 meters, especially in the oldest piles (11 years), which is consistent with the findings of Amir et al. (2022), who found the decrease of aerobic bacteria in stockpiled soils and the time since stockpiling to be correlated. Therefore, it may be the case that the harsh conditions attributed to the stockpile's deep layers develop with the time of storage.

The presumed anaerobic conditions of the deep strata of the stockpiles have been evidenced by the abundance of methane in the deep layers of stockpiles (>1m), indicating the presence of methanogenic archaea (Williamson & Johnson, 1990; Fischer et al., 2022), which are strict anaerobic microorganisms. Also, the studies of Amir et al. (2022) on the microbial communities of the stockpiled soils found the anaerobic bacterial community did not decrease with increasing stockpile depth, which may suggest that the anaerobic conditions are present in most parts of the piles.

1.3.5 Effect of stockpiling and associated disturbances on fungal guilds and trophic modes.

Soil fungi are known to play crucial roles in forest health and regeneration (Frąc et al., 2018; Policelli et al., 2020), to establish complex symbiotic relationships with other organisms (Averill et al., 2014; Ramsfield et al., 2020), and are important in soil structuring processes (Miller & Jastrow, 1992). Furthermore, the re-establishment of native mycorrhizal fungi improves the restoration of disturbed forest ecosystems (Neuenkamp et al., 2019; Koziol et al., 2022), including post-mining sites (Wang, 2017). Therefore, determining the consequences of stockpiling on fungal diversity is of paramount relevance at the time of planning ecosystem restoration.

Disturbances in soil physical and chemical attributes affect fungal associations with plant roots (Jasper et al., 1987; Fadaei et al., 2021) and soil disturbances are among the factors leading to a

significant mycorrhizal fungal decrease in the forests (Arnolds, 1991). This could explain the negative effect of soil stockpiling and associated disturbances on arbuscular mycorrhizal (AM) (Stark & Redente, 1987; Ezeokoli et al., 2019a). AMF spores decrease with stockpile increasing depth (Amir et al., 2022); however, time since stockpiling may be the main factor determining the decrease in AM fungi capacity to infect roots in the soils (Abdul-Kareem & McRae, 1984; Miller & Jastrow, 1992).

Relatively fewer works have examined the impact of soil stockpiling on ericoid mycorrhizal fungi (ERM). The findings of Fadaei et al. (2021), indicate that ERM fungi follow the same trends as AM fungi, since stockpiling decreased the root colonization and diversity of ERM in stockpiled soils (Fadaei et al., 2021). Likewise, ERM fungi infectivity is reduced in early reclaimed sites, although they seem to recover over time (Hutton et al., 1997; Ngugi et al., 2020). Therefore, the initial decrease of the ERM fungi in stockpiled and reclaimed sites may reflect the response of the mycorrhizal fungi to the removal of suitable host plants in these soils (Miller & Jastrow, 1992).

Ectomycorrhizal fungi (ECM) dominate in boreal ecosystems (Tedersoo & Nara, 2010). The distribution of the ECM fungi in the boreal mixed wood forest is highly influenced by the native vegetation of the region (Read, 1991). The AM fungi have been described as “generalist” since they tend to establish symbiotic relationships with a broad range of plant species (Richardson et al., 2000; Fitter, 2005; Tedersoo & Nara, 2010). Consequently, the AM fungi's non-specific plant associations promote the colonization of non-native plants, like weeds and forbs (Read., 1991; Bunn et al., 2015). Moreover, a higher proportion of AMF and ECM fungi in reclaimed and undisturbed soils respectively have been reported (Ramsfield et al., 2020), and ~80% of plants growing in post-mining sites are associated with AMF (Wang, 2017). So, the shift in the dominant

type of mycorrhizal fungi from ECM to AMF could explain the reported dominance of invasive plant species in the stockpiled soils (Buss et al., 2020) and reclaimed sites (Dhar et al., 2018).

There is a decrease in ECM and an increase in saprotrophic fungal groups following the disturbance generated by soil stockpiling and associated processes (Sukdeo et al., 2018). These findings align with other studies reporting the dominance of ectomycorrhizal fungi in the undisturbed boreal mixed-wood forests while in the adjacent reclaimed sites, saprotrophic fungal groups are dominant (Ramsfield et al., 2020). The dominance of ECM fungi over the saprotrophic fungi in the unmined forest has been attributed to an antagonistic relationship between these two fungal groups (Orwin et al., 2011; Averill et al., 2014), in which the ECM fungi control the saprophytic decomposition of litter through the limitation of available nitrogen in the soil, thus increasing soil carbon storage (Averill et al., 2014; Jacobs et al., 2018). Therefore, the shift from ECM to saprotrophic fungi in disturbed soils seems to originate in the increased amount of available carbon resulting from soil aggregate destruction by soil extraction and handling (Six et al., 1998; Wick et al., 2008). An additional factor explaining the shift in fungal guilds between disturbed and undisturbed ecosystems is the removal of forest vegetation as part of the mining and industrial activities (Strohmayer, 1999; Ghose, 2001), which decreases the population of ECM fungi (Pec et al., 2017; Rodriguez-Ramos et al., 2021) therefore eliminating the control of ECM over the saprotrophic fungi.

1.3.6 Effect of stockpiling and associated disturbances on functions played by microbial communities.

Microbial communities are key players in nutrient cycling and other soil functions (Madsen, 2011; Maestre, et al., 2016; Nannipieri et al., 2020). For example, heavily degraded soils usually have a considerably lower amount of nutrients like nitrogen and phosphorus (Cooke & Johnson, 2002;

Li, 2006), which may be connected to the fact that disturbances of the biological communities may disrupt the cycling of these nutrients (McClain et al., 1998; Foster & Bhatti, 2005). Disruption of nutrient cycling may persist for many years (Amazonas et al., 2011). Therefore, the recovery of nutrient cycling dynamics, and the microbes involved, is part of ecosystem restoration (Falk et al., 2006; Huang et al., 2012).

Nitrogen is fundamental for life and is the main nutrient limiting primary production in soils (Vitousek & Howarth, 1991; McGuire et al., 1995). Some of the stages of the nitrogen cycle are performed exclusively by microbes (Jetten et al., 2008). There is a decreased nitrogen mineralization in stockpiled soils (Harris & Birch,1989). Genes involved in the nitrogen cycle, such as *nirS* (a marker for denitrification), *nifH* (a marker for nitrogen fixation), and *amoA* (a marker for ammonia oxidation), increased with increasing reclamation time (Zahraei, 2015), thus indicating that the functions of the microbial communities in the nitrogen cycle are restored following reclamation.

Phosphorus has been regarded as the second most limiting nutrient for primary production (Vitousek & Howarth, 1991), and in some cases may be as limiting for primary production as nitrogen (Elser et al., 2007). Phosphate solubilization and mineralization mediating organisms play an essential role in the cycling of phosphorus (Alori et al., 2017; Liang et al., 2020; Tian et al., 2021). Soil stockpiling negatively impacts microbial phosphate solubilization (Mashigo, 2018). However, a metagenomic study revealed that the abundance of genes involved in phosphorus solubilization increased following a mining site restoration (Liang et al., 2020). Together the reviewed literature consistently indicates that the cycling of nitrogen and phosphorus are altered by soil disturbances, like stockpiling, but these cycles respond positively to reclamation treatment.

1.4 Structure of the microbial communities in disturbed ecosystems

1.4.1 Microbial community assembly processes

The recovery of community structure and the ecosystem to their pre-disturbance condition is expected as the most likely outcome of restoration (Cutler et al., 2017; Jurburg et al., 2017). However, community structure might be resistant to restoration (Suding et al., 2004; Lankau et al., 2014; Calderón et al., 2017) or move to incomplete restoration or alternative ecosystem states (Scheffer et al., 2001; Falk et al., 2006). Better knowledge of processes that influence the succession and structuring of microbial communities following disturbances like soil stockpiling, might help to predict the outcome of restoration projects.

The mechanisms that determine the structure and patterns of the succession of microbial communities are a source of debate (Dumbrell et al., 2010; Nemergut et al., 2013; Kane et al., 2020). Stochastic or neutral theories posit that individuals within a community are competitively, ecologically, and functionally identical, therefore differences in traits do not influence the abundance and speciation rates (McGill et al., 2006; Zhou & Ning, 2017), and rely on the relevance of random extinction, birth/death rate, colonization, and drift as the most influential factors in community assembly (Harpole, 2010; Nemergut et al., 2013). On the other hand, niche-based processes are centered on deterministic theories (e.g., trade-offs, environmental filtering, priority effects) that are important determinants of community assembly (Dumbrell et al., 2010; Chase & Myers, 2011; Nemergut et al., 2013).

More phylogenetically clustered microbial communities are those that are shaped by selective processes like environmental filtering (Horner-Devine & Bohannan, 2006; Stegen et al., 2012; Dong et al., 2021), and therefore their phylogenetic relatedness is evidenced by traits that allow

them to occupy a given habitat. The stochastic processes, however, are determinant in communities with a regional species pool larger than locally interacting species, (i.e., higher ratio gamma diversity: alpha diversity) and in which selection is weak (Chase & Myers, 2011). However, both stochastic and deterministic factors influence the structure of microbial communities (Dumbrell et al., 2010; Caruso et al., 2011; Chase & Myers, 2011). Speciation and dispersal seem to be the processes that incorporate new species in the community, whereas selection and drift seem to be the processes that determine the relative abundances of the microbial groups (Vellend, 2010; Kane et al., 2020).

Additionally, other deterministic community assembly processes commonly found in the literature, are the historical contingencies or legacy effects, defined as the influences of conditions or processes that originated in the past on the current community structure, (Zhou & Ning, 2017). The legacy effect of vegetation continues to be determinant in the structure of microbial communities even two years after the vegetation was removed (Elgersma et al., 2011), and the negative effect of an invasive plant on arbuscular mycorrhizal fungi continues to be present in the soil, even six years after the invasive plant was removed (Lankau et al., 2014). However, the relevance of legacy effects on soil communities will depend on the frequency and intensity of the disturbance the soil went through (Jacquet & Altermatt, 2020).

1.4.2 Ecological succession and community assembly processes in naturally and anthropogenically disturbed ecosystems.

The impact of naturally occurring disturbances on the succession and structure of soil microbial communities has been documented (Wilhelm et al., 2013; Whitman et al., 2019). These include the impact of forest fires (Holden et al., 2016; Knelman et al., 2017; Smith et al., 2017) and glacial

retreat (Wilhelm et al., 2013; Cline & Zak, 2014; Schütte et al., 2019). Microbial communities show considerable resilience to disturbances (Sun et al., 2016), and the severity of the natural disturbance determines the structure of the community (Smith et al., 2017; Whitman et al., 2019), including the switch from ectomycorrhizal to arbuscular mycorrhizal fungi in boreal forest soils (Treseder & Lennon, 2015). Similarly, after forest fires the microbial community succession is initially determined by stochastic processes, but later deterministic factors seem to gain more influence over the shaping of the community structure (Ferrenberg et al., 2013; Yuan et al., 2019).

Anthropogenic ecosystem disturbances may generate drastic shifts in microbial community composition and change ecosystem dynamics (Rodriguez-Ramos et al., 2021). Fungal and bacterial communities seem to follow different successional patterns after soil disturbance (Banning et al., 2011; Sun et al., 2016; Sun et al., 2017; Gorzelak et al., 2020), and display contrasting structure patterns, depending on the time since stockpiling (Gorzelak et al., 2020) and reclamation (Sun et al., 2017). Both deterministic and stochastic factors are important in the assembly of soil microbial communities following disturbance (Dumbrell et al., 2010; Chase & Myers, 2011). Some researchers found that the effect of deterministic processes associated with environmental factors (i.e., pH, organic matter, and texture), are important for community assembly (Li et al., 2014; Gao et al., 2020; Wang et al., 2020), and are more important than stochastic factors (Gao et al., 2020; Kane et al., 2020). Similarly, the community composition of the disturbed soils influences the capacity of newly introduced communities to colonize the soil, which indicates that deterministic processes like priority effects (i.e., the impact of early-colonizing taxa in the community structure) are crucial in the community assembly (Calderón et al., 2017). However, Osburn et al. (2019) found that both stochastic and deterministic factors shape

the structure of bacterial communities, but stochastic processes seem to be more influential for the structure of fungal communities.

1.4.3 Disturbance factors associated with the shift in the microbial communities of the soil.

Soil microbial communities' structure and diversity are significantly influenced by several factors, including biotic, chemical, and anthropogenic activities (Kowalchuk et al., 2002; Fierer et al., 2007; Weil & Brady, 2017). In the case of stockpiled soils, the reduced level of nitrogen and organic carbon is generally the main factor affecting the microbial communities (Ghose & Kundu, 2004; Sheoran et al., 2010; Shrestha & Lal, 2011). Another important factor affecting the microbial community composition is the lack of oxygen in the piles, generated by the soil compaction associated with the increase in bulk density and storage time (Johnson et al., 1991; Ezeokoli et al., 2019; Block et al., 2020), since the anoxic conditions generally select the dominance of microbial populations with anaerobic metabolisms and reduce the levels of aerobic microbial groups.

Several factors are correlated with the structure of microbial communities in reclaimed and undisturbed ecosystems. For instance, pH, nitrogen, and plant cover are the most important predictors of the microbial community structure in reclaimed and undisturbed soils in the AOSR (Dimitriu & Grayston, 2010). More precisely, the plant cover is the main factor that determines the dominant microbial communities in the undisturbed boreal forest (Read, 1991; Masse et al., 2017), whereas pH, nitrogen deposition, and clay content correlate with the dominant groups in reconstructed soils (Masse et al., 2017), like those soils generated by reclamation.

1.4.4 Effect of soil disturbances on microbial diversity

Soils constitute an intricate web of habitats that hosts extensive microbial communities implicated in essential ecosystem functions (Fierer, 2017; Tecon & Or, 2017). Fierer et al. (2017) have argued

that soil should not be considered a single environment since the interplay of chemical, physical, and spatiotemporal factors in the soil creates heterogeneous and patchy environments (Fenchel, 2002; Tecon & Or, 2017). The unparalleled vast biological diversity in the soils lies within microbes (Kowalchuk et al., 2002). The characterization of this diversity and the mechanisms that structure the microbial communities is a challenging task, since most soil microorganisms (>99%), have not been cultured (Zhou et al., 2010), and the concept of microbial species is rather diffuse (Kowalchuk et al., 2002).

The importance of microbial diversity in the ecosystem has been generally taken for granted by most soil microbial ecology works since increased microbial diversity has intuitively been considered a positive sign of ecosystem health, but no rationale for such a perception is usually provided. However, more diverse bacterial communities are generally more resistant to ecosystem disturbances (Eisenhauer et al., 2012; Ezeokoli et al., 2019). Likewise, microbial communities in which few species are highly dominant (uneven) are less resilient to environmental stress (Wittebolle et al., 2009). Similarly, plant richness (i.e., number of species) is correlated with bacterial richness in the soils (Lamb et al., 2011; Schlatter et al., 2020), and therefore microbial richness and diversity may influence the types of plants that populate certain ecosystems (Read, 1991; Tedersoo & Nara, 2010). Therefore, microbial diversity is a determinant factor to consider at the time of implementing ecosystem restoration programs.

1.4.5 Microbial diversity and ecosystem multifunctionality

Soil microbial communities are fundamental in the functioning of soil ecosystems (Chodak et al., 2009), and their activities are essential for healthy soil (Tecon & Or, 2017). Some of the functions played by microbes are (1) consumption and production of atmospheric gasses like CO_2 and methane, (2) mediation of nutrient cycling, (3) soil organic carbon regulation and litter

decomposition, and (4) plant growth promotion (Delgado-Baquerizo et al., 2016; Fierer, 2017). Therefore, microbes are key components of the ecosystem's multifunctionality, which is defined by Byrnes et al. (2014) as the range of processes and services that are provided simultaneously by the ecosystem. Several factors are predictors of ecosystem multifunctionality, (e.g., climate, temperature, and pH), and the fact that microbial diversity could also be a driver of ecosystem multifunctionality has also been considered (Delgado-Baquerizo et al., 2016).

The relationship between ecosystem function and microbial diversity has been reviewed previously (Cardinale et al., 2011; Byrnes et al., 2014; Wang et al., 2019). There is a growing body of literature suggesting that ecosystem multifunctionality is promoted by microbial diversity (Delgado-Baquerizo et al., 2016; Wagg et al., 2019; Shi et al., 2021), microbial community evenness (Wittebolle et al., 2009), and species richness (Maestre et al., 2012) in terrestrial ecosystems. For instance, microbial diversity (both fungal and bacterial) is positively correlated with ecosystem function, and microbial diversity seems to be a significant predictor of ecosystem multifunctionality (Delgado-Boquerizo et al. 2016). Therefore, any perturbation in the microbial community that decreases microbial diversity, could also affect essential functions played by microbes, thus impacting ecosystem multifunctionality. However, decreased diversity impairs multifunctionality only in portions of the community with low functional redundancy (e.g., N_2 fixing and nitrifying communities), whereas functions with high redundancy, like soil basal respiration, are not affected by the decreased microbial diversity (Wertz et al., 2006; Li et al., 2021).

Stockpiling leads to decreased soil microbial diversity (Gould & Liberta, 1981; Ghose, 2001; Ezeokoli et al., 2019), and specific stockpiling factors like the age since stockpiling (Ghose, 2001), nutrient scarcity (Ezeokoli et al., 2019) and stockpile depth (Harris et al., 1989; Williamson &

Johnson, 1990; Johnson et al., 1991) are factors associated with decreased microbial diversity. Soil handling and mixing are also factors responsible for the deterioration of soil structure, thus impacting soil biological properties (Paterson et al., 2019; Block et al., 2020). Disturbances caused by soil mixing generate a decrease of >20% in bacterial richness in the soil and modify the community composition (West & Whitman, 2022). Therefore, stockpiling could affect important ecosystem functions played by microbes.

1.5 Microbial communities as indicators of soil health and condition

Soil health has been defined as “the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans” (USDA-NRCS, 2021). Soil quality, on the other hand, has been defined as "the capacity of a soil to function, within ecosystem and land-use boundaries, to sustain biological productivity, maintain environmental quality, and promote plant and animal health"(Doran & Parkin, 1994). Even when the two concepts look similar and are usually used interchangeably, soil health deals with the integrity and balance of the soil community, stability, resilience, and self-regulation of the soil ecosystem (Weil & Brady, 2017), whereas soil quality refers to the physico-chemical or edaphic attributes of the soil that determine soil productivity (Curell, 2012; Maikhuri & Rao, 2012). Soil health and quality measures may facilitate the prediction of the efficacy of a reclamation process using a particular stockpiled soil (Ezeokoli et al., 2019). Such an assessment usually includes physical and chemical factors (Schoenholtz et al., 2000; Cardoso et al., 2013). Likewise, the response of the soils’ biological components to the disturbance has been proposed as an accurate tool to determine soil health status (Doran and Zeiss, 2000; van Bruggen and Semenov, 2000), the magnitude of soil degradation, and ecosystem response to soil management practices designed to revert the disturbance (Harris, 2003).

Since soil microbes are sensitive to alterations in soil characteristics (Mummey et al., 2002; Benintende et al., 2008; Masto et al., 2009), they can respond rapidly to changing soil conditions (Izquierdo et al., 2005; Muñoz-Rojas, 2018). Thus, the health status of the soil is reflected by shifts in microbial communities (Ohsowski et al., 2012; Waterhouse et al., 2014), Therefore, it is likely that the composition of microbial communities can be used as a measure of the impact of soil stockpiling on soil health (Costantini et al., 2016; Ezeokoli et al., 2021). Microbial community structure may, therefore, shed light on the usefulness of the stored soils in the reclamation of disturbed ecosystems.

Anthropogenic disturbances have a greater impact on microbial communities than climate variations (Hermans et al., 2020), and the bacterial community structure can be used to predict soil physicochemical characteristics and determine the magnitude of anthropogenic disturbances (Hermans et al., 2017). Similarly, changes in the abundance of specific bacterial taxa in the soil are correlated with changes in the soils caused by specific disturbances (Jiménez-Bueno et al., 2016; Kim et al., 2021), and the fertility of the soil can be determined based on the presence and abundance of arbuscular mycorrhizal fungi (Syibli et al., 2013), and ectomycorrhizal fungi (Kranabetter et al., 2009). Similarly, microbial communities are accurate indicators of disturbed and undisturbed ecosystems during post-mining reclamation (Mummey et al., 2002). The disturbance generated by stripping and salvaging is associated with the presence of several specific fungal groups (Sukdeo et al., 2018; Amir et al., 2022), and the arbuscular mycorrhizal fungi composition can be used to differentiate between recent and mature reclamation sites (Ezeokoli et al., 2020). These findings seem to indicate that stockpiling creates a microbial community that diverges from that generated by the natural range of variability, and that could be profiled as indicators of soil disturbance.

1.6 Research project

To examine the magnitude of the impact of stockpiling on soil microbial communities, I assessed the turnover in microbial communities, and their putative functions in a chronosequence of 0.5-28 years old stockpiled soil from two oil-extraction locations in northern Alberta. I characterized the communities by marker gene sequencing and correlated them with environmental metadata to shed light on the factors that could explain the variability in the microbial communities.

1.6.1 Research questions and hypotheses

My Ph.D. thesis aims to shed light on the following questions: (i) How does time since stockpiling and stockpile depth affect soil microbial community structure and diversity? (ii) What environmental factors shape the stockpiled soil microbial communities? (iii) which assembly processes determine the beta diversity of the microbial communities in the stockpiled soils? and (iv) Does soil stockpiling impact the trophic modes and key functions played by microbes in soils?

I hypothesize that the bacterial and fungal communities of the stockpiles and the undisturbed soils are significantly divergent from each other and that the stockpile age and depth account for most of the divergence between the two soils. Furthermore, since microbes play critical roles in multiple ecosystem services, I hypothesize that the impact of stockpiling on microbial community structure also affects the functions played by soil microbial communities. Additionally, I hypothesize that selective processes associated with the harsh conditions attributed to soil stockpiling will drive the turnover and structure of microbial communities in stockpiled soils.

1.6.2 Methods

1.6.2.1 Methods and experimental design

We collected samples from stockpiles and undisturbed soils in two different mining locations in northern Alberta: Horizon (surface-mining site) and Wolf Lake (*in-situ* oil sands thermal extraction site). At the Horizon site, we sampled stockpiles of 0.5, 1.5, 5, and 7 years old, whereas, we sampled stockpiles of 2, 11, and 28 years old at the Wolf Lake site. From each of these stockpiles, we collected samples at five different depths (0-10 cm, 10-20 cm, 20-30 cm, 80-90 cm, and >300 cm). We sampled three replicate pits at each sampling location. The measured edaphic parameters included: pH, conductivity, water content, total carbon, total nitrogen, soil texture, and aggregate size. I extracted DNA in triplicate from soil samples of the eight stockpiles and six nearby undisturbed soils used as reference sites. Following PCR amplification, the V4 16S rRNA DNA region for the prokaryotic communities and the ITS-2 DNA region for the fungal communities' marker genes were sequenced. The data generated in the procedures described above was analyzed using multivariate statistics to test the hypotheses.

1.6.3 Significance and implications of the study

If these hypotheses are supported, it might indicate that the disturbance generated by the stockpiling makes microbial communities and their functions different from the communities generated by the range of natural variability. Even when we are not certain about the consequences this could have in the trajectories followed by the reclaimed soil, several reports have demonstrated that the disturbances generated by stockpiling on the soil may persist even after the soil has been applied in the reclamation of post-mining sites. If microbial communities of the stockpiled soils and the functions they play are different from those in the undisturbed soil, the land reclamation goal of returning the disturbed post-mining site to its pre-disturbance conditions may not be achieved using stockpiled soil as a reclamation substrate.

CHAPTER 2

Impact of stockpile depth and storage time on soil microbial communities

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2.1 Introduction

2.1.1 Oil sand extraction activities in the Athabasca Oil Sand region

Covering an area of 142,200 km^2, the Athabasca Oil Sands region (AOSR) is one of the three most important oil reserves in the world and is a key component of the Canadian economy (Government of Alberta, 2021). In this region, the superficial oil sand deposits are exploited using open-pit mining (Mackenzie, 2013). In contrast, the deposits in the deeper soil layers are extracted employing steam-assisted gravity drainage (SAGD) operations and other thermal in *situ* extraction methods (Johnson and Miyanishi, 2008). The environmental impact associated with oil-mining activities generates vast zones of disturbed land (Rowland et al., 2009). As an example, in the year 2020, the ecosystem disturbance generated by the oil-sand open-pit mining activities in the AOSR expanded to more than 900 km^2 (Alberta Environment and Parks, 2022). Similarly, the landscape fragmentation associated with *in-situ* oil sands extraction methods (Jordaan et al., 2009), is characterized by a scattered landscape of smaller patches of total area (Opdam et al., 1993), which is detrimental to biodiversity (Fahrig, 2003).

2.1.2 Land reclamation and soil stockpiling

The goal of land reclamation is to restore mined lands to their pre-disturbance conditions (Bohrer et al., 2017), which includes the rehabilitation of ecosystem function, services, and land use capability (Ezeokoli et al., 2019). Surface soils are a valuable resource in land reclamation, since they are generally rich in nutrients, native plant propagules, and microbial communities (Mushia et al., 2016, Buss et al., 2020). Therefore, surface soils are often mandated to be retained for use in the reclamation of disturbed ecosystems, especially at sites where mining has concluded (Paterson et al., 2019). There is not always a nearby post-mining site available for reclamation; as a result, topsoil may remain stockpiled (stored) for long periods (Strohmayer, 1999). Longer storage times can lead to a severe disturbance of soil structure and chemical properties (Harris and Birch, 1989; Izquierdo et al., 2005), which may compromise stockpiled soil quality and probably its usefulness as a reclamation substrate (Harris et al., 1989; Birnbaum et al., 2017).

2.1.3 Microbial communities as indicators of soil health

Soil health has been defined as "the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans" (NRCS-USDA, 2021). Soil health assessment may be used to facilitate the prediction of the efficacy of a reclamation process using a particular stockpiled soil. Such an assessment usually includes physical and chemical factors of the soil (Cardoso et al., 2013; Stewart et al., 2018). Likewise, the response of the soils' biological components to the disturbance has been proposed as an accurate tool to determine soil health status (Doran and Zeiss, 2000; van Bruggen and Semenov, 2000).

There is a growing body of literature addressing the effects of soil stockpiling on plant communities (Golos et al., 2016, Buss et al., 2018) and invertebrates (Boyer et al., 2011, Ezeokoli et al., 2021). However, relatively little attention has been paid to the impact of soil stockpiling on

microbial communities or to specific stockpiling factors such as depth and storage time on microbial communities' structure, dynamics, and diversity. Similarly, few studies have assessed the potential of microbial communities as bioindicators of stockpiled soils' health. Many of the studies to date are based on cultivation techniques (Persson and Funke, 1988; Harris et al., 1989; Johnson et al., 1991). However, since most of the microbes are not able to be cultured under laboratory conditions (Amann et al., 1995; Harwani, 2013), these studies may be biased toward the culturable microbial fraction. Other studies have assessed the enzymatic activity of stockpile microbial communities (Harris and Birch, 1989; Waterhouse et al., 2014), but due to the high functional redundancy commonly found in the soils, these activities may be impossible to attribute to any specific microbial lineage (Wittebolle et al., 2009; Maron et al., 2018). Yet other approaches have included the assessment of the microbial biomass in the stockpiles as bioindicators (Waterhouse et al., 2014; Block et al., 2020), which usually generates limited information about the community composition (Fierer et al., 2021) due to its low taxonomic resolution. Therefore, these studies may not accurately represent the status of the microbial communities in the stockpiles. On the other hand, since the 16S ribosomal RNA is universal in prokaryotes, the profiling of this marker gene can be used to examine the structure of soil microbial communities, independent of their physiological state or culturability. This approach allows a more robust and representative study of the microbial communities and more precise detection of their fluctuations in response to changes in the soil conditions, which may be used to gain insights on the impact of soil disturbances, like soil stockpiling, on microbial communities.

2.1.4 General approach and objectives of the research

To determine the impact of stockpiling on soil microbial communities, we assessed the prokaryotic microbial communities of stockpiled topsoil generated from two oil extraction locations in

northern Alberta, Canada. The objectives of the study were: 1) to determine whether the disturbance generated by the soil stockpiling affects the structure and diversity of prokaryotic soil microbial communities, making them different from the communities of nearby undisturbed soils used as reference, and 2) to ascertain the extent to which stockpile storage time and stockpile depth are important predictors of variability between the microbial communities of the stockpiles and the reference soils. My findings may provide insights into the consequences of stockpiling for soil biodiversity, and community dynamics, which may shed light on the effectiveness of soil microbial communities as markers of soil health and the usefulness of stockpiled soils in the reclamation of post-mining sites. In turn, this may lead to the improvement of stockpiling protocols and practices and help optimize conditions for surface soil stockpiling and their successful use in post-mining restoration operations.

2.2 Methods

2.2.1 Study sites

Samples were collected from stockpiled and undisturbed soils in two locations in the central region of the boreal forest in Alberta: (1) Canadian Natural Resources Limited Horizon (CNRL Horizon), an oil sands surface mining site north of Fort McMurray, Alberta (57.337 °N, 111.755 °W), and (2) Canadian Natural Resources Limited Wolf Lake (CNRL Wolf Lake), a thermal *in-situ* oil-sands extraction site near Cold Lake, Alberta (54.695 °N, 110.730 °W). At both sites, LFH organic soil and mineral upland topsoils were stored in stockpiles of various ages and heights (Buss et al., 2020). Stockpiles at the CNRL Horizon site were generated with excavators and were flat to pile or hill-shaped, covering 10 hectares on average. The stockpiles at the CNRL Wolf Lake site were generated with dozers, covered 0.3 hectares on average, and were pile or hill-shaped (Buss et al.,

2020). The vegetation atop the stockpiles was mostly composed of herbaceous species to a combination of woody/herbaceous native species and herbaceous-invasive species (Stratechuk, 2020).

The undisturbed soils were used as reference sites whose conditions reflect the combination of soil type and vegetation characteristic of the mixed-wood natural subregion of the boreal forest in Alberta, and therefore were chosen as targets for the ideal reclamation outcomes. To avoid the effects of the anthropogenic disturbances generated by the mining activities in the Horizon site, the reference locations were situated approximately 5-6 kilometers away from the stockpiles. For the reference sites in Wolf Lake, the more fragmented disturbance generated by the *in-situ* oil-sands extraction allowed each of the four reference sites to be located closer to their respective stockpiling site (approximately 150 meters away). The reference sites at both Horizon and Wolf Lake were characterized by an upland canopy of white spruce and trembling aspen (Stratechuk, 2020).

2.2.2 Field sampling methods

Sampling took place in August 2018. A total of seven stockpiles and five reference soils (two in the Horizon site and three in the Wolf Lake site) were sampled. At the Horizon site, we sampled stockpiles 0.5, 1.5, 5, and 7 years old; at the Wolf Lake site, we sampled stockpiles 2, 11, and 28 years old. At both sites, stockpile ages were chosen based on availability. Five depths were sampled for each stockpiled soil: 0-10 cm, 10-20 cm, 20-30 cm, 80-90 cm, and >300 cm. The sampling took place in three replicate pits per depth layer, except for the 10-20 cm and >300 cm samples, which were sampled from only one pit per stockpile. Sampling pits were spaced ~20 meters apart from each other to account for the variability in the soils. The pits were dug using a sterile pickaxe and a shovel to a depth of 90 cm, while an excavator was used for depths of > 300

cm. Soil samples were collected from the sampling face of the pit in sterile plastic bags and transported to the laboratory in coolers. Samples were then passed through a 4 mm sieve to homogenize them and remove large debris. Samples were stored in sealed sterile plastic bags at -80 °C. Following preliminary analysis of sample grouping, samples were grouped in three age categories: young (i.e., 0.5-, 1.5-, and 2.0-year-old stockpiles), intermediate (i.e., 4- and 7-year-old stockpiles), and old (i.e., 11- and 28-years old stockpiles). Similarly, soil samples were categorized into the following depth groups: surface (0-10 cm and 10-20 cm), intermediate (20-30 cm), and deep (80-90 cm and >300 cm).

2.2.3 Soil chemical and physical parameters

The physical and chemical properties of each soil sample were measured as follows: pH and electrical conductivity (EC), were determined using the methods described by Kalra and Maynard, (1991). Gravimetric water content was determined as previously outlined in Topp et al. (2008). Soil texture was identified according to the hydrometer method (Kaddah, 1974). Total soil carbon and total nitrogen content were determined using a Thermo-Finnigan Delta V Advantage Continuous Flow Isotope Ratio Mass Spectrometer, at the Natural Resources Analytical Lab (NRAL) at the University of Alberta.

2.2.4 DNA extraction and amplification

DNA was extracted from triplicate 0.25 g randomized subsamples of homogenized soil using the DNeasy PowerSoil kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The triplicate DNA samples were then combined to make a single representative sample. DNA concentration was measured with a Qubit dsDNA HS Assay Kit according to the manufacturer's protocol (ThermoFisher Scientific, Canada). The V4 16S rRNA region was amplified using the Earth Microbiome Project primer pair 515F and 806R (Caporaso et al., 2011). The PCR thermal

cycle was 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. The extracted DNA samples, a lab-constructed mock community (consisting of genomic DNA of 10 different bacterial strains, see Figure S2-1), and the respective extraction blanks (Table S2.1) were sequenced in randomized plate order, based on the 16S rRNA V4 gene, using an Illumina MiSeq platform with the 250-bp paired-end kit (V2 500-cycle PE Chemistry, Illumina, USA), by Microbiome Insights (Vancouver, Canada).

2.2.5 Data analyses

Demultiplexed FASTQ files corresponding to the DNA amplicons generated by Illumina sequencing were processed in a stepwise workflow (quality-filtering, trimming, dereplication, merging of paired-end reads into amplicon sequence variants (ASV), and chimera identification and removal). These analyses were performed in "R version 3.6.1" (R Core Team, 2019), using the "DADA2 1.16.0" pipeline (Callahan et al., 2016). The reverse amplicon sequences showed lower quality than the forward ones (as expected from DNA amplicons generated by Illumina sequencing), so we trimmed the last 50 bp out of 250 bp for the reverse amplicons and the last 10 bp out of 250 bp of the forward amplicons. In sum, 95.1% of the reads passed the quality filter step. After the dereplication and merging processes, 16,334 ASVs were generated, of which 4.64% ASVs were removed as chimeras, reducing the number of unique ASVs to 15,576. At this point, we applied the taxonomy assignment, using the "SILVA 132 release ribosomal RNA gene database" (Quast et al., 2012). We then used the "Decontam 1.6.0" R package (Davis et al., 2017), which allowed us to identify and eliminate 27 likely contaminant ASVs. After eliminating the sequences classified as mitochondria and chloroplasts, we ended up with 14,173 ASVs, of which 14,071 ASVs were assigned to the domain Bacteria and 102 ASVs were classified into the domain

Archaea. The reads were then rarefied via random subsampling to 7,420 per sample, corresponding to the lowest sample read count (see the rarefaction curve in Figure S2-2).

2.2.6 Statistical analyses

Statistical analyses were conducted in "R studio version 3.6.1" (R Core Team, 2019) unless otherwise specified. To determine the extent of the divergence between the stockpiles and reference soils, the difference among the stockpile's depth layers, and the difference among stockpiles according to their storage time, we compared the ASV composition of each sample using Bray-Curtis dissimilarity as the distance matrix. The results of the comparisons were visualized using Non-Metric Multidimensional Scaling (NMDS) ordination with the R package "Microeco 1.04.1" (Liu et al., 2021), and with a hierarchical cluster dendrogram using the "ward" agglomeration method from "stats 4.3.0" package (R Core Team, 2019). The significance of the dissimilarities among the contrasted groups was tested with permutational multivariate analysis of variance (PERMANOVA) using the Adonis function in the "Vegan 2.5-6" package (Oksanen et al., 2013). To establish the degree of dissimilarity between the microbial communities of the stockpiles and reference soils and the extent of the variation in microbial communities according to the stockpile age and depth layers was conducted an analysis of similarities (ANOSIM) in "Vegan 2.5-6" (Oksanen et al., 2013). The normality of the datasets was assessed using the Shapiro-Wilk test. The impact of stockpiling on diversity was assessed by diversity (Shannon-Wiener), richness (Chao1 and observed ASV) using "Phyloseq 1.30.0" (McMurdie and Holmes, 2013), and Pielou's evenness using the "Microbiome 1.8.0" package (Lahti and Shetty, 2017). The significance of the dissimilarities in alpha diversity between the groups was assessed through an analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post hoc test, using the "Agricolae 1.3.2" R package (Mendiburu and Yaseen, 2020). Similarly, the significance

of the differences in microbial taxon abundance in the compared soil types, depth layers, and age categories was determined using the Mann–Whitney–Wilcoxon test or Kruskal-Wallis with Dunn's post hoc test, depending on the number of groups contrasted, using the "Microeco 1.04.1" (Liu et al., 2021). The differences in soil edaphic parameters among samples were visualized in a principal component analysis (PCA) biplot.

2.3 Results

2.3.1 The similarity between microbial communities of the stockpiles and the reference soils

Microbial communities of the reference soils and stockpiles differed significantly ($p<0.001$) (Fig. 2.1A). The main taxa that account for the observed difference are a higher relative abundance of Bacteroidetes and Proteobacteria in the stockpiles and a higher relative abundance of Verrucomicrobia ($p<0.001$) and Acidobacteria ($p<0.01$) in the reference soils (Fig. S2-3; Fig. S2-4). Thaumarchaeota was the dominant archaeal phylum in both reference sites and the Horizon stockpiles, whereas the Euryarchaeota was the dominant archaeal phylum in the Wolf Lake stockpiles (Fig. S2-5). Also, the Crenarchaeota were found exclusively in the stockpiles (both Wolf Lake and Horizon). Nanoarchaeota were present in both stockpiles and the Wolf Lake reference sites, but not in the Horizon reference sites (Fig. S2-5). Overall microbial diversity (Shannon-Wiener), taxon richness (Chao1 and Observed), and evenness (Pielou) were significantly higher ($p<0.001$) in the stockpiles than in the reference soils (Fig. 2.2). Together, these findings seem to indicate that soil stockpiling causes a divergence of the prokaryotic microbial communities of the soils.

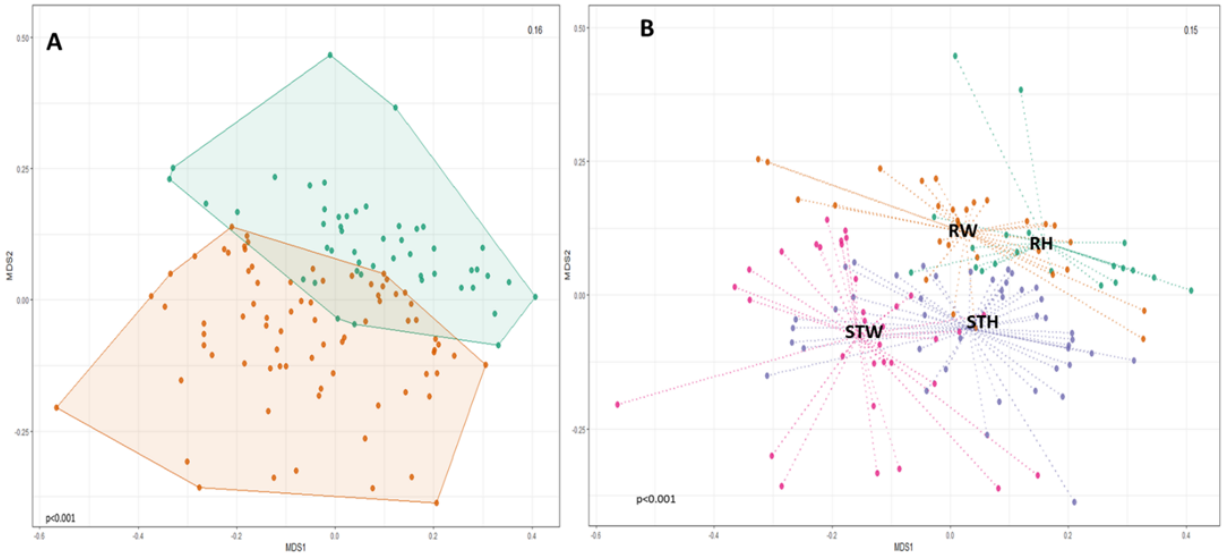

Figure 2.1. NMDS ordination plot showing the dissimilarities (Bray Curtis) between the microbial communities of the stockpiles and the reference soils. A: The communities of stockpiles (orange polygon) and reference soils (green polygon) are shown. B: The dissimilarities of soil microbial communities in the two stockpiling locations and the two reference sites are shown. The position of the centroids is indicated by the acronyms corresponding to each sampling site. RH: Reference site Horizon (green circles), RW: Reference site Wolf Lake (orange circles), STH: Stockpile Horizon (purple circles), STW: Stockpile Wolf Lake (pink circles).

The microbial communities of the two reference sites (Wolf Lake and Horizon), were more similar to each other than they were to their respective stockpiled samples (Fig. 2.1B). Likewise, the microbial communities of the two stockpiling sites were more similar to each other than they were to their corresponding reference soils (Fig. 2.1B). These findings were supported by the analysis of similarities (ANOSIM) (Table 2.1).

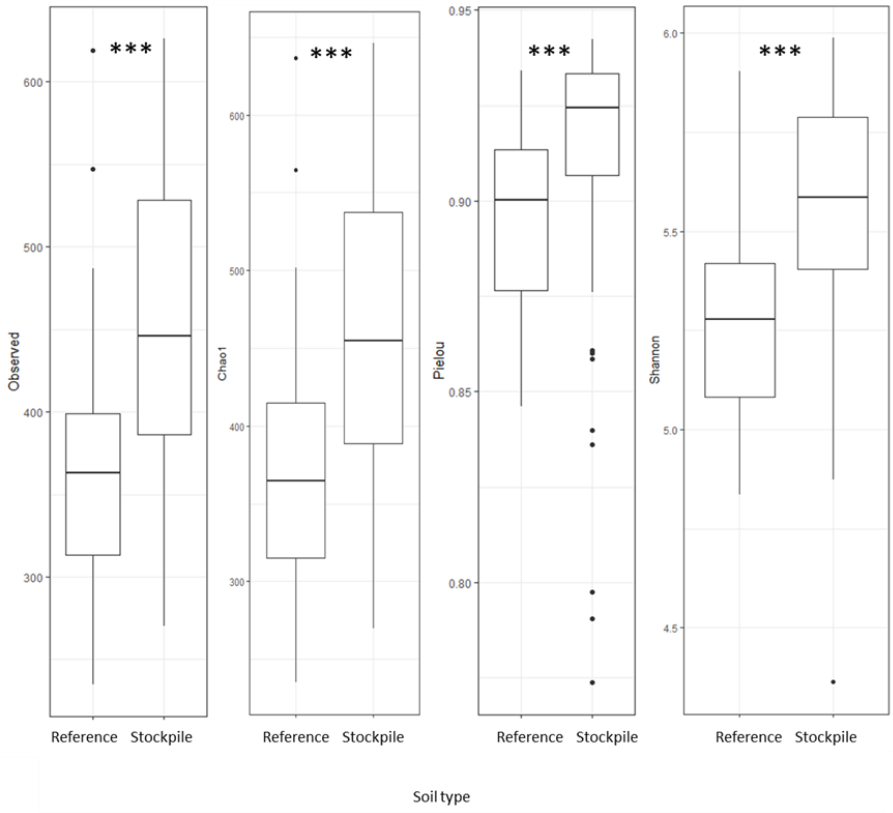

Figure 2.2. Alpha diversity metrics of the microbial communities of the stockpiles and their reference soils. The asterisks indicate the significance of the difference between the two groups: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

Even though the microbial communities of the majority of the stockpile samples were quite different from the communities in the reference samples, a subset of the stockpile soil samples overlapped with the range of natural variability (i.e., variability in community and ecosystem dynamics in non-human disturbed systems) (Fig. S2-6A; Fig. S2-6B). We, therefore, attempted to determine the characteristics of the stockpiled soils samples that overlapped, and those that did not overlap, with the range of natural variability, and whether these factors were related to stockpile age or stockpile depth.

Table 2.1. Results of the analysis of similarities (ANOSIM) for the two stockpiles and the corresponding reference soils in terms of the microbial community.

Soil Type		R-score	p-value
Reference WL	Reference Horizon	0.142	<0.016
Reference WL	Stockpile WL	0.344	<0.001
Reference Horizon	Stockpile Horizon	0.238	<0.001
Stockpile Horizon	Stockpile WL	0.143	<0.001

2.3.2 Stockpile age and microbial communities

The younger (i.e., 0.5, 1.5, and 2.0 years) and older (i.e., 11 and 28 years) stockpiles were highly divergent from the reference soils (Fig. 2.3, Table S2.2). Interestingly, microbial communities of intermediate-aged stockpiles (i.e., 5 and 7 years) were more similar to the reference soils (Fig. 2.3; Fig. S2-6A and Table S2.2). Proteobacteria, Bacteroidetes, and Actinobacteria were higher in the 0.5 years old stockpiles, Proteobacteria and Bacteroidetes continued to be higher in the 1.5- and 2.0-years old stockpiles whereas Verrucomicrobia were significantly higher in the 5 and 7-year old stockpiles. The 28-year-old stockpiles were characterized by an increase in Firmicutes, Proteobacteria, and Actinobacteria (Fig. S2-7). Crenarchaeota was the dominant archaeal phyla in the 0.5 years old stockpile, Euryarchaeota was dominant in the 2 and 11 years old stockpiles, whereas the Thaumarchaeota was more abundant in the 1.5, 5, 7, and 28 years old stockpiles (Fig. S2-8). Likewise, the overall microbial richness (Observed and Chao1) and evenness (Pielou) in stockpiles of all ages was different ($p<0.05$) from that of the reference soils, whereas the diversity

(Shannon-Wiener Index) in younger stockpiles was different ($p<0.05$) from the reference soils (Fig. S2-9).

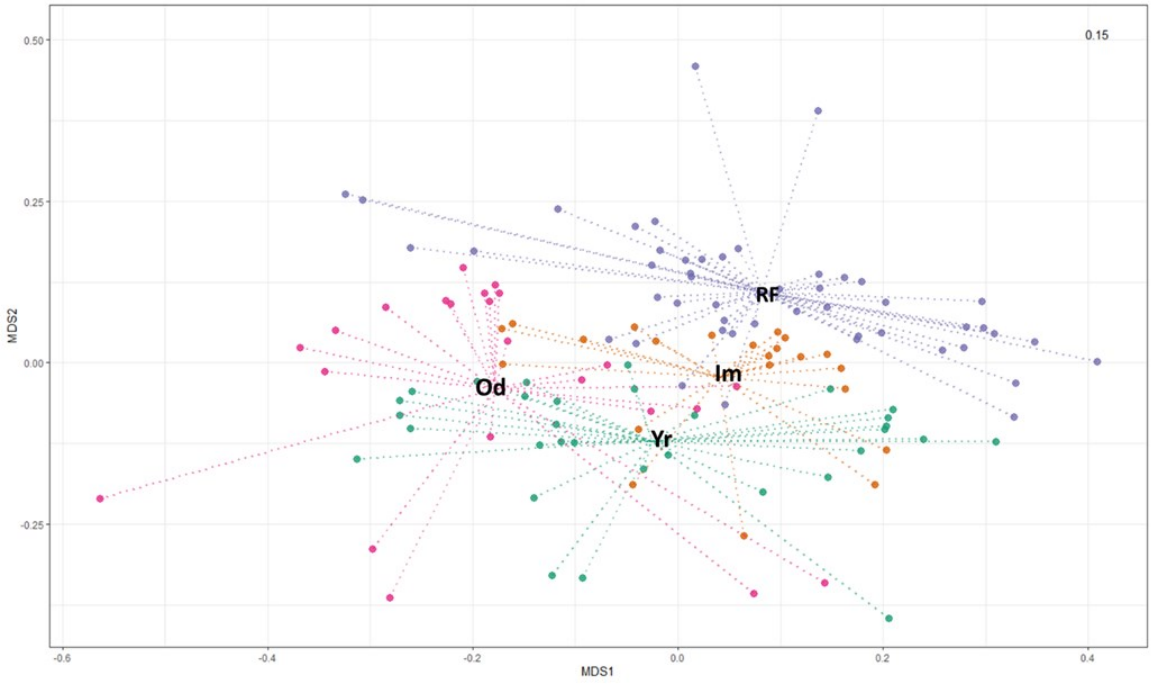

Figure 2.3. NMDS ordination plot representing the dissimilarities (Bray-Curtis) among soil samples according to the age of the stockpiles. The position of the centroids is indicated by the acronyms corresponding to the age category of the samples. Yr =Younger (0.5, 1.5, and 2 years), Im = Intermediate (5 and 7 years), Od = Old (11 and 28 years), Rf = Reference (undisturbed) soils.

2.3.3 Stockpile depth and microbial communities

Microbial communities of the stockpiles and their reference target soils diverged even at equivalent soil depths (Fig. 2.4 and Table S2.4), providing further support for the role of stockpiling generating a shift in soil microbial communities. Likewise, microbial communities in the stockpile surface layers (i.e., 0-10 cm, 10-20 cm), and intermediate (i.e., 20-30 cm) were not significantly different from each other but differed ($p<0.001$) from the communities in the deep (i.e., 80-90 cm and >300 cm) stockpile layers (Fig. 2.4). The effect of the depth of the stockpiled soils in the microbial communities varied depending upon stockpile age. Microbial communities in surface

and intermediate layers were different from communities in the deep layers of the 1.5 and 2.0- and 28-year-old stockpiled soils ($p<0.05$), whereas the surface and deep layers of stockpiles 0.5, 5.0, 7.0, and 11 years old, were not significantly different (Fig. S2-6A, and Table S2.3).

Overall microbial diversity (Shannon-Wiener Index) and taxon evenness (Pielou's Index) decreased dramatically in the 80-90 and >300 cm layers of the stockpiles relative to the surface and intermediate depths (Fig. S2-10). However, the Shannon-Weiner diversity and Pielou's index did not vary in the 80-90 cm depth of the reference soils relative to the surface and intermediate depths.

Acidobacteria and Verrucomicrobia were significantly higher ($p<0.05$) in the stockpiled surface and intermediate depth soils when compared with the >300 cm stockpiled soil layer. In the stockpile's deepest layers (i.e., >300 cm), Proteobacteria were lower than in the surface layers but continued to be one of the dominant taxa (Fig. S2-11). Firmicutes and Bacteroidetes were significantly higher ($p<0.05$) in the deeper stockpile soils than in the surface and intermediate depths. The surface of the stockpiles was dominated by the archaeal families *Nitrososphaeraceae* and *Nitrosotalaleaceae,* while methanogens of the archaeal families *Methanorregulaceae, Methanobacteriaceae,* and *Methanosarcinae* were considerably higher in the > 300 cm depth layer (Fig. S2-13). This significant difference in the prokaryotic community composition likely reflects the contrasting conditions between the surface and deep stockpile layers (Fig. S2-12). The reference soils, on the other hand, showed a significantly higher ($p<0.05$) proportion of Acidobacteria, Proteobacteria, and Verrucomicrobia in the 0-10 cm depth when compared with the 80-90 cm layer, while Actinobacteria was significantly higher in the deeper strata of these soils (Fig. S2-14). Although Firmicutes and Bacteroidetes were also dominant phyla in the reference soils, they were not significantly different between the surface and deeper reference soil layers.

However, my results regarding the >300 cm stockpiles should be interpreted with caution, since samples for these soil layer (and for the 10-20 cm), were not sampled in triplicate, unlike for the other sample depths, which could reduce the statistical power of the tests performed or led to incorrect inferences regarding the communities in these soil layers.

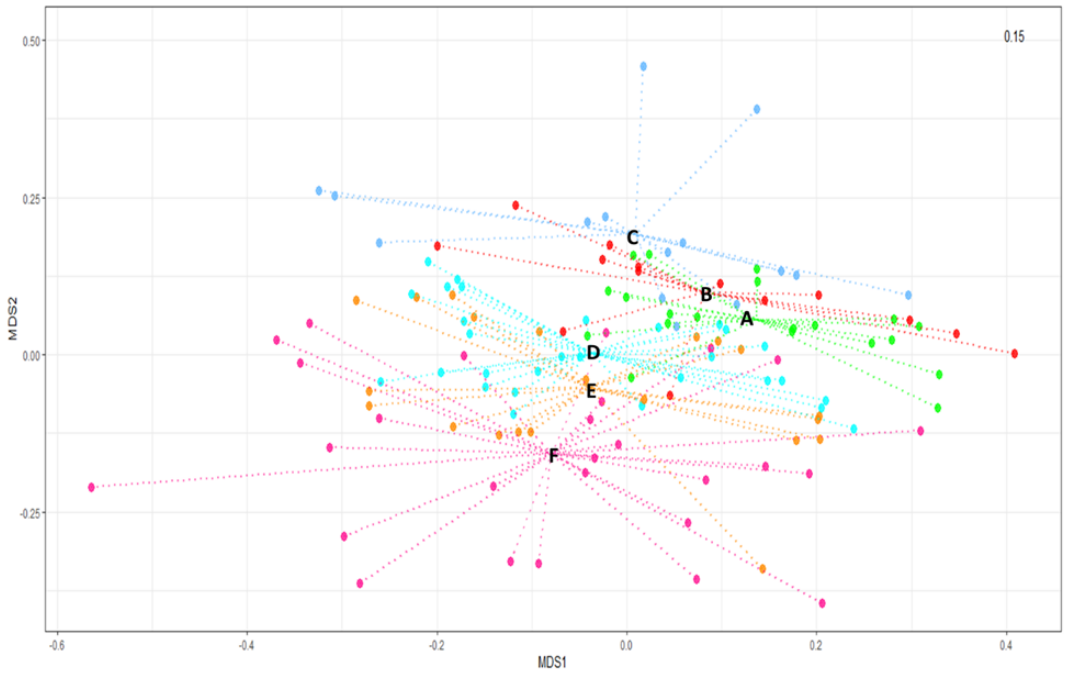

Figure 2.4. NMDS ordination plot showing the dissimilarities (Bray-Curtis) of microbial communities according to the depth of the stockpiles and reference soils. The position of the letters indicates the location of the centroids. The lines radiating from the centroids represent the distances between the centroids of the depth categories and the location of each sample in the ordination space. A= surface layer reference soils (0-10 cm and 10-20 cm), B = Intermediate layer reference soils (20-30 cm), C = deep layer reference soils(80-90 cm), D = Surface layer stockpiles(0-10 cm and 10-20 cm), E = Intermediate layer stockpiles(20-30 cm), F = Deep layer stockpiles(80-90 cm and >300 cm).

2.4 Discussion

Similar to previous studies (Fresquez, 1984; Birnbaum et al., 2017), my study shows that stockpiling generates an important shift in the structure of the microbial communities of the soil. The success of land reclamation may be contingent upon the adequate health and function of the soil used (Johnson et al., 1991), and microbes play vital roles in soil structure and functions impacted by soil stockpiling (Mashigo, 2018). Given that the disturbance generated by stockpiling on soil attributes and biological components may persist after the soils have been applied in reclamation (Fowler et al., 2015; Dhar et al., 2019), the impact of stockpiling on soil microbial communities we report in the present study may affect the desired outcomes of land reclamation.

The divergence between the microbial communities of the stockpiles and the reference soil can be interpreted as a direct effect of the variation in soil texture and other edaphic factors, as suggested by Johnson et al. (1991) and described in similar studies (Claassens et al., 2006; Dimitriu et al., 2010). The study indicates that stockpiles and their reference counterparts were segregated into two groups by the variation in soil texture and pH (for some of the stockpiles; Fig. S2.15). These factors have been shown to be important predictors in the structuring of microbial communities in disturbed and restored ecosystems (Bach et al., 2010; Dimitriu and Grayston, 2010).

Likewise, the relatively higher abundance of the bacterial phyla Bacteroidetes and Proteobacteria in the stockpiles than in the reference soils seems to correlate with an increase in the levels of nutrients like carbon and nitrogen in the soil (Fierer et al., 2007). Such a correlation between the variability of the microbial communities with nutrient availability has been widely documented (Zhou et al., 2002; Hansel et al., 2008). The phyla that increase are likely copiotrophic groups that dominate in nutrient-rich environments (Fierer et al., 2007), like some of the younger stockpiled soils targeted by the study (Fig. S2-16), where the higher proportion of carbon and nitrogen likely

result from the destruction of the soil aggregates during soil removal, handling, and stockpiling (Six et al., 1998; Wick et al., 2009).

The higher overall community diversity we observed in stockpiled soils relative to undisturbed soils (Fig. 2.2) is similar to some previous reports indicating higher overall diversity in disturbed systems, relative to undisturbed counterparts (Persson and Funke, 1988; Ngugi et al., 2018). However, my results differ from those of Fresquez (1984) and Ezeokoli and colleagues (2019), who found higher microbial diversity in reference soils than in disturbed ones. In this case, soil stockpiling generates a shift in soil microbial diversity, but does not reduce the overall microbial diversity.

The process of soil stockpiling appears to promote the establishment of a shared microbial community that is distinct from the native soil communities. Microbial communities in the two stockpiling locations (Horizon and Wolf Lake), which were more than 400 km apart, were more similar to each other than to their reference undisturbed soils, which were between 150 m and 6 km of their respective stockpiles. To the best of my knowledge, this is the first report of such similarity between microbial communities of distantly located stockpiling sites. However, since the two stockpiling locations are in the AOSR, the trends observed in the study may not be able to be extrapolated to stockpiles more generally.

2.4.1 Effect of storage time on soil microbial diversity

The general recommendation for stockpiling is to store the soil for as short a time as possible in order to mitigate the impact of the disturbance on soil health (Dhar et al., 2019; Patterson et al., 2019). However, my data indicate that after stockpiling, a “rest period” prior to using as a reclamation substrate may be beneficial, so long as that rest period does not extend for too long.

The divergence between the younger stockpiles and the reference soils might be attributed to the remaining effects of the disturbance caused by the soil stripping and stockpiling. The disruption of the soil structure for these samples was recent, and the effects of such a disruption may persist for more than six months (Birnbaum et al., 2017; Paterson et al., 2019), or in this case, more than 2 years. Some studies indicate that the relative abundance of selected fungal groups (Jasper et al., 1987; Birnbaum et al., 2017) and fungal community structure (Gorzelak et al., 2020) are initially impacted by stockpiling, but recover to resemble the communities in the reference soils within 5-10 years. Bacterial communities of stockpiles are more similar to the reference soils within the first 2-3 years of storage (Gorzelak et al., 2020), thus indicating a faster recovery than the 5-7 years that we saw in my research. Therefore, bacterial communities may recover more rapidly than fungal communities, which suggests that the optimal rest period might vary depending on which community is targeted. Furthermore, microbial community recovery time may vary from site to site, indicating that each site should be optimized with respect to rest time. Nevertheless, my data indicate that minimizing stockpiling time may not be optimal for reclamation and that several years of storage may improve reclamation outcomes.

The convergence of the microbial communities of the 5-7 years old stockpiles with that of the reference soils is likely caused by the interaction of multiple factors. The recovery of soil properties, including soil aggregates and the decrease in light fraction carbon (LFC), considered the most available carbon pool for microbial decomposition, is a proxy of the microbial community restoration after 3 years of stockpiling (Wick et al., 2009) and fungal communities within 5-10 years of disturbance (Birnbaum et al., 2017). Also, vegetation is likely associated with soil community restoration and has been deemed as fundamental in shaping soil microbial communities (Hanif et al., 2019; Krishna et al., 2020). According to studies carried out by Buss et

al., (2020) on the same stockpiles and reference sites targeted in my study, the plant community composition of the 7-year-old stockpile was the most similar to that in the reference soils. Hence, the restoration of microbial communities in the 5-7 year period may occur concomitantly with the recovery of the plant community.

The divergence of the oldest stockpiles from the reference soils may be explained by variations in physicochemical parameters that occur during storage in stockpiles (Izquierdo et al., 2005; Anderson et al., 2008). Even though the oldest stockpiles and reference soils were not different in most of the measured physicochemical parameters, the analysis shows that they differ significantly in terms of pH and salinity (Fig. S2-16). Therefore, these factors and potentially other unmeasured edaphic and climatic factors may account for the disparity between the microbial communities of the two systems.

2.4.2 Revisiting the stockpile "shelf life" concept

Earlier studies have suggested that topsoil has an expiry date (Kundu and Ghose, 1997; Ghose, 2001; Ghose, 2004). These sources use the concept of "shelf-life" to refer to the progressive deterioration of soil's desirable characteristics, including the microbial community, with the time of storage, until the stockpiled soil eventually becomes infertile and probably unsuitable for further use. However, based on my findings, we argue that the divergence of the stockpiles with the reference soils does not follow a linear progression with regard to the storage time, rather microbial communities of the stockpiles seem to recover to the levels of the undisturbed soils after a given storage period and subsequently diverge again. Also, there was no decrease in soil microbial diversity or species richness with increasing storage time (Fig. S2-9), as proposed by the shelf-life concept. Therefore, instead of shelf-life, there seems to be an "optimum usage time" for the stored soil in reclamation activities. However, a note of caution is due when comparing my findings to

previously published results, since the initial characteristics of the topsoil, stockpiling practices, vegetation cover, climatic conditions in which the soil was stockpiled, and differences in sample sizes may differ from those found by the resent study, which could generate a different response in terms of the microbial community (Weber et al., 2016) and recovery patterns.

Since the central goal of restoration of the boreal ecosystems is the creation of diverse, self-sustaining, and resilient native communities (Government of Canada, 2016), the existence of a specific age-range at which the stockpiled soil's microbial communities recover to resemble the undisturbed soil, and therefore can be used optimally in the reclamation of disturbed ecosystems, points to recommending a "rest period" following soil stockpiling, with optimal times to be determined, and toward a better understanding of potential stress or failure points in the use of stockpiled soils for reclamation.

2.4.3 Stockpile depth and microbial communities

There is a correlation between microbial diversity and ecosystem function (Delgado-Boquerizo et al., 2016; Calderón et al., 2017), indicating that a decrease in overall diversity could have detrimental effects on ecosystem restoration. Stockpile depth has negative effects on microbial diversity (Harris et al., 1989; Johnson et al., 1991), leading to recommendations to reduce stockpile height (Block et al., 2020). We also observed a dramatic decrease in bacterial and archaeal diversity at depth in the stockpiles (Fig. S2-10; Fig. S2-12). This decrease in microbial diversity with depth is likely due to the harsh conditions predominant in the deep portions of the stockpiles (Abdul-Kareem and McRae., 1984; Johnson et al., 1991). Some studies assert that the main factor associated with the severe conditions in the deep stockpile layers is the reduced oxygen level, which generates anaerobic zones (Harris et al., 1993). Even though the oxygen concentration in the stockpiles was not determined by the present study, the fact that anaerobic bacterial taxa, such

as *Clostridium, Peptococcus,* and *Paludibacter* as well as methanogenic archaeal groups like *Methanosarcinaceae* and *Methanobacteriaceae* were significantly higher and dominated in the 80-90 cm and >300 cm stockpile strata, could be indicative of anoxic conditions deeper in the stockpiles. Other studies suggest that the decrease in microbial diversity in the deep soil layers could be associated with reduced biomass and nutrients (Paterson et al., 2019). Therefore, the significant reduction in the levels of carbon and nitrogen in the >300 cm depth layer of the stockpiles (Fig. S2-17) could be associated with the decrease in microbial diversity. My findings seem to indicate that stockpiled soils from deeper layers are problematic for reclamation.

When assessing the combined effects of stockpile storage time and depth on microbial communities, the stockpiles in the intermediate age range (5-7 years), did not differ with depth. As a result, soils from deeper layers within these stockpiles maintain their overall community composition and appear to be "healthier" than deeper soils from either newer or the oldest stockpiles (except 0.5 and 11-year-old stockpiles). This finding further supports the idea that intermediate-aged stockpiles are ideal for reclamation, as surface and deep soils that closely resemble the reference soils can be used for reclamation.

Conclusions

The results of my study provide important insights into the impact of soil stockpiling on microbial communities and support the potential of bacterial communities as suitable indicators of soil health. The study revealed marked dissimilarities in the microbial communities of the stockpiles and their reference soils. There appears to be an optimum usage time for the stockpiled soils following a "rest period", in which the negative effects of depth and storage time are minimal. The findings contribute to the understanding of the consequences of stockpiling on soil biology and will be useful for reclamation specialists to improve stockpiled soil usage protocols for post-

mining restoration operations. My recommendation includes the reduction of the stockpile height, allowing soils to “rest” following stockpiling, and restriction of the soil stockpiling time. Further research should focus on the universality of the rest period and maximum age parameters determined here, the specific determinants of diversity loss with depth, and the effects of stockpiling on other soil biology components and markers of soil health.

Data availability

The sequencing data used in this study was deposited in the National Center for Biotechnology Information (NCBI) of the National Library of Medicine under the accession number PRJNA944787

CHAPTER 3

Impact of stockpiling on soil fungal communities and their functions

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3.1 Introduction

Fungi constitute an important proportion of the soil microbial community (Rodriguez-Ramos et al., 2021) and are of paramount importance in forest ecosystems where they play crucial roles in forest health and regeneration (Frąc et al., 2018), establish complex symbiotic relationships with multiple organisms (Genre et al. 2020; Ramsfield et al. 2020), are chief decomposers of organic matter and mediate the availability of nutrients (Zanne et al., 2020). Mutualistic interactions between mycorrhizal fungi and plants are critical for plant productivity and nutrition (Read & Perez-Moreno, 2003) and are key predictors of plant diversity and plant community composition (van der Heijden et al., 1998; Wardle et al., 2004).

Fungal communities and the symbiotic associations they establish are negatively affected by soil disturbances (Goss & De Varennes, 2002; Fadaei et al., 2021), and the mycorrhizal fungi are particularly impacted by disturbances like those generated by surface soil mining (Jasper et al., 1987), and associated activities like vegetation removal (Arnolds 1991; Prada-Salcedo et al., 2021). These disturbances cause a reduction in the infectivity of mycorrhizal fungi (Jasper et al., 1989) and a change in mycorrhizal community composition (Schnoor et al., 2011), which ultimately affects plant growth and productivity (Goss & de Varennes, 2002), the stability of soil organic matter (Averill & Hawkes, 2016), and promotes the colonization of disturbed systems by

invasive plant species (Bunn et al., 2015). Disturbances that affect soil health and plant community dynamics may impact fungal communities and their functions (Rodriguez-Ramos et al., 2021), thus compromising ecosystem multifunctionality and challenging the success of ecosystem reclamation and restoration (Bunn et al., 2015).

Activities related to oil sand mining have generated extensive areas of disturbed land in northern Alberta (Rowland et al., 2009; Dimitriu et al., 2010), where mining sites must be reclaimed to equivalent land capability once the mining operations have concluded (Paterson et al., 2019). Due to the high nutrient quality and richness in biological components commonly found in the topsoil (Golos et al., 2016; Mushia et al., 2016), oil extraction companies are legally required to store the surface soil extracted during the mining activities, for its use in the reclamation of post-mining sites (Paterson et al., 2019). However, the removed topsoil may remain stored for long periods (Persson & Funke 1988), and the time and conditions in which topsoil remains stockpiled lead to a severe disturbance in terms of structure and chemical properties (Harris & Birch 1989; Anderson et al., 2008), which may compromise soil usefulness as a restoration substrate (Visser et al. 1984; Strohmayer 1999).

Understanding the effect of soil stockpiling on the fungal communities of the soil may shed light on the functions and services that are affected by this disturbance. Some studies have assessed the impact of soil stockpiling on fungal-specific functional groups (Birnbaum et al., 2017; Fadaei et al., 2021), on fungal total biomass (Johnson et al., 1991; Block et al., 2020), on fungal physiological profile (Visser et al., 1984), or fungal spores (Jasper et al., 1987; Harris & Birch 1989). However, due to the limited taxonomic resolution accounted for by these studies, most of them fail to accurately represent the fluctuations in the fungal community composition and their

function in response to the disturbance generated by soil stockpiling and associated factors such as stockpile age or depth.

Microbial communities are sensitive to alterations in soil characteristics (Masto et al. 2009), they can respond rapidly to changing soil conditions (Izquierdo et al., 2005; Muñoz-Rojas 2018) and their shifts usually reflect the magnitude of the environmental disturbance (Ohsowski et al., 2012; Waterhouse et al., 2014). Consequently, fungal communities' response to the disturbance caused by soil stockpiling can be used as an effective indicator of soil health (Hartmann et al., 2012; Costantini et al., 2016), defined as "the continued capacity of soil to function as a vital living ecosystem that sustains plants, animals, and humans" (NRCS-USDA 2021).

In the present study, I profiled the ITS2 fungal marker gene in stockpiled soils of different ages and depths, and in undisturbed soils used as reference, to examine the fluctuations and variability in fungal community structure and putative functions in response to the disturbances associated with soil stockpiling. The objectives were 1) to determine whether stockpiling makes fungal communities different, in terms of structure and function, from those generated by the range of natural variability; and 2) to determine how stockpiling factors, such as soil storage time and stockpile depth, affect the fungal community diversity, composition, and functions. Based on the literature and my previous studies, I hypothesize that soil stockpiling generates microbial communities significantly different from those in their undisturbed counterparts and that stockpile depth is detrimental to fungal diversity, and their functions. As for the effect of stockpile age on fungal communities, do not expect a decrease in fungal diversity or their functions with increasing soil storage time. The results of my research will contribute to the understanding of the consequences of stockpiling on soil biology and health, which will be useful for reclamation

specialists to generate the optimal conditions for surface soil stockpiling and use in post-mining restoration operations.

3.2 Methods

3.2.1 Sampling

Samples were taken from seven stockpiles and five undisturbed soils in the central region of the boreal forest in Alberta. The sites sampled were (1) CNRL Wolf Lake (54.695 °N, 110.730 °W), where oil sands are extracted by steam-assisted gravity drainage (SAGD) methods, and (2) CNRL Horizon (57.337 °N, 111.755 °W), north of Fort McMurray, where oil sands are extracted by surface mining. At both sites, the stockpiles were composed of mineral upland topsoil and LFH (i.e., litter, fibric, and humic).

The two locations used as reference were undisturbed sites that were characteristic of the mixed wood natural subregion of the boreal forest in northern Alberta, (in terms of soil, climate type, and vegetation) and therefore represent the natural state of the ecosystem and could be considered as the benchmark conditions for a successful reclamation. The reference sites at Horizon were located 5-6 kilometers away from the stockpiles, while the reference sites at Wolf Lake were located 150 meters away from their respective stockpiles. The higher distance between the reference soils and stockpiles at the Horizon site was selected to avoid the disturbance generated by the surface mining activities used in the oil extraction activities at the Horizon site, whereas the shorter distances between the stockpiles and reference soils in the Wolf Lake site were due to the comparatively more fragmented disturbance that is produced by the *in-situ* oil-sands extraction. The vegetation found at both the Wolf Lake and Horizon reference sites was characterized by an upland canopy of trembling aspen and white spruce (Stratechuck, 2020).

Details of the sampling process can be found in Hernandez et al.(2024). Briefly, in August 2018, we sampled seven stockpiles at the two mining sites and a total of five reference soils (3 at Wolf Lake and 2 at the Horizon site). The age of the stockpiles sampled were 0.5, 1.5, 5.0, and 7.0 years old, at the Horizon site and 2.0, 11.0, and 28.0 years old at the Wolf Lake site. For each stockpiled soil, we sampled 0-10 cm, 10-20 cm, 20-30 cm, 80-90 cm, and >300 cm depth. For each sampling point, three replicate pits were excavated (excluding the 10-20 cm and >300 cm, from which only one pit was sampled). The replicate pits were spaced approximately 20 meters apart from each other. Soil samples were then homogenized, and large debris was removed using a 4 mm sieve. The sieved soil samples were stored at -80°C, in sterile plastic bags. The determination of the physical and chemical parameters of each soil sample was performed as indicated in Hernandez et al.(2024).

3.2.2 DNA extraction and amplification

We extracted DNA from triplicate randomized homogenized subsamples of 0.25 grams of soil each, using the DNeasy Power soil kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The extracted triplicate DNA samples were combined to make a single DNA sample. To determine the DNA concentration, we used a Qubit dsDNA HS Assay Kit (Thermofisher Scientific, Canada) following the manufacturer’s protocol. The internal transcribed spacer 2 (ITS2) region was amplified using the fITS7-ITS4 primer pair (Ihrmark et al., 2012), and the following PCR program: 98°C for 30 seconds followed by 35 cycles of 98°C for 5 seconds, 55°C for 5 seconds and 72°C for 15 seconds, and a final extension at 72°C for 1 minute. The length of the PCR product was determined using gel electrophoresis. The DNA samples, the extraction blanks (see Figure S3-1-B), and a lab-constructed mock community (a combination of DNA from previously identified fungal groups (see Figure S3-1-A) were sequenced in randomized plate order,

with an Illumina MiSeq platform with the 250-bp paired-end kit (V2 500-cycle PE Chemistry, Illumina, USA), using the commercial sequencing services of Microbiome Insights (Richmond, Canada).

3.2.3 Data analyses

DNA amplicons generated by Illumina sequencing, as demultiplexed FASTQ format files went through quality-filtering, dereplication, merging of paired-end reads into amplicon sequence variants (ASV), and identification and removal of chimeras, using the "DADA2 1.16.0" pipeline (Callahan et al., 2016) in "R version 3.6.1" (R Core Team 2019). In total, 71% of the reads passed the quality filtering process; of these, 6.7% of the reads were discarded after the dereplication and merging steps. Finally, 3% of the reads were discarded as chimeric, ending up with a total of 2,929,616 paired-end reads and 7,483 unique ASVs. We assigned taxonomy using the UNITE reference fungal database (Abarenkov et al., 2010). Then 39 likely contaminant ASVs were eliminated using the "Decontam 1.6.0" R package (Davis et al., 2017). ASVs were rarefied via random subsampling to 6,569 reads per sample (Fig. S3-2), which was the lowest read count per sample. To infer the ecological function and trophic mode of the ASVs, we used the FUNGuild database (Nguyen et al., 2016), which included only the ASVs whose likelihood of being the assigned function/trophic mode was "probable" or "highly probable", as suggested by Nguyen et al. (2016).

3.2.4 Statistical analyses

The statistical analyses were performed in "R studio version 3.6.1" (R Core Team 2019). The determination of the normality of the datasets was done by applying the Shapiro-Wilk test. The differences in community composition between the fungal communities of the stockpiles and reference soils, among the soil depth layers, among the ages of the stockpiles, and the fungal guilds

and trophic modes, were determined based on Bray-Curtis dissimilarities, generated from the comparison of the ASV composition of the samples. The statistical significance of the dissimilarities between the fungal communities was tested using permutational multivariate analysis of variance (perMANOVA) with 999 permutations, using the "ADONIS" function of the r-package "Vegan 2.5-6" (Oksanen et al., 2013). The degree of the dissimilarities between the fungal communities of the conditions assessed was tested using the analysis of similarities "ANOSIM" function also in "Vegan 2.5-6". The distances between communities (Bray-Curtis), were visualized using Non-Metric Multidimensional Scaling (NMDS) ordination using "Vegan 2.5-6". A random forest model developed using the R package "Microeco 0.6.0" (Liu et al., 2021), identified the key fungal taxa that constitute the dissimilarities between the stockpiles and reference soils. The correlation between the key fungal taxa, guilds, and trophic modes with soil parameters was explored with a Pearson correlation using the "Microeco 1.04.1" r-package (Liu et al., 2021). The effect of stockpiling on alpha diversity indicators (Shannon-Weaver diversity index, Chao1 richness estimator, observed ASV richness) was assessed using "Phyloseq 1.30.0" (McMurdie & Holmes 2013), and Pielou's evenness was determined using the "Microbiome 1.8.0" package (Lahti et al., 2019). The statistical significance of alpha diversity dissimilarities was assessed using the analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post hoc test, using the package "Agricolae 1.3.2" (Mendiburu & Yaseen 2020). To determine the significance of the differences in taxa abundance, guilds, and trophic modes among the conditions of interest, we used Mann–Whitney U-test and Kruskal-Wallis with Dunn's post hoc tests, from the "Microeco 1.04.1" package.

3.3 Results

3.3.1 Fungal communities of the stockpiles and the reference soils.

The NMDS ordination revealed a significant difference ($p<0.001$) between the fungal communities of the stockpiles and the reference soils (Fig. 3.1). Further, a pairwise comparison of the fungal communities of the stockpiling sites and their respective reference soils indicated that the dissimilarities between the stockpiles and their reference soils were larger than the dissimilarities between the communities in the two stockpiling sites (Wolf Lake and Horizon; Fig. S3-3). Together these results indicated that the fungal communities generated by the disturbance associated with stockpiling were distinct from the communities found in the range of natural variability observed in the undisturbed samples.

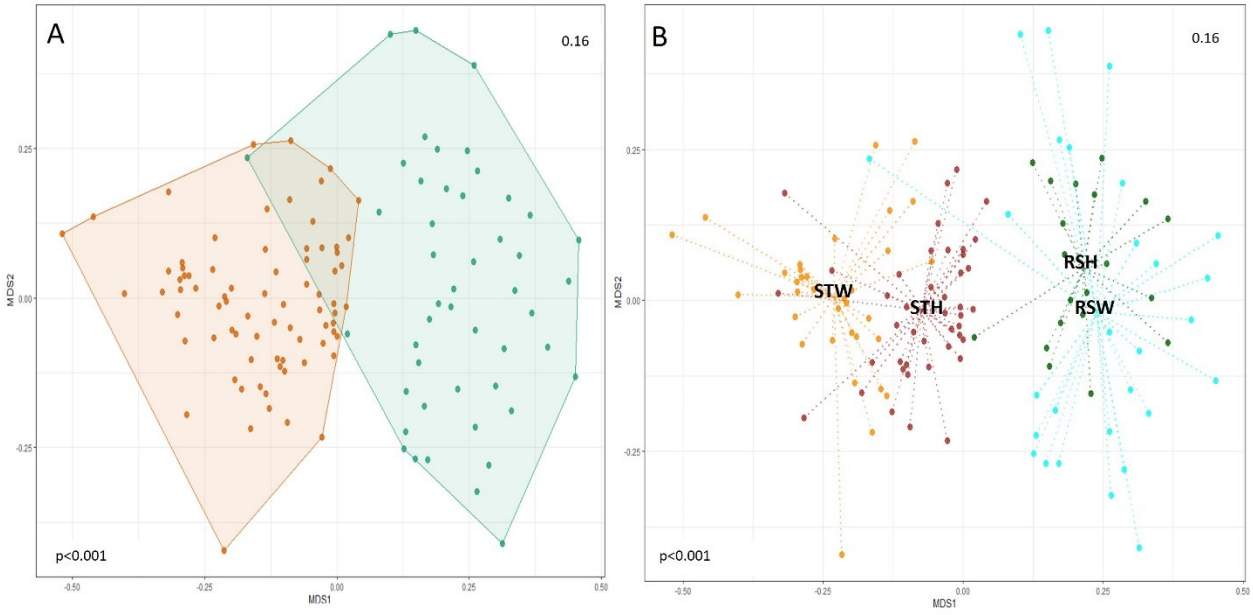

Figure 3.1. NMDS ordination representing the similarity (Bray-Curtis) between the fungal communities of the stockpiles and those in the reference soils. A: Fungal communities of reference soils (green polygon) and stockpiles (orange polygon) are shown. B: Representation of the dissimilarities of soil microbial communities in the two stockpiling locations (STH and STW) and the two reference sites (RSH and RSW). The acronyms corresponding to each sampling site

indicate the position of the centroids. RSH: Reference site Horizon (green circles), RSW: Reference site Wolf Lake (blue-cyan circles), STH: Stockpile Horizon (brown circles), STW: Stockpile Wolf Lake (orange circles). The significance of the difference between reference soils and stockpiles was assessed using PERMANOVA.

Glomeromycota and Ascomycota were significantly higher ($p<0.001$) in the stockpiles, whereas Basidiomycota and Mucoromycota were significantly more abundant ($p<0.001$) in the reference soils (Fig. 3.2). Some of the key fungal genera that accounted for the differences between the stockpiles and their reference soils were basidiomycete genera *Russula, Cortinarius, Piloderma, Inocybe,* and the ascomycete genera *Wilcoxina* and *Archaerrhizomyces*, all of them were dominant in the reference soils, while in the stockpiles the ascomycete genera *Pseudeurotium, Neobulgaria,* and *Pseudogymnoascus* were significantly higher (Fig. 3.3). Some of the fungal genera that accounted for the dissimilarities between stockpiles and reference soils were correlated with soil texture and water content (Fig. S3-4). However, most of these indicator genera did not correlate significantly with any of the other edaphic factors included in the study. Taxon richness (Chao1 estimated and Observed ASV) was significantly ($p<0.05$) higher in the stockpiles. Still, there were no significant differences in either overall diversity (Shannon-Weaver) or taxon evenness (Pielou) (Fig. 3.4). Therefore, the conditions promoted by the stockpiling generated a shift in the fungal communities of the soil, affecting fungal species richness, but not overall diversity.

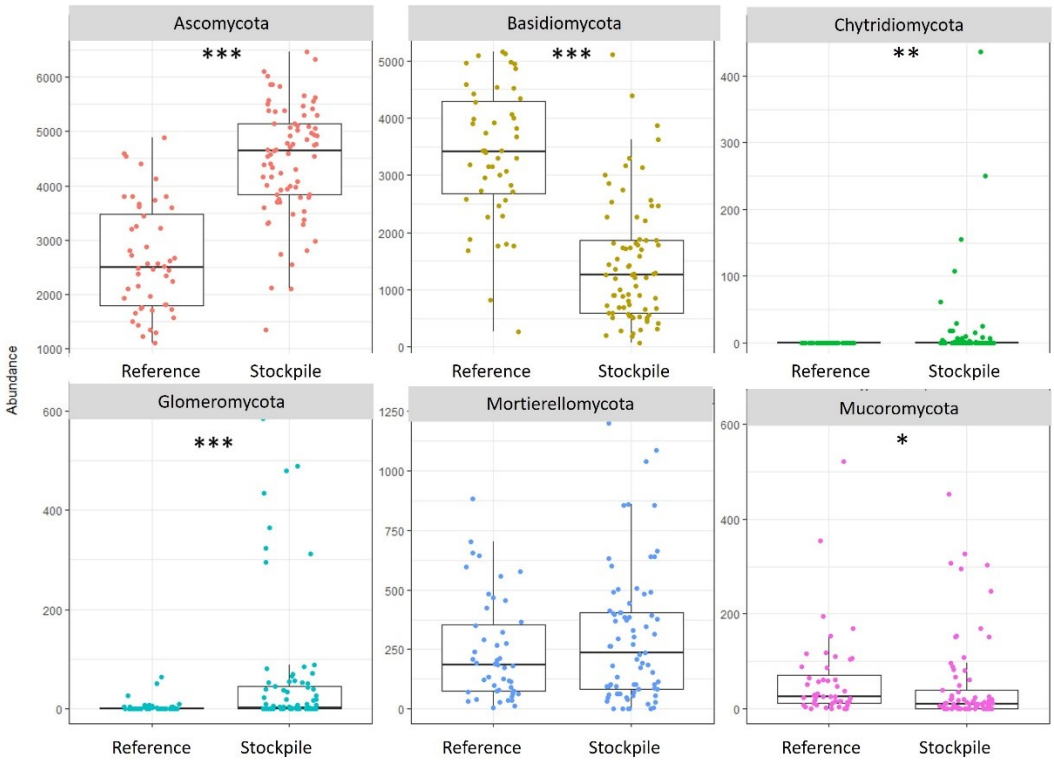

Figure 3.2. Relative abundance of the main fungal phyla found in the stockpiles and soils used as references. The significance of the difference between the compared groups is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

The study of fungal function inferred by the FUNGuild database (Nguyen et al. 2016), assigned guild and trophic mode to 58.15% of the ASVs and revealed that the "saprotrophs" dominated significantly in the stockpiles, whereas the "symbiotrophs" were significantly higher in the reference soils. (Fig. S3-5). The ectomycorrhizal, lichenized, and endophytic fungi were higher in the reference soils, but the endomycorrhizal fungi (ericoid mycorrhizae, orchid mycorrhizae, and arbuscular mycorrhizae) were higher in the stockpiles (Fig. 3.5). Among the ectomycorrhizal fungi, the genera *Russula* (Basidiomycota), *Cortinarius* (Basidiomycota), *Cenococcum* (Ascomycota), and *Clavulina* (Basidiomycota), were found both in stockpiles and reference soils (although *Russula* was significantly higher in the reference soils), *Endogone* (Mucoromycota) and

Chaetospermum (Basidiomycota) were found exclusively in the stockpiles, while *Tuber* (Ascomycota) and *Elaphomyces* (Ascomycota) were found in the reference soils only. The saprotrophic fungi were positively correlated ($p<0.001$) with soil nutrients like carbon and nitrogen in the stockpiles, whereas the ectomycorrhizal fungi were negatively correlated ($p<0.001$) with sand, nitrogen, and carbon ($p<0.05$) in the stockpiles (Fig. S3-6).

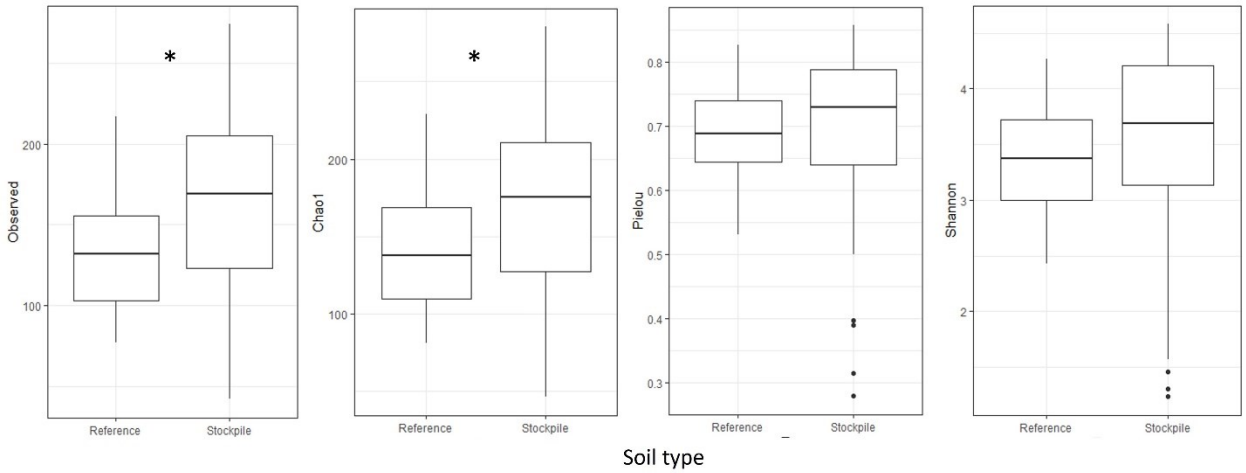

Figure 3.3. Alpha diversity metrics corresponding to the stockpiles and the reference soils. The significance of the difference in the diversity metrics between groups is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

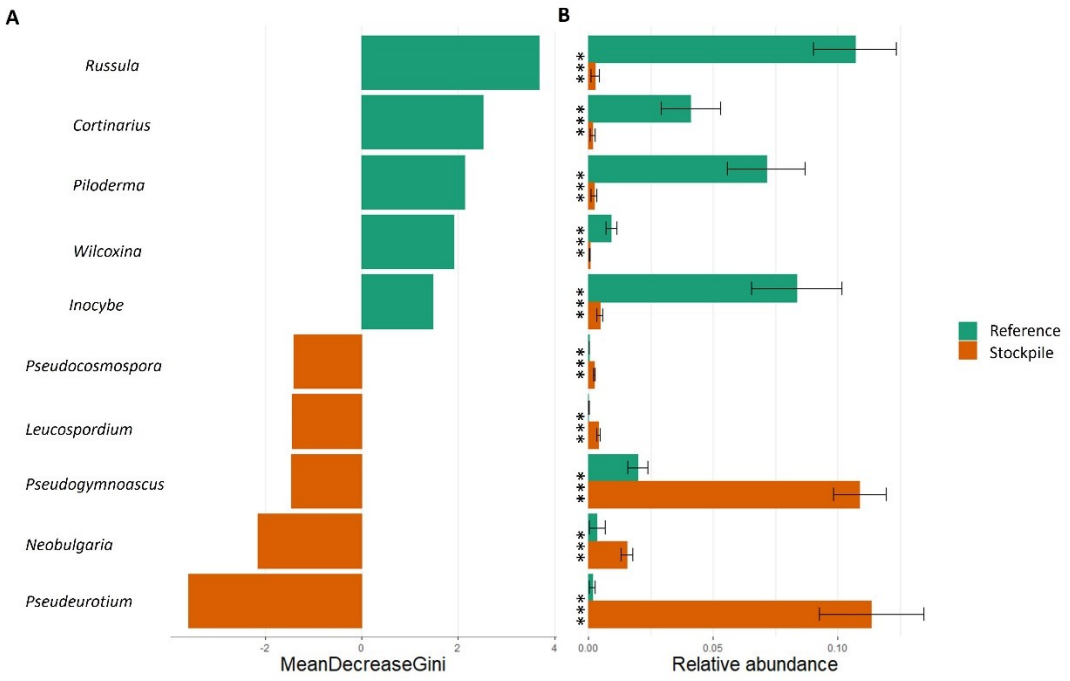

Figure 3.4. Results of the random forest modeling indicating the main fungal taxa that characterize stockpile and reference soils (respectively) (A), and the relative abundance of these taxa (B) in the two studied soils. "Mean decrease gini" or "decrease in heterogeneity" is a measure of how homogeneous each soil category gets when a given taxon is included in the soil category (stockpiled or reference soil). The "mean decrease gini" will be higher when a given taxon better characterizes or represents a given soil category. The significance of the abundance of fungal groups between the contrasted groups is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

For the Wolf Lake site, all stockpiles were significantly different from their respective reference soils; however, the youngest (2 years) and oldest (28 years) stockpiles were the most dissimilar to their undisturbed counterparts (Fig. S3-7). For the Horizon site, all stockpiles (0.5, 1.5, 5, and 7 years old) were dissimilar to their reference soils to a similar degree.

Depending on the stockpile age, some fungal taxa differed in their relative abundance. The genus *Pseudogymnoascus* (Ascomycota) was dominant in the 0.5, 5, and 7 years old stockpiles, while

Pseudeurotium (Ascomycota), dominated in the 2 and 11 years old stockpiles (Fig. S3-8). The genera *Lacrymaria* (Basidiomycota) and *Coprinus* (Basidiomycota) were more abundant in the 7-year-old stockpile, whereas *Tetracladium* (Ascomycota) and *Chaetomium* (Ascomycota) were more abundant in the youngest and oldest stockpiles respectively. Aside from the 5-year-old stockpile, the Shannon-Weaver diversity index and species richness (observed ASV and Chao1 estimated) did not change significantly when compared with their respective reference soils (Fig. S3-10). Therefore, fungal species richness and diversity were not significantly affected by the soil storage time. The results of the functional analysis of the fungal communities showed that the youngest stockpile (0.5 years) had the highest proportion of fungi in the orchid and ericoid mycorrhizal fungal guilds, whereas the arbuscular mycorrhizal fungi were highest in the 2-year-old stockpiles, but were also present in the 5, 7, 11, and 28-year-old stockpiles (Fig. S3-11).

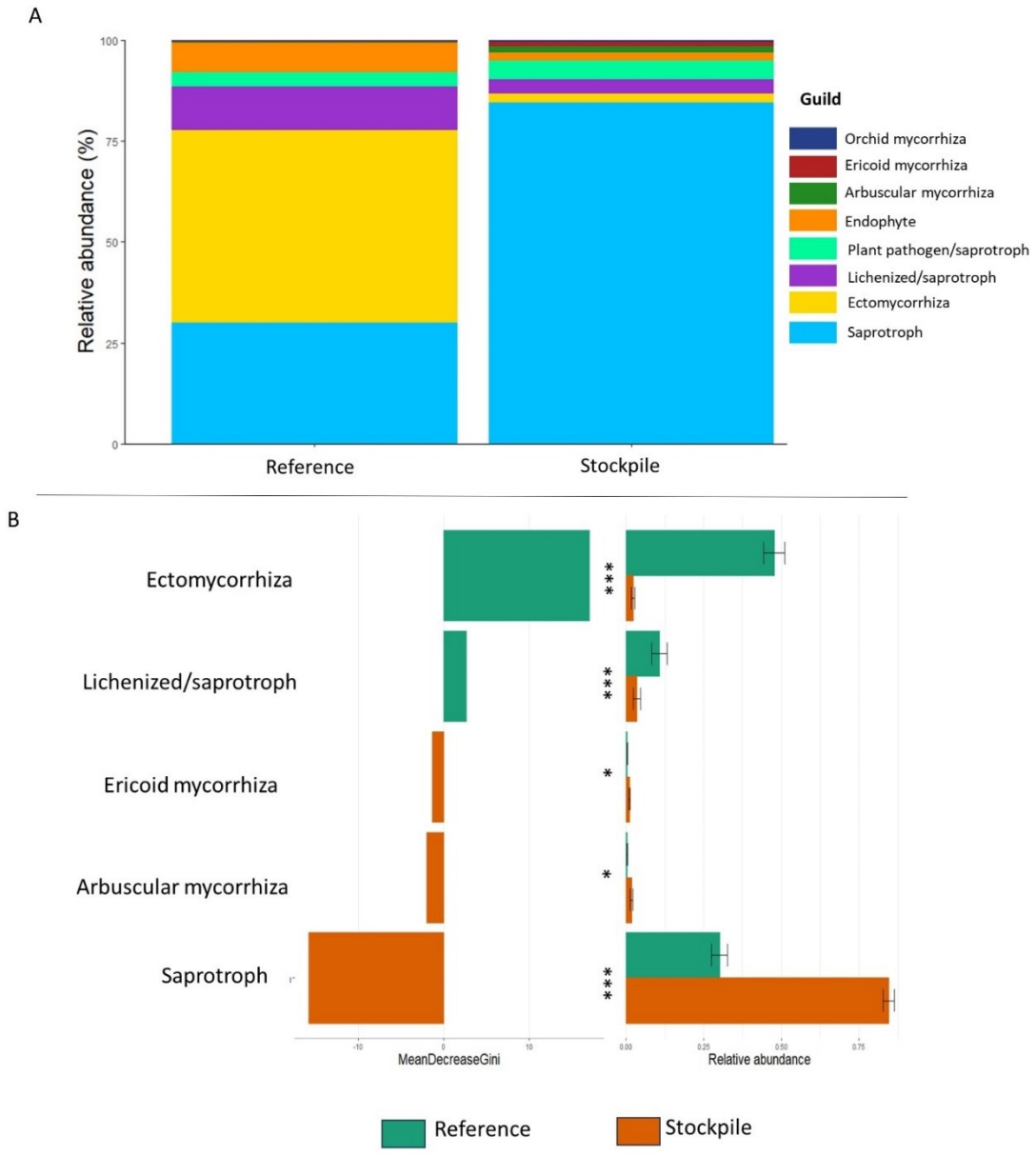

Figure 3.5. Fungal guilds in the stockpiles and the reference soils. A) Random forest model indicating the main fungal guilds that characterize the stockpiles and reference soils (respectively), and, B), the relative abundance of the main fungal guilds in stockpiles and reference soils. "Mean decrease gini" or "decrease in heterogeneity" is a measure of how homogeneous each soil category gets when a given guild is included in the soil category (stockpiled or reference soil). The "mean decrease gini" will be higher when a guild better characterizes a given soil category. The significance of the difference between the compared groups is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

3.3.2 Stockpile depth and fungal communities

The fungal communities of the stockpiles were different from those in the reference soils ($p<0.01$) even at the same soil depth (Fig. S3-12). Furthermore, the fungal communities of the stockpile surface (0-10 cm, 10-20 cm, and 20-30 cm), and deep layers (80-90 cm and >300 cm) were distinct (Fig. S3-12). The main identified fungal genera that dominated in the stockpile surface were *Pseudeurotium* (Ascomycota), *Pseudogymnoascus* (Ascomycota), and *Mortierella* (Mucoromycota), whereas *Pseudeurotium* was also the most abundant in the deep stockpile layer.

Additionally, the analysis of alpha diversity indicators according to the depth of the stockpile revealed that fungal diversity (Shannon-Weaver), evenness (Pielou), and richness (Observed ASVs) experienced a significant decrease in the 80-90 cm and >300 cm of the stockpiles when compared with those in the 0-10 cm of the stockpiles (Fig. S3-13). In the case of the reference soils, richness (Observed ASV and Chao1 estimated), evenness (Pielou), and diversity (Shannon-Weaver), did not change significantly throughout the 0-90 cm depth soil layer. The arbuscular mycorrhizal fungi were more abundant in the surface (0-10 cm, 10-20 cm, and 20-30 cm) of the stockpiles (mainly in the Wolf Lake stockpiles) and were not found in the deep layer (80-90 and >300 cm) of all stockpiles (Fig. S3-9). The >300 cm depth stockpile soil had the lowest diversity of fungal guilds, followed by 80-90 cm. However, the fungal guild diversity in the deepest layer of the reference soils (80-90 cm) did not decrease.

3.4 Discussion

Soil fungi play key roles in boreal forest ecosystems (Lindahl et al., 2007; Ramsfield et al., 2020), where they represent an important proportion of soil biota (Dimitriu et al., 2010; Treseder &

Lennon 2015) and are involved in complex relationships with plants (Visser 1995; Clemmensen et al., 2013; Frąc et al., 2018). Therefore, disturbances affecting plants and soil integrity may affect soil fungal communities (Fadaei et al., 2021; Rodriguez-Ramos et al., 2021), which aligns with my results indicating that the fungal communities in the disturbed stockpiled soils differed significantly from the range of natural variability found in undisturbed soils, both in terms of taxonomic composition and function, as we originally hypothesized. Furthermore, some of the fungal groups whose abundance declined significantly in the stockpiles, and that accounted for the dissimilarities between stockpiles and reference soils (i.e. *Cortinarius, Piloderma,* and *Russula*), are markers of undisturbed conditions in sub-boreal forest soils (Sukdeo et al., 2018). Since the impact of stockpiling on fungal functions persists after the soil has been used in reclamation (Stahl et al., 1988), the disturbance associated with stockpiling may lead to undesired reclamation trajectories (Dhar et al., 2019), and represents a challenge to the goals of land reclamation of returning disturbed ecosystems to their pre-disturbed condition (Naeth et al., 2013; Ezeokoli et al., 2019).

Stockpiling normally decreases soil fungal diversity (Ezeokoli et al., 2019; Gorzelak et al., 2020), and the reduction in microbial diversity (including fungal diversity) has been linked to a suppression of ecosystem functions (Delgado-Baquerizo et al., 2016). However, we observed no reduction in diversity (Shannon-Weaver) resulting from stockpiling; in fact, there was an increase in species richness in the disturbed stockpiled ecosystems in my study. This increase in diversity may have been related to the dominance of invasive plants on these stockpiles (as reported by Buss & Pinno (2019), since there is a direct correlation between the invasive plant diversity and richness in soil biota (Pyšek et al., 2012; Lekberg et al., 2013; Gaggini et al., 2018). The increase in fungal richness may also have been associated with the high amount and variety of organic matter in the

targeted disturbed ecosystem (Santonja et al., 2017; Otsing et al., 2018) made available during the process of soil stripping and stockpiling (Wick et al., 2009).

3.4.1 Functional implications of the variation in fungal guilds and trophic modes

Generally, arbuscular mycorrhizal fungi (AMF) tend to dominate fungal communities in disturbed soils, while ectomycorrhizal fungi (EMF) tend to dominate in undisturbed soils (Dimitriu & Grayston 2010; Anthony et al., 2017; Ramsfield et al., 2020), which is consistent with my results. EMF are generally associated with the native vegetation of ecosystems like the boreal mixed wood forest (Read, 1991; Toljander et al., 2006). On the other hand, the generalist nature of many AMF allows them to establish symbiotic relationships with a wider array of plant species (Richardson et al., 2000; Fitter, 2005). The AMF association helps invasive/non-native plants in their colonization (Richardson et al., 2000; Nuñez & Dickie 2014; Frąc et al., 2018), which would explain why a significant proportion of the vegetation of post-mining sites forms symbiotic relationships with AMF (Wang, 2017) including forbs (Bunn et al., 2015) and herbs (Read, 1991). This is consistent with the findings of Buss and Pinno (2019), that the plant communities of the stockpiles from my study were dominated by invasive plants (including forbs and weeds). Similarly, the colonization of the disturbed sites by invasive plants could explain the reduction of "symbiotrophic" fungi in the stockpiles, reported here, since invasive plants may have a disruptive effect on fungal mutualistic relationships (Stinson et al., 2006; Bever et al., 2010; Anthony et al., 2017). This theory was supported by the significantly lower amount of lichenized and ectomycorrhizal fungi detected in the stockpiles relative to the reference soils. Considering that one of the objectives of land reclamation is "to create a self-sustaining environment with local vegetation" (Government of Canada, 2016), the fact that AMF is associated with the dominance of invasive plants in the stockpiles may challenge the suitability of the stockpiled soil to be applied in reclamation.

The abundance of saprotrophic fungi and their carbon degradation activities are correlated to the nutrients in soil litter (Zhang et al., 2018). This is consistent with the significant correlation we observed between the saprotrophic fungi and the proportion of carbon and nitrogen in the stockpiled soil. Therefore the higher abundance of saprotrophic fungi in the stockpiles relative to the reference soils reported here might have resulted from elevated nutrients liberated from organic matter such as plant litter (Martos et al., 2009) and dead fungal biomass (Frey 2019) that remained in the stockpiled soils following tree clearing, stripping, and soil mixing processes. Likewise, the dominance of the fungal phylum Ascomycota in the stockpiles could be explained by the high affinity shown by species of this phylum for litter-rich environments (Zhang et al., 2018).

The increase of saprotrophic fungi in stockpiled soils coincides with a dramatic decrease in the abundance of EMF (Sukdeo et al., 2018), which is consistent with my findings. Pec et al., (2017) and Rodriguez-Ramos et al., (2021), attribute the shift from EMF to saprotrophic fungal guilds in boreal forests to the removal of the overstory (e.g., clear-cut logging), one of the steps carried out before soil can be salvaged (Ghose, 2001). On the other hand, the decreased saprotrophic fungi and the increased EMF relative abundances in the reference soils could be explained by decreased carbon decomposition, imposed by the EMF, which limits the growth of saprotrophic fungi (Averill et al., 2014; Jacobs et al., 2018). The mechanisms under this amensalistic relationship are analyzed by Orwin et al., (2011) and Averill and Hawkes (2016), who indicate that the EMF decreases the saprotrophic litter decomposition by limiting soil nitrogen and thus reducing the enzymes needed by saprotrophs to degrade organic matter, therefore enhancing soil carbon storage. This leads to an indirect inhibition of the proliferation and dominance of the saprotrophic fungal groups in undisturbed soils.

3.4.2 Impact of Stockpile Depth and Age on Soil Fungal Communities

Conditions in the deeper layers of the stockpiles have been described as “inhospitable”(Abdul-Kareem & McRae 1984; Harris et al., 1993; Anderson et al., 2008) and there are concerns that such conditions may affect the diversity and community composition of the soil (Block et al., 2020). The shifts in fungal community composition, the decrease in alpha diversity indexes (richness, evenness, and diversity), and guilds and trophic modes between surface and deep stockpile layers reported by the study, could be a consequence of the reported harsh conditions in the deep stockpile strata, or to the decrease in litter and root biomass with increasing stockpile depth (Paterson et al., 2019), since multiple fungal groups are commonly involved in symbiotic relationships with plant roots or decompose organic material mainly derived from plants (Orwin et al., 2011). This argument is further supported by the fact that such a decrease in diversity indexes and fungal guilds did not seem to have occurred in the deep layers of the reference soils (80-90 cm). Therefore, findings suggest that using surface stockpile soils (0-10 cm and 20-30 cm) would be more convenient for reclamation than soil in the deeper layers of the stockpiles. Based on this, we recommend reclamation specialists preferentially use the upper ~80 cm of the stockpiled soils in reclamation practices. Then the next ~80 cm stockpile soil layer should be used in reclamation only after some time when it has recovered from the above-described harsh conditions. Doing this, we expect that microbial community diversity and probably their functions could be restored to the levels found in the stockpiled soil surface.

The impact of time since stockpiling on soil microbial communities is a common concern (Kundu & Ghose 1997; Birnbaum et al., 2017), therefore a reduction of the storage time of the soil has been generally recommended (Kundu & Ghose 1997; Dhar et al., 2019; Paterson et al., 2019) to achieve the desired outcomes of land reclamation. However, several studies of fungal communities

in stockpiles during the time of storage (Birnbaum et al., 2017; Gorzelak et al., 2020; Ngugi et al., 2020) indicate that the fungal communities appear to change dramatically initially due to disturbance, but recover to resemble those in the reference soils after several years (i.e, 5-10 years *sensu* Gorzelak et al., 2020). The increase in the dissimilarities between some of the younger stockpiles and their reference soils reported here seems to align with this argument. However, a "recovery" of the fungal communities was not detected in any of the storage times analyzed.

Orchid mycorrhizal fungi (OMF) are necessary for seed germination of a wide variety of plants (McCormick & Jacquemyn., 2014), and may help them colonize novel habitats in their primary succession (De Long et al., 2013). The relatively higher proportion of OMF in the youngest stockpiles could reflect their influence on the germination and establishment of plant seeds after the stockpiling disturbance. Similarly, studies have noticed a correlation between soil disturbance and the presence of plant pathogenic fungi (Shi et al., 2019), which is consistent with the higher abundance of plant pathogenic fungi in the younger stockpiles. Also, the fact that lichenized fungi did not proliferate until the 2nd year of soil stockpiling, shows their sensitivity to soil disturbances (Boggess et al., 2024).

Conclusions

The research set out to examine the impact of stockpiling on soil fungal communities and their potential function. We showed that stockpiling affects fungal communities of the soils, making them distinct from the range of natural variability observed in undisturbed reference communities. These distinctions may be challenging if the goal of land reclamation is the restoration of the disturbed ecosystems to equivalent land capability. My research has also shown that, unlike the undisturbed reference sites, stockpile fungal communities are stratified by depth. Trait-based fungal community analysis suggested that stockpiling was associated with a shift in the function

of fungal communities of the soils. This shift in functional guilds has important implications for ecosystem functions such as carbon/nitrogen cycling and invasion of the disturbed soils by invasive plants. In sum, the findings of the study contribute to the knowledge of the impact of soil stockpiling on soil biology and shed light on the understanding of the usefulness of the stockpiled soil as a reclamation substrate.

CHAPTER 4

Influence of predictors and assembly processes on the structure of microbial communities in disturbed soils

4.1 Introduction

The impact of anthropogenic disturbances on the ecosystem may drastically modify soil attributes (Falk et al., 2006; Kane et al., 2020), and are associated with shifts in soil community composition (Rodriguez-Ramos et al., 2021). An example of an extreme disturbance that impacts soil structure, integrity, and biology is soil stockpiling, a legally mandated process by which topsoil is excavated and salvaged (stockpiled) at the start of the mining operations (Strohmayer, 1999; Ghose, 2001; Fadaei et al., 2021). The stockpiled soil is then used as a substrate for the reclamation of post-mining sites. However, the stockpile age and depth influence the suitability of the stockpile soils for use in reclamation (Kundu & Ghose, 1997; Block et al., 2020; Buss et al., 2020).

Under ecosystem restoration practices, like land reclamation, the recovery of the ecosystem to its pre-disturbance condition is usually the desired outcome (Cutler et al., 2017; Jurburg et al., 2017). However, community structure might be difficult to restore (Suding et al., 2004; Lankau et al., 2014; Calderón et al., 2017), or move to incomplete restoration or alternative ecosystem states (Scheffer et al., 2001; Falk et al., 2006). Therefore, insights into processes driving the dynamics of community assembly may be used to predict recovery trajectories followed by the ecosystem during reclamation and to define successful reclamation programs in post-mining sites (Kane et al., 2020).

The dynamics of succession and the structure of the communities are governed by deterministic (Niche-based) and stochastic (Neutral) processes (Vellend, 2010; Nemergut et al. 2013). Niche-based processes assume that deterministic factors like environmental filtering (i.e. selection of taxa by abiotic factors) are the primary determinants of community assembly and variability. Neutral theories, on the other hand, attribute the variability in microbial communities primarily to stochastic factors like drift (random replacement of organisms in a system) and dispersal (random movement of organisms through space) (Nemergut et al., 2013; Stegen et al., 2013). The relative importance of deterministic and stochastic processes in the dynamics of microbial succession in naturally and anthropogenically disturbed ecosystems has been widely documented (Wilhelm et al., 2013; Whitman et al., 2019; Kane et al., 2020), and continues to be a source of debate (Dumbrell et al., 2010; Nemergut et al., 2013). There is an interplay between stochastic and deterministic processes in the assembly of microbial communities (Yan et al., 2016; Kane et al., 2020). Therefore, the integration of novel microbial taxa in the community is mediated by dispersal, whereas the relative abundance of microbial groups in the community is mainly influenced by selective processes and drift (Vellend, 2010; Kane et al., 2020). Similarly, more extreme environmental conditions lead to the dominance of deterministic processes in microbial communities (Yan et al., 2016). However, the influence of stochastic processes in the community increases when extreme conditions wane (Yan et al., 2016).

Microbial communities are essential in the dynamics and functioning of the soil (Chodak et al., 2009; Delgado-Baquerizo et al., 2016a), they play fundamental roles in nutrient cycling, and climate regulation (Delgado-Baquerizo et al., 2016b; Fierer et al., 2021). Likewise, due to their close and complex associations with plants (Liao et al., 2008; Averill et al., 2014), microbial communities may influence the vegetation that populates disturbed and undisturbed ecosystems

(van der Heijden et al., 1998; Averill et al., 2014; Nuñez & Dickie, 2014). Therefore, microbial communities are key players in ecosystem restoration (Bach et al., 2010), and the knowledge of the influence of predictors and assembly processes in the patterns of succession of microbial communities following disturbance is important to understanding the course and potential outcomes of ecosystem restoration.

In the present study, I aim to determine the predictors associated with the variability in the microbial communities of stockpiled soil from different ages and soil depths, generated from two oil-sand extraction sites in Alberta, Canada. I further analyze the relative influence of various deterministic and stochastic assembly processes on microbial communities. Based on the inhospitable conditions commonly attributed to soil stockpiling, I predict that: (1), deterministic factors associated with environmental filters (e.g., pH and nutrient availability), will drive most of the variability in the microbial communities in the stockpiles; (2) the relative importance of the environmental filters in the community turnover will increase with increasing stockpile depth and storage time; (3) the influence of environmental filters on the community composition will depend on the abundance of each microbial taxon in the community (i.e. the importance of environmental filters will differ for the more abundant and the less abundant taxa in the community); and (4) microbial taxa that dominate in stockpiled soils will be associated with the non-native plants and edaphic factors that characterize these disturbed soils. The main objectives are (i) to identify the factors that explain the β diversity between stockpiled and reference soils, to determine whether specific soil disturbances generated by stockpiling (i.e. increased compaction, decreased pH, increase in nutrient availability), have a key influence in the structure of microbial communities of the soil, and (ii) to identify the microbial taxa correlated with the disturbance generated by soil stockpiling and that therefore may serve as indicators of soil condition. From a broader

perspective, our research will contribute to the body of literature on the ecological and evolutionary processes influencing the assembly of microbial communities in disturbed soils.

4.2 Methods

4.2.1 Sampling sites

Details of the sampling can be found in Cabrera-Hernandez et al. (2024). Briefly, we sampled stockpiled soils located in two oil sand extraction sites in Alberta, Canada. The sampling sites were (1) CNRL Horizon (57.337 °N, 111.755 °W), and (2) CNRL Wolf Lake (54.695 °N, 110.730 °W). Two locations representative of the Alberta mixed-wood natural subregion were used as references representing the undisturbed state of the ecosystem. A total of five reference soils (3 at the Wolf Lake site and 2 at the Horizon site) and seven stockpiles were sampled in August 2018. At the Horizon site, four stockpiles aged 0.5, 1.5, 5, and 7 years old were sampled. At the Wolf Lake site, three stockpiles of 2, 11, and 28 years old were sampled. Samples were taken from each stockpile at 0-10 cm, 20-30 cm, 80-90 cm, and >300 cm depths. Three replicate pits (separated by ~20 meters from each other) were collected from each sampling point, except for the >300 cm depth; only one pit per site was sampled at this depth. The collected soils were homogenized and processed through a 4 mm sieve to remove large debris, and frozen in plastic bags at -80°C.

4.2.2 Soil chemical and physical properties

The determination of pH, electrical conductivity, aggregate size, gravimetric water content, total nitrogen, and total carbon and soil texture, was carried out as outlined in Cabrera-Hernandez et al. (2024).

4.2.3 Plant community

A detailed description of the methods used in the study of the plant community composition and functional groups in stockpiles and reference soils can be found in Buss et al. (2020), from which the plant community datasets were obtained (i.e., plant relative abundance and classification into functional groups). Briefly, trees were classified according to the species that were able to become part of the overstory (Buss et al., 2020). The plant community composition of stockpiles and reference soils was determined by counting the individuals belonging to functional groups woody (trees and shrubs), grass (sedge, grass, and rush), and forbs (native, non-native, and stockpile forbs), classified according to Flora of Alberta (Moss & Packer, 1994). Native forbs were those occurring in forested areas but could also be found in the stockpiles, whereas stockpile forbs were defined as plant species found exclusively in the stockpiles (Buss et al., 2020).

4.2.4 DNA extraction and amplification

DNA was extracted in triplicate from randomized homogenized soil subsamples of 0.25 grams, using the DNeasy Power soil kit (QIAGEN, Hilden, Germany) according to the protocols outlined by the manufacturer. The triplicate DNA samples were mixed into a single DNA sample per pit. DNA concentration was determined using a Qubit dsDNA HS Assay Kit (Thermofisher Scientific, Canada). For Bacteria and Archaea, we amplified the 16S rRNA gene V4 region using the primer pair 515F and 806R (Caporaso et al., 2011). The fungal internal transcribed spacer 2 (ITS2) marker region was amplified with the fITS7-ITS4 primer pair (Ihrmark et al., 2012). The PCR cycle used in the amplification of the 16S rRNA gene was 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension at 72°C for 10 min. For the amplification of the fungal ITS region, we applied the following PCR program: 98°C for 30 seconds followed by 35 cycles of 98°C for 5 seconds, 55°C for 5 seconds, and 72°C for 15 seconds,

and a final extension at 72°C for 1 minute. The length of the PCR product was determined by electrophoresis. The extracted DNA samples were sent for Illumina sequencing along with the mock community and extraction blanks. The sequencing was carried out using an Illumina MiSeq platform with the 250-bp paired-end kit (V2 500-cycle PE Chemistry, Illumina, USA), by Microbiome Insights (Vancouver, Canada).

4.2.5 Data analyses

DNA amplicons from the Illumina sequencing were processed and merged into amplicon sequence variants (ASV), following the "DADA2 1.16.0" pipeline (Callahan et al., 2016) in "R version 3.6.1" (R Core Team, 2016). For the prokaryotes, 95.1% of the reads passed the quality filtering. Following dereplication and merging, 16,334 ASVs were generated, of which 4.64% of the ASVs were discarded as chimeras, reducing the number of ASVs to 15,576. We used the "SILVA 132 release ribosomal RNA gene database" (Quast et al., 2012) to assign taxonomy. We identified and eliminated the sequences considered mitochondria and chloroplasts, ending up with 14,071 ASVs assigned to the domain Bacteria and 102 ASVs classified into the domain Archaea. The reads generated were rarefied to 7,420 per sample via random subsampling.

For the fungi, 71% of the reads passed the quality filtering process. Of these, 3% of the reads were discarded as chimeric, and 2,929,616 paired-end reads (7,483 unique ASVs), were generated. The UNITE reference fungal database (Abarenkov et al., 2010) was applied for taxonomy assignment. The fungal ASVs were rarefied to 6,569 reads per sample using random subsampling. The FUNGuild database (Nguyen et al., 2016) was used to infer the function and trophic mode of the fungal ASVs, retaining only those ASVs whose likelihood of belonging to the function assigned by the database was "probable" or "highly probable", as suggested by the creators of the database (Nguyen et al., 2016).

4.2.6 Statistical analyses

The statistical analyses were performed in "R studio version 3.6.1" (R Core Team, 2016) unless otherwise stated. Dissimilarities in microbial community composition between stockpiled and reference soils were determined by applying Bray-Curtis and UNIFRAC dissimilarities, using "Phyloseq 1.30.0" (McMurdie & Holmes, 2013). To determine the statistical significance of the dissimilarities between the microbial communities we used permutational multivariate analysis of variance (PERMANOVA) with 999 permutations, from the "ADONIS" function of the R-package "Vegan 2.5-6" (Oksanen et al., 2013). The phylogenetic distance (Faith's) between stockpiled and reference soil was determined using the R-package "Picante 1.8.2" (Kembel et al., 2020). The influence of regional species pool (γ-diversity) on β-diversity was determined with a correlation analysis of the Bray-Curtis distances between sample communities, using the R package "Mecodev 0.2.0" (Liu et al., 2021). To shed light on the impact of stockpiling in the co-occurrence of microbial groups and the keystone taxa (i.e. Microbial groups with key importance in the structure of the community) in the soil, we applied a co-occurrence network using "Igraph" (Csardi. 2013), within the "Microeco" R package environment (Liu et al., 2021), and visualized with "Gephi" (Bastian et al., 2009). The keystone taxa were determined based on their number of significant (*P*-value < 0.01) and strong (Spearman $r > 0.6$ or $r < -0.6$) correlations with other microbial groups in the community. The correlation between the physical, chemical, edaphic, and biological factors was determined using Spearman correlation with the "corrplot 0.92" R-package (Wei et al., 2021). The influence of the predictors on the microbial communities was assessed with a Mantel test of the Spearman correlation coefficient in the R-package "Microeco 1.04.1" (Liu et al., 2021). To determine the most important predictors associated with the microbial community turnover, and the proportion of the variations in the community that are explained by these predictors we used a

canonical correspondence analysis (CCA), from the R-package “Vegan 2.5-6” (Oksanen et al., 2013). A forward selection model was used to identify the predictors that significantly explain the variation in the community, also using the “Vegan 2.5-6” R-package.

4.2.7 Ecological modeling to infer the assembly processes influencing the structure of microbial communities

4.2.7.1 Neutral model

We used Sloan’s community model (Sloan et al., 2006), to determine if the distribution of the microbial community (mean relative abundance and occurrence) follows the neutral community assembly model. This approach is based on the premise that the most abundant taxa (in the metacommunity) are more frequently dispersed by chance and therefore will be more widespread than the less abundant taxa, which are more likely to be affected by drift (Burns et al., 2016). Therefore, the model predicts that widespread ASVs (i.e. those with a higher frequency of occurrence) are those more abundant in the metacommunity. To calculate the fit of the community to the neutral model we used the R-package “minpack.lm 1.2-3” (Elzhov et al., 2023), a 95% confidence interval around the model was achieved by 999 bootstraps, using the R-package “HMisc 4.6.0” (Harrell, 2019).

4.2.7.2 Phylogenetic signal

To determine the influence of selective(niche-based) pressures on community structure and turnover, we assessed the phylogenetic signal, which examines the traits or habitat preferences among closely related taxa (Revell et al., 2008), to find out if ASV’s phylogenetic distances and niche distances (i.e. correlation of taxa with environmental conditions) are significantly correlated (i.e., niche conservation). The phylogenetic signal was assessed with a Mantel test correlogram, from the “Microeco 0.17” R-package (Liu et al., 2021), employing a Spearman coefficient to

correlate the taxa in the microbial communities and several edaphic parameters (i.e., Total carbon, total nitrogen, and pH).

4.2.7.3 Null model

To determine the processes behind the turnover in the microbial community structure in disturbed soils, the study followed the conceptual framework proposed by Vellend (2010) and made operational by Stegen et al. (2013) and Stegen et al. (2015). The model infers the ecological relatedness between taxa based on their measured phylogenetic relatedness, employing β-mean nearest taxonomic distance (βNMTD). The dissimilarities between the observed βNMTD and null βNMTD model (999 randomizations) generate the β-nearest taxon index (βNTI), measured as standard deviations from the null model distribution. Then, βNTI < -2 indicates that community turnover is significantly lower than expected and is interpreted as influenced by homogeneous selection, i.e. increased community similarity caused by similar environmental conditions. On the other hand, βNTI > 2, indicates that community turnover is significantly higher than expected, which corresponds to the influence of heterogeneous/variable selection, i.e. decreased community similarity caused by variation in environmental conditions. Similarly, the Raup–Crick Bray (RCBray) β-diversity metric, identifies the difference between observed and null distribution Bray-Curtis values (999 randomizations), assessed as standard deviations between +1 and -1. The fraction of pairwise comparisons in which βNTI < 2 and an RCbray > +0.95 is considered to be influenced by dispersal limitation (Stegen et al., 2013, 2015; Zhou & Ning, 2017a), i.e. high compositional variability in the community due to a low rate of dispersal. On the other hand, βNTI < 2 and RCbray < -0.95, have been estimated to define the effect of homogenizing dispersal, i.e. low compositional turnover due to high dispersal rates. However, the model considers that drift alone is acting on the community when βNTI < 2 and RCbray < 0.95 (Stegen et al., 2013; Liu et

al., 2021). Both βNMTD and Bray–Curtis-based Raup–Crick (RCBray) indexes were determined using the R packages "Picante 1.8.2" (Kembel et al., 2020) and "Microeco 1.04.1" (Liu et al., 2021).

4.2.7.4 Effect of assembly processes in microbial groups depending on their relative abundance.

To determine if the assembly processes influencing the less abundant taxa in the community were different from those governing the turnover in the community in general, we performed the null model analysis on the "rare" ASVs, which we define as those with relative abundance < 0.1% in all samples, according to an approach followed by similar studies (Chen & Wen, 2021). In this case, no archaeal ASV meets the conditions of being < 0.1% in every sample, so only bacterial and fungal ASVs were used in this part of the analysis.

4.2.7.5 Niche breadth

To shed light on the differential tolerance of the microbial communities to environmental factors and assembly processes, we calculated the Levins' niche breadth (Levins, 1968) for Bacteria, Archaea, and fungi in the disturbed soils, using the R-package "spaa 0.2.2." based on the distribution of each taxon in different environments/samples. The significance of the dissimilarities in niche breadth among Bacteria, Archaea, and fungi, was assessed through an analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post hoc test, using the "Agricolae 1.3.2" (Mendiburu, 2021).

4.3 Results

4.3.1 Microbial communities in disturbed soils

As we previously described (Cabrera-Hernandez et al., 2024), microbial communities of disturbed and reference soils were significantly different (Fig. 4.1 and Fig. 4.2), which could be explained by the difference in the abundance of the most important phyla found in the two studied soil types (Fig.S4-1). Compared to the communities in the undisturbed reference sites, stockpiling generates bacterial, archaeal, and fungal communities that were less phylogenetically clustered (Fig. 4.2). Similarly, the co-occurrence network analysis revealed that stockpiling correlated with a shift in the soil keystone taxa, (Fig.S4-9), since in the reference soils Ascomycota and Basidiomycota were the predominant keystone taxa, involved in 24.6% and 22.3% of the connections with other microbial groups, respectively. In the stockpiles, Proteobacteria and Ascomycota became the main keystone taxa, involved in 24.4% and 22% of the connections with other microbial taxa, respectively (Fig. S4-9). Also, taxa/nodes and modules in the stockpiled soils were more interconnected than those in the reference soils (Fig. S4-9); which suggests that complexity in the soil microbial communities increases after the disturbance. The correlation between the β-diversity and γ-diversity, indicated that the regional species pool was significantly important for the prokaryotic community turnover, but not for the fungal community turnover (Fig. S4-13).

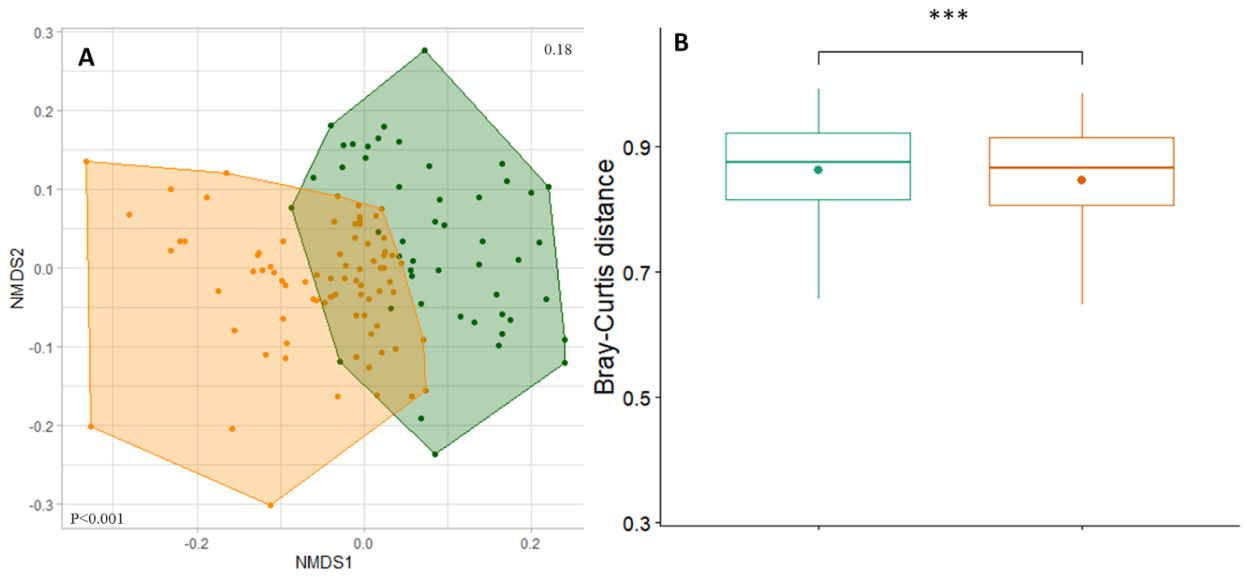

Figure 4.1. Comparison of the microbial communities of the disturbed (orange) and reference soils (green). 1A. NMDS ordination showing the UNIFRAC dissimilarities between the microbial communities (Bacteria, Archaea, and fungi) of the studied soils. B. Bray Curtis dissimilarity between samples(based on microbial communities) in the two soil types contrasted. The significance of the dissimilarities is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$= ***.

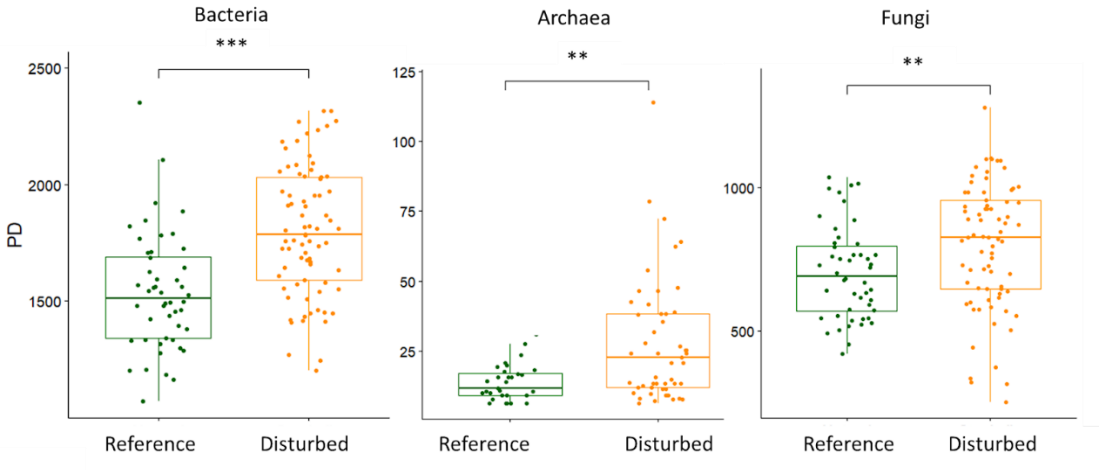

Figure 4.2. Phylogenetic diversity (Faith's) index for the bacterial, archaeal, and fungal communities of the disturbed and reference soils. The asterisks indicate the significance of the difference between the two groups: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = *$p \leq 0.001$ = ***.

4.3.2 Predictors associated with the shift in microbial communities of the disturbed soil.

Some of the microbial groups that account for the difference between disturbed (stockpiled) and reference soils were Basidiomycota and Verrucomicrobia (Fig. S4-1). In the soil surface(0-10 cm and 20-30 cm), these taxa showed a positive correlation ($p < 0.05$) with the native vegetation of the reference soils but correlated negatively ($p<0.05$) with the increase in most of the soil nutrients and non-native plant functional groups. On the other hand, the Bacteroidetes, Proteobacteria, and Ascomycota were positively correlated ($p<0.05$) with some of the non-native plant groups and nutrients in the soil but correlated negatively with forest trees and native functional groups of the reference soils (Fig. S4-4 and Fig. S4-5A). In the deep layers of the soil (80-90 cm and >300 cm), the influence of nutrients (i.e., carbon and nitrogen), decreases, but the importance of soil aggregate size increases.

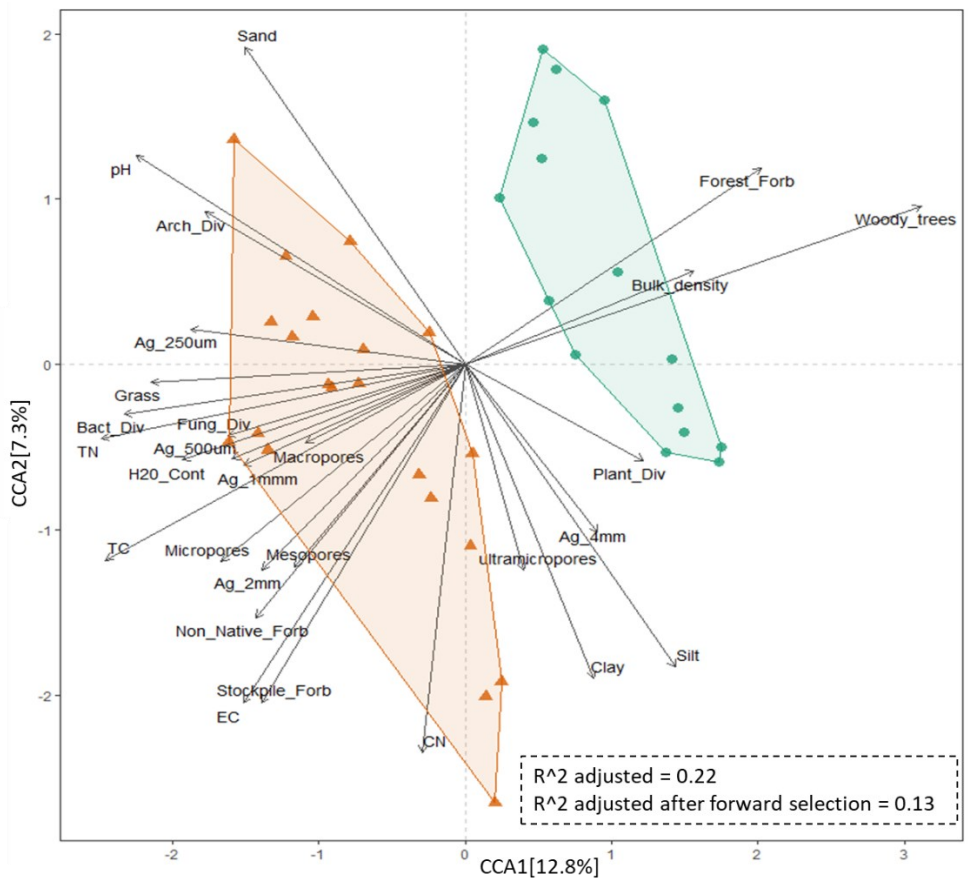

Figure 4.3. CCA ordination of the influence of environmental and biological factors in the variation of microbial communities between 0-10 cm profile of reference sites (green) and disturbed soils (orange). Abbreviations of some parameters included are: Arch_Div = archaeal diversity, Fung_Div = Fungal diversity, Bact_Div = bacterial diversity, EC= electroconductivity, TC = total carbon, TN = Total nitrogen, H20_cont = water content, Ag = Aggregate size, CN = carbon to nitrogen ratio. After forward selection the factors that significantly ($p<0.001$) explain the variability in the microbial communities were: Woody trees, forest forbs, stockpile forbs, total carbon, clay, and macropores. These factors together significantly explain ~ 13% of the variability in the system.

The diversity index (Shannon-Weaver) was correlated to multiple predictors in all layers of the stockpiled soil. In the surface soils, bacterial and fungal diversity correlated positively with soil total carbon, nitrogen, soil pores, and non-native plant functional groups. However, bacterial and

fungal diversity correlated negatively with forest native functional groups. Also in the surface soils, archaeal diversity was negatively correlated with plant diversity and clay, but positively with pH and nitrate (Fig.S4-3A and S4-3B). In the deeper stockpile layers (80-90 cm and >300 cm) the influence of plant community on microbial diversity weakens. However, bacterial and fungal diversities were mostly correlated with soil nutrients (i.e., carbon and nitrogen) soil pores, and texture.

The amount of community variability explained by the measured factors was ~13% in the 0-10 cm stockpile depth, (Fig. 4.3), ~15% in the 20-30 cm stockpile depth (Fig. S4-2A), and ~17% in the combined deeper stockpile depths (Fig. S4-2B). Most of the variability between the disturbed (stockpiled) and reference soils was not represented by the CCA analysis, leading me to hypothesize that the variability not explained by the forward selection model could either result from additional environmental/edaphic factors not accounted for by the study or from stochastic community assembly processes.

4.3.3 Assembly processes likely influencing the turnover of microbial communities.

The results of Sloan's community model for communities in stockpiled soils indicated that bacterial communities fit the neutral model better than archaeal communities (Fig. S4-12). The fungal community assembly does not seem to fit a neutral community assembly model, except for fungal communities in the >300 cm depth (Fig. S4-12). Consistently, no significant phylogenetic signal was found between microbial communities and the edaphic factors measured (Fig. S4-11), which reflects a likely lack of niche conservation across phylogenetically related taxa (Münkemüller et al., 2012).

Likewise, the null model analysis revealed that stochastic community assembly processes were considerably more important than deterministic processes for the assembly of microbial communities in disturbed soils (Fig. 4.4). Archaeal, bacterial, and fungal communities were shaped by different stochastic processes, and the relative influence of these assembly processes varied along the time the disturbed soil remained stockpiled (Fig. 4.4). In terms of soil storage time, homogenizing dispersal and dispersal limitation were the main processes influencing the turnover in bacterial communities, but dispersal limitation became more important for communities in the oldest stockpiled soil (i.e. 28 years old stockpile). The influence of drift varied but was the main factor influencing the fungal communities through soil storage time. Homogenizing dispersal was the main driving process for the archaeal communities in younger and the oldest stockpiles, whereas drift dominated the archaeal communities in the stockpiled soils in the 5,7, and 11 years of storage. Although communities were mostly influenced by stochastic processes through soil storage time, among the deterministic processes, variable selection was the most influential process for the bacterial communities(especially for the 28 years stockpile) and homogenizing selection was the most important for most fungal communities in the stockpiles independently of the storage time.

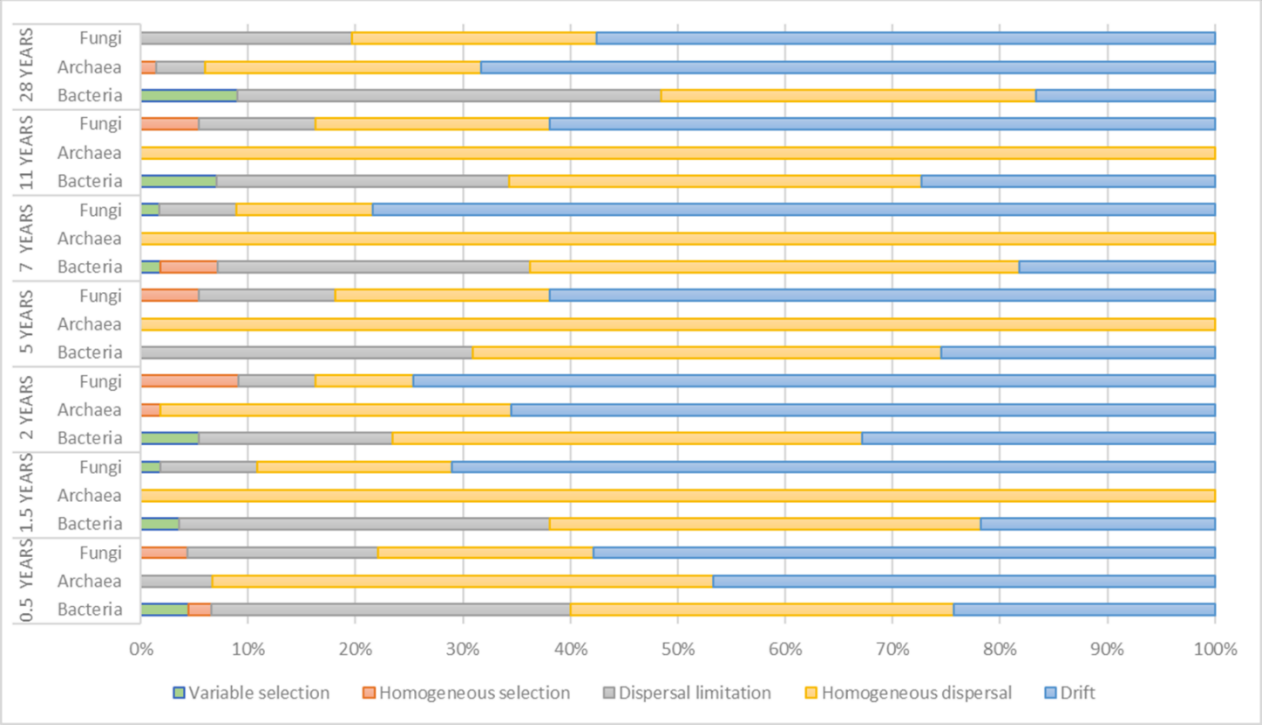

Figure 4.4. Processes shaping the microbial communities of disturbed soils across the time since disturbance. The deterministic assembly processes considered were variable selection and homogeneous selection. The stochastic processes were homogeneous dispersal, dispersal limitation, and drift.

Similarly, the analysis showed that the relative influence of assembly processes shaping microbial communities fluctuated depending on the soil depth (Fig. 4.5) and the relative abundance of the taxa in the communities (Fig.S4-7). Regardless of soil depth, the bacterial communities were mostly affected by drift, homogeneous dispersal, and dispersal limitation, but the influence of drift was considerably higher for the rare bacterial taxa (i.e., taxa with a relative abundance of <1% in every sample) than for the general bacterial community (Fig. 4.5; Fig.S4-7). Also, homogenizing selection was the second most influential factor (following drift) for the rare bacterial taxa in the > 300 cm depth. Fungal communities were mostly influenced by drift (Fig. 4.4; Fig. 4.5) at all soil depths, whereas homogenizing dispersal was the leading process influencing the rare fungal taxa

in the community (Fig.S4-7) regardless of soil depth. The archaeal communities were primarily affected by homogeneous dispersal at all soil depths.

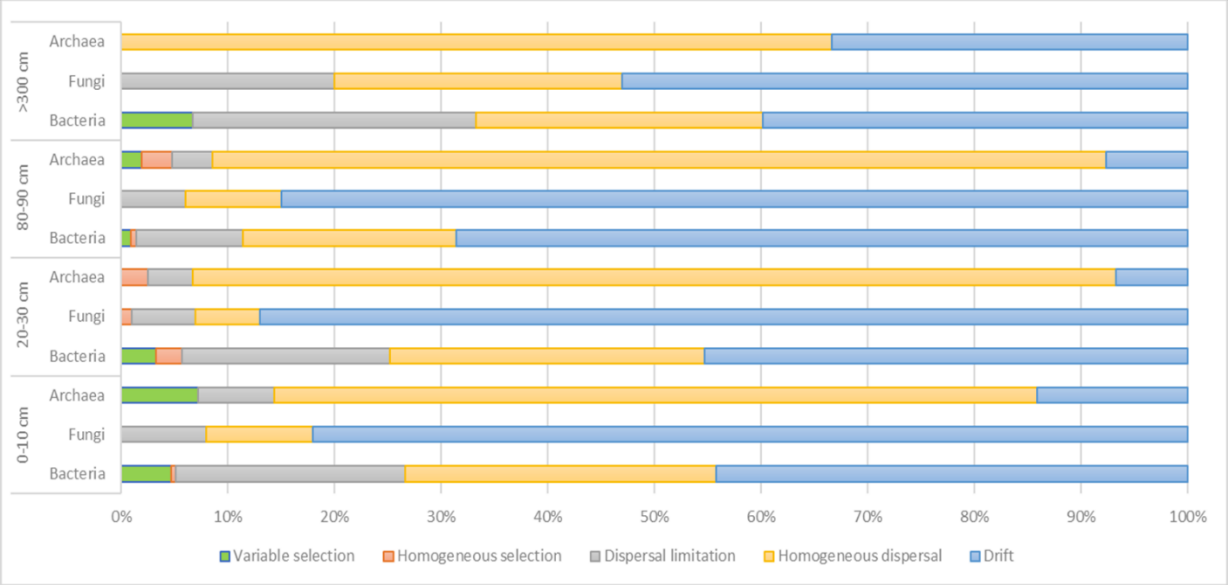

Figure 4.5. Processes shaping the microbial communities of disturbed soils across the soil depth profile. The deterministic assembly processes considered were variable selection and homogeneous selection. The stochastic processes are homogeneous dispersal, dispersal limitation, and drift.

When examining the factors shaping the fungal guilds and trophic modes in disturbed and reference soils, we found that the saprotrophic fungi were mostly influenced by drift, while homogenous dispersal was the primary factor shaping the communities for the mycorrhizal fungi (i.e., ectomycorrhiza and endomycorrhiza) (Fig. S4-6).

Interestingly, like the microbial communities in the stockpiled soils, the microbial communities in the reference soils were mainly influenced by stochastic processes (Fig. S4-8). Drift was the dominant assembly process for the fungal communities in all reference soil depths, whereas

homogenizing dispersal drift and dispersal limitation were the most influential processes for the prokaryotes. These results may indicate that stochastic assembly factors maintain their influence on the community even after the disturbance generated by the stockpiling.

Bacterial communities in the stockpiled soils exhibited a significantly larger ($p<0.05$) niche breadth than fungi and Archaea, but the niche breadth for the fungal communities was significantly higher ($p<0.05$) than that of the archaeal communities (Fig. S4-10). This supports the idea of bacterial communities being more versatile than fungal communities when it comes to niche preferences and tolerance.

4.4 Discussion

4.4.1 Microbes as markers of soil disturbance

Anthropogenic disturbances impacting vegetation or soil structure may affect soil microbial communities (Fadaei et al., 2021; Rodriguez-Ramos et al., 2021), generating a substantial turnover in community composition (Lauber et al., 2008; Cabrera-Hernandez et al., 2024), function (Liang et al., 2020), and their co-occurrence patterns (Sun et al., 2017), which aligns with the results shown here. Interestingly, the less phylogenetically clustered communities generated by the stockpiling may be an indication of the reduced strength of the selective pressures in community assembly and suggests that the variability described was generated by the colonization of microbial taxa distantly related to the native microbial groups in the community.

Two microbial groups could be delineated based on the factors they associate with, these were: Group 1. Microbial taxa with a copiotroph lifestyle (Fierer et al., 2007), which correlated positively with soil disturbance (e.g., increase in nutrients and invasive plant species). Group 1 includes Proteobacteria, Bacteroidetes, and Ascomycota. Group 2: Microbial taxa positively associated

with the conditions present in the undisturbed (reference) soils (e.g. native vegetation and low nutrients in the soil), with an oligotrophic lifestyle (Fierer et al., 2007; Kranabetter et al., 2009). Group 2 includes Verrucomicrobia and Basidiomycota. Interestingly, the fact that both microbial groups seemed to be negatively correlated with most of the factors that are strongly and positively associated with the opposite group, suggests that these factors are important predictors of community turnover. Since these microbial groups seem to be sensitive to the fluctuations of soil parameters and vegetation, they may be used as efficient indicators of soil conditions.

The positive correlation exhibited by microbial taxa to specific plant functional groups may indicate that microbes growing in the stockpiled soils may ease the establishment of invasive plants (van der Heijden et al., 1998; Nuñez & Dickie, 2014; Frąc et al., 2018), or plants could promote the success of specific microbial groups (Ladygina & Hedlund, 2010; Mitchell et al., 2010) in the stockpiled soils. This may occur when non-native plants release litter that is usually richer in nitrogen and carbon than that generated by the native vegetation (Liao et al., 2008), thus promoting the dominance of copiotrophs or r-strategists microbial groups.

High microbial diversity has been usually regarded as a positive sign of soil health (Delgado-Baquerizo et al., 2016b), and soil resilience to disturbance (Wittebolle et al., 2009; Eisenhauer et al., 2012). However, increased diversity can also be an indication of disturbance, when its increase is due to the arrival of invasive species that populate soil after the disturbance (Hobbs & Huenneke, 1992; Buss et al., 2020). My results seem to support this idea since both bacterial and fungal diversities were positively correlated with non-native/invasive plant functional groups but negatively correlated with native plant functional groups. We cannot ascertain the directionality of the correlation we describe here. However, since both plant and microbial groups differed from

those found in the reference natural ecosystems, diversity may not act as a positive indicator of ecosystem health for stockpiled soils.

4.4.2 Environmental filters explain a small proportion of the variability in the disturbed soil.

Predictors commonly associated with microbial community turnover following disturbance include pH (Dimitriu & Grayston, 2010; Zhalnina et al., 2015; Zhou et al., 2021), nutrient availability (Fierer et al., 2007; Paterson et al., 2019), plant community composition (Mitchell et al., 2010), plant diversity (Zak et al., 2003), and soil texture (Bach et al., 2010). These factors can act as environmental filters and drive deterministic processes that influence the patterns of community assembly, through the selection of taxa that share a repertoire of phenotypic traits that confer fitness to particular environmental conditions (Kraft et al., 2015). The relative influence of specific environmental filters associated with disturbance may vary with soil depth (Philippot et al., 2021), and may fluctuate as the communities develop (Wang et al., 2012; Hargreaves et al., 2015; Yan et al., 2016), which explains the correlation of the dominant microbial groups with edaphic factors that were generally different at each soil depth. The drivers that dominate in the deeper stockpile depths could be associated with the extreme conditions in that soil layer (Johnson et al., 1991; Kundu & Ghose, 1997; Ghose & Kundu, 2004), namely, anoxic conditions lead to the dominance of fermentative and anaerobic microbes that may decrease the soil pH with their metabolic products. The drivers that are influential in the surface (i.e., total carbon and CN), may result from the plant litter and exudates yielded by the vegetation (Liao et al., 2008).

The influence of deterministic factors on the assembly of microbial communities is stronger in more extreme conditions (Yan et al., 2016). Therefore, I hypothesized that the harsh conditions commonly attributed to soil stockpiles like low pH and scarce nutrients (Johnson et al., 1991; Kundu & Ghose, 1997; Strohmayer, 1999), would make environmental selection the primary

factor governing the turnover in the microbial communities. However, the predictors that most significantly explained the turnover in the microbial communities only accounted for ~1/5 of the variability in the microbial communities, suggesting that the source of most of the variability lies in unmeasured environmental factors in the community or in factors that are associated with neutral variability (stochastic assembly processes) (Stegen et al., 2012; Zhou & Ning, 2017a).

4.4.3 Microbial succession following soil disturbance is primarily governed by stochastic processes.

The dominance of stochastic processes in the assembly of microbial communities has been widely documented (Stegen et al., 2012; Chen et al., 2019), and could be explained since stochastic processes dominate when extreme conditions are weak (Yan et al., 2016). In such conditions increased stochastic processes lead to the assembly of a community that is less phylogenetically clustered (Yan et al., 2016; Tripathi et al., 2018; Zhou et al., 2021), which is consistent with my findings. The lack of niche conservation and the key influence of neutral processes in the community might indicate that factors associated with soil management (i.e., soil stripping, mixing, piling, homogenizing, and compaction), which increase or reduce the dispersal of microbes in the communities, are more responsible for the community turnover in the stockpiles than the commonly attributed extreme conditions associated to environmental filters. The similarity in the assembly processes influencing the microbial communities in the stockpiled soils and those in the undisturbed soils could indicate that the stockpiling disturbance may alter the strength of stochastic factors, but the influence of these assembly factors on microbial communities is maintained. Furthermore, the higher influence of the γ-diversity on the structure of prokaryotic communities indicates that besides local assembly processes (i.e., stochastic or deterministic processes), the size of the regional species pool is also an important factor governing the turnover

in these communities (Xing et al., 2024), which generally occurs when selective pressures are low (Chase & Myers, 2011).

The relative influence of stochastic processes varies depending on the microbial group affected (Farjalla et al., 2012; Chen & Wen, 2021), as reported here, which might be related to the relative abundance and size of the microbes (Farjalla et al., 2012; Logares et al., 2018; Chen & Wen, 2021), or to their physiological status (Kivlin et al., 2014; Choudoir & DeAngelis, 2022). The variability of the influence of stochastic and deterministic factors according to the soil depth profile or to the time since disturbance aligns with reports that have found a similar pattern (Stegen et al., 2012; Wu et al., 2018; Liu et al., 2021). Bacterial communities were mainly shaped by homogeneous dispersal and dispersal limitation (Chen et al., 2020; Chen & Wen, 2021; Huang et al., 2022), which occurs since soil stripping and mixing at the start of the mining activities homogenizes soil conditions and structure (Wick et al., 2009; Shrestha & Lal, 2011; Block et al., 2020) and its microbial communities (West & Whitman, 2022). However, after stockpiling, the conditions in some parts of the soils may vary drastically, creating more compact, anoxygenic, or nutrient-depleted zones (Johnson et al., 1991; Ghose & Kundu, 2004; Sheoran et al., 2010; Block et al., 2020). This may explain the increased influence of dispersal limitation in the oldest and deepest stockpiled soil, since dispersal limitation imposes physical and physiological barriers (e.g., through soil compaction) for microbe distribution (Kivlin et al., 2014), as shown here. On the other hand, the importance of drift for fungal communities has been documented (Huang et al., 2022; Zhang et al., 2023), and may be explained by the relatively lower population size of fungi compared to Bacteria in the soils (Wu et al., 2018; Zhang et al., 2023), which makes fungal communities more prone to vary due to random birth-death or extinction events under weak selective pressures.

The relatively lower effect of dispersal on fungal communities may be explained by the fact that dispersal is stronger for smaller organisms (Shurin et al., 2009; Wu et al., 2018), like bacteria and archaea. Also, the relative ease of dispersal shown by bacteria may be due to their tolerance to environmental conditions as well as to their higher metabolic flexibility, compared to fungi (Finlay, 2002; Farjalla et al., 2012; Wu et al., 2018). Bacteria, therefore, may be more widespread in a diverse array of environments where they are transported (Chen et al., 2020; von Meijenfeldt et al., 2023). The flexibility of Bacteria to live in a wide array of environmental conditions is supported by their superior niche breadths, compared to Archaea and fungi (Fig. S4-10).

Functionally redundant groups seem to be more affected by ecological drift (Zhou & Ning, 2017) which may explain the relatively high influence of this stochastic process on saprotrophic fungal groups. Also, the efficacy of dispersal can be affected by priority effects (Zhou & Ning, 2017b; Debray et al., 2021). Therefore, the weak dispersal effect on the assembly of the saprotrophic fungi could be due to the antagonism exerted by some mycorrhizal fungi (Orwin et al., 2011; Averill et al., 2014), based on the reduction of the saprotrophic litter decomposition in the soils (Janowski & Leski, 2022), which greatly limits saprotrophic fungi distribution (Averill & Hawkes, 2016). Conversely, vegetation is one of the main factors determining the dispersal of mycorrhizal fungi (Janowski & Leski, 2022), which could be understood by the complex relationship these fungal guilds establish with plants (van der Heijden et al., 1998; Scott et al., 2019), and may explain the high influence of homogeneous dispersal found in the mycorrhizal fungi by my study. The fact drift was the main assembly process influencing the fungal communities, but homogenizing dispersal was the chief factor influencing the mycorrhizal fungi, suggests that the assembly processes affecting the functional groups in the communities could differ from processes shaping communities according to taxa.

4.4.4 The influence of assembly processes varies according to taxa relative abundance.

The strength of the assembly processes will be reflected by the relative abundance of the taxa in the community (Liu et al., 2021). Thus, deterministic assembly mechanisms will shape the most abundant taxa in the community, whereas the rare taxa will be more affected by stochastic assembly mechanisms such as drift (Liu et al., 2021). Likewise, due to their low number, rare taxa are more prone to be influenced by drift. In contrast, the dominant taxa will more likely be affected by random dispersal due to their high number, which explains their widespread distribution (Sloan et al., 2006; Chen & Wen, 2021). The fact that the main assembly processes influencing the rare taxa at each stockpile depth were different from the main assembly processes shaping the structure of the community in general, suggests that abundant and rare taxa are differentially affected by stochastic factors, as has been proposed by ecological theories regarding community assembly (Sloan et al., 2006; Zhou & Ning, 2017b).

Conclusion

The study provides insights into the predictors and assembly dynamics of microbial communities in stockpiled soils. In both disturbed and undisturbed soils, certain microbial groups and their diversity indexes were correlated with the environmental conditions including edaphic parameters and vegetation, indicating that microbial taxa within each of these groups may be used as effective markers of soil conditions. Therefore, their relative abundance may indicate the effectiveness of soil restoration practices including reclamation. Also, my findings indicate that microbial communities in the stockpiles were primarily structured by neutral assembly processes, however, most literature indicates that deterministic factors are assumed to be the main drivers of microbial communities in disturbed soils. Likewise, fluctuations of the relative strength of neutral processes according to the microbial group affected (Bacteria, Archaea, or fungi), taxon relative abundance,

or fungal functional group corresponds to expected patterns of stochastic assembly described by ecological theories. Further studies should address the assembly dynamics of the microbial functional diversity in disturbed soils to determine whether the processes influencing the phylogenetic and taxonomic diversity are different from those influencing the microbial functional groups (as shown here for the fungi). The knowledge of the processes influencing the assembly of microbial communities following disturbance may serve to understand and predict the reclamation trajectories followed by communities in restored ecosystems. This knowledge will help determine soil restoration strategies, like those implemented in the reclamation of post-mining sites using stockpiled soils.

CHAPTER 5
Conclusions and Future Directions

5.1 Summary of findings

The detrimental effects of stockpiling on soil integrity, health, and quality have been widely documented (Harris et al., 1989a; Kundu & Ghose, 1997; Block et al., 2020). I proposed that the disturbance associated with stockpiling generates a shift in soil microbial communities (Bacteria, Archaea, and fungi), and their functions. Consistent with my hypothesis and with the body of literature regarding the impact of stockpiling on soil biology (Birnbaum et al., 2017; Buss et al., 2020), the results of my studies indicate that stockpiling has adverse effects on soil microbial communities, making them different from that in the reference soils. Likewise, supporting my initial hypothesis, even when the two stockpiling sites (i.e, Wolf Lake and Horizon) were separated by more than 400 km, microbial communities were more similar to each other than to their respective reference soils, suggesting that the conditions created by stockpiling disturbance were similar, disregarding the geographical distance or location of the two sites, and these conditions promoted the establishment of a specific microbial community that significantly diverges from that generated by the range of natural variability.

Based on the relevance of microbial communities in soil function (Delgado-Baquerizo et al., 2016; Fierer et al., 2021) and restoration (Bach et al., 2010; Averill & Hawkes, 2016), and since disturbance may persist after the stockpiled soil is used as a reclamation substrate (Dhar et al., 2018), I conclude that the impact of soil stockpiling on microbial communities may represent a challenge for the goals of land reclamation.

My results indicate that stockpiling generated a shift in key microbial taxa (i.e. taxa with an important role in the maintenance of the community structure). The most important microbial groups that accounted for the difference between the stockpiles and their reference soils were correlated with some of the factors of the disturbance generated by the stockpiling, like the increase in soil nutrients, likely made available by the degradation of soil aggregates during the soil striping and stockpiling (Wick et al., 2009). Similarly, bacterial and fungal diversities were positively correlated with the presence of non-native/invasive plant functional groups. Some of these plant functional groups were among the most important predictors of the change in the microbial communities, thus shedding light on the importance of vegetation for the variability in microbial communities found between stockpiles and their reference soils. However, microbial communities may be responsible for the plant community composition in these systems as well. For example, there was a higher proportion of arbuscular mycorrhizal fungi (AMF) in stockpiles and ectomycorrhizal fungi (EMF) in reference soils. Compared to the EMF, the AMF may generally associate with a wider range of plant species (Richardson et al., 2000; Fitter, 2005; Corrales et al., 2018), thereby potentially easing the establishment of non-native plant functional groups in disturbed ecosystems (Richardson et al., 2000; Nuñez & Dickie, 2014; Frąc et al., 2018). Thus, the differential distribution of mycorrhizal fungi could explain the dominance of the invasive vegetation found in the stockpiles and the native plant communities that dominate in the reference soils (Buss et al., 2020).

The comparison of the fungal functional guilds and their trophic modes in stockpiles and reference soils revealed that there was a shift in the dominance of ECM in the reference soil to saprotrophic fungi in the stockpiled soils. Due to the complex association between ECM and the boreal forest vegetation (Clemmensen et al., 2013; Policelli et al., 2020), nitrogen is sequestered in the reference

soils (Averill & Hawkes, 2016; Lindahl et al., 2021; Tedersoo & Nara, 2010), which leads to the accumulation of organic matter in the forest soil. In the process of stockpiling, this nitrogen is released during overstory removal (Pec et al., 2017; Rodriguez-Ramos et al., 2021), carried out as part of the mining activities (Johnson & Miyanishi, 2008). The associated accumulation of plant-derived organic matter in the stockpiles (Martos et al., 2009) may have promoted the growth of saprotrophic fungi. The antagonism between these two fungal groups could respond to the limitation of saprotrophic fungi colonization exerted by the ECM. Therefore, the shift in microbial communities generated by soil stockpiling is also manifested at the level of the functions played by microbial communities in the soil.

Stockpile depth has consistently been mentioned as one of the factors negatively associated with the decrease in microbial diversity in stockpiled soils (Block et al., 2020; Harris et al., 1989b; Johnson et al., 1991; Strohmayer, 1999). My results strongly support these conclusions since bacterial, archaeal, and fungal communities in the stockpile surface (0-10 cm, 10-20 cm, and 20-30 cm) were dramatically different from the communities in the deeper stockpile layers (80-90 cm and >300 cm), and this shift in community composition was accompanied by an important decline in fungal, bacterial, and archaeal diversity as well as the fungal functional guilds and their associated trophic modes in the deeper stockpile layers. The reasons for such a general decrease in diversity function and community composition could be attributed to the hostile conditions commonly found in the deeper sections of stockpiled soils (Abdul-Kareem & McRae, 1984; Harris et al., 1989b; Anderson et al., 2008), which are characterized by low nutrient and biomass production (Paterson et al., 2019) and anoxic zones (Johnson et al., 1991; Block et al., 2020). A close analysis of the microbial communities of the deeper stockpile layers shows that these soil depths were mainly dominated by anaerobic and facultative aerobic prokaryotic groups (e.g.,

methanogens and fermenters), as well as saprotrophic fungi. These microbial groups are clear indicators of the sub-oxic conditions that likely dominate the deeper regions of the stockpiles. Therefore, the increased depth of the stockpiled soils changes microbial community composition and is detrimental to microbial diversity, and function. Therefore, the use of soil at this depth as a reclamation substrate could be problematic.

Soil storage time has been regarded as a negative factor for soil microbial communities, associated with a general decline in soil desirable properties (Johnson et al., 1991; Kundu & Ghose, 1997; Strohmayer, 1999). Some sources have considered that soil has an "expiry date", after which the decline in soil health attributes, including microbial communities, makes it unsuitable to be used as a reclamation substrate(Kundu & Ghose, 1997; Ghose & Kundu, 2004). My findings shed light on the impact of stockpile age on microbial communities, showing that fungal and prokaryotic communities follow different trajectories through soil storage time. My results indicate that prokaryotic communities in the younger and older stockpiles diverged significantly from the reference soils. However, bacterial communities of intermediate-aged stockpiles (i.e., 5 and 7 years) became more similar to the reference soils. This may indicate that even when stockpiling initially generates a significant disturbance, the stockpile bacterial communities recover to resemble those communities in the reference soils within a few years; however, this recovery seems to be transitory, since after prolonged storage, the communities could differentiate again from those in the reference soils. This finding also seems to indicate that, in terms of bacterial communities, there is an optimal age range for the stockpiled soils to be successfully used as a reclamation substrate.

The fungal communities, on the other hand, exhibited notable differences with the reference soils along the chronosequence time points, but for some of the younger (i.e., 2 years old) and the oldest

(i.e., 28 years old) stockpiles, these differences seemed to increase. The fact that the differences between fungal communities in stockpiles and reference soils remained significant across the chronosequence may indicate that the fungi are more sensitive to the impact of the disturbance generated by soil stockpiling than the Bacteria. Likewise, the higher abundance of the endomycorrhizal fungi (i.e., arbuscular mycorrhizae, ericoid mycorrhizae, and orchid mycorrhizae) in some of the younger stockpiles suggests that these guilds proliferate when the disturbance generated by the stockpiling activities is recent and may play a fundamental role in the course of the secondary succession, probably easing the establishment of invasive plant communities in the stockpiled soils, as discussed above and evidenced by the higher proportion of non-native plant functional groups in the stockpiles (Buss et al., 2020). The fact that fungi seemed to be more sensitive to stockpiling than prokaryotes is also supported by the shift in key soil microbial taxa from fungi to Bacteria generated by stockpiling, as revealed by the co-occurrence analyses.

Although multiple studies have indicated that soil storage time is associated with a decrease in microbial alpha diversity (Kundu & Ghose, 1997; Ghose & Kundu, 2004), I found the opposite trend, with most bacterial alpha diversity indexes increasing significantly with storage time. On the other hand, the fungal community alpha diversity generally did not change significantly with soil storage time, (excepting the 2 years old stockpile). Therefore, in my system, stockpiling time seemed to impact microbial community composition but did not decrease the microbial diversity.

Prior studies have generally attributed harsh conditions to stockpiled soils (Johnson et al., 1991; Strohmayer, 1999; Block et al., 2020). This led me to hypothesize that the variability in the microbial communities of the stockpiles and their divergence with the communities of the reference soils could be explained by the physical, chemical, and biological factors responsible for

the conditions found in the stockpiles. Contrary to my expectations, the measured environmental and edaphic factors only explained less than 20% of the variability in the system, and most of such variability was influenced by neutral community assembly processes, including drift and dispersal. However, the relative influence of the neutral assembly processes is different for each microbial group and tends to fluctuate according to soil depth and storage time. So, fungal communities were primarily influenced by drift, archaeal communities by homogeneous dispersal, and bacterial communities by dispersal limitation and homogeneous dispersal. The relatively high influence of drift on the fungi could be due to their relatively lower population size, compared to bacterial communities (Wu et al., 2018; Zhang et al., 2023). Intriguingly, the assembly processes explaining the β-diversity between fungal guilds and trophic modes in the stockpiles show that the mycorrhizal fungi are primarily assembled by homogeneous dispersal, which is facilitated by the vegetation (Janowski & Leski, 2022). On the other hand, saprotrophic fungal communities are primarily governed by drift, which could be explained by the high influence drift has in functional redundant groups (Zhou & Ning, 2017).

The relative importance of the above-mentioned neutral assembly processes was different for the rare microbial taxa (i.e., Those with a relative abundance of <1% in every sample). So, the influence of drift for the rare bacterial taxa was considerably higher than the influence this factor represented for the bacterial communities in general. Fungal communities were chiefly influenced by drift, but the less abundant fungal taxa were mainly influenced by homogenizing dispersal. The relatively higher influence of drift on the rare bacterial taxa could be due to the greater effects of random death, colonization, and extinction events on less abundant groups (Sloan et al., 2006; Chen & Wen, 2021). The high influence of dispersal on the rare fungal taxa, on the other hand,

may suggest that these fungal taxa belong to the mycorrhizal functional group, whose dispersion is facilitated or limited by vegetation (Janowski & Leski, 2022).

5.2 Contributions to the field

I. My preliminary results confirm that stockpile depth drastically changes microbial community composition and diversity. Some of the microbial groups affected are responsible for important ecosystem services and play key roles in the type of vegetation that populates the soil. Therefore, the use of deeper stockpiled soils from between 80-90 cm and >300 cm, could compromise the goals of land reclamation, especially if the disturbance persists in the soil after it has been used as a reclamation substrate.

II. The results of my study differ from the dominant "shelf-life" ideas (i.e. that soil desirable characteristics deteriorate with increasing stockpile storage time), since:

I). In general, alpha diversity metrics did not seem to be reduced by soil storage time.

2). There was not a linear progress of the dissimilarities between stockpiled and reference soils with increasing stockpile age.

3). The dissimilarities between prokaryotic microbial communities of stockpiles and reference soils increased for younger and older stockpiles but decreased for intermediate-age stockpiles.

IV. The shift in fungal communities generated by stockpiling also impacts fungal functions. The endomycorrhizal fungi (i.e., ericoid mycorrhiza, arbuscular mycorrhiza, and orchid mycorrhiza) were more abundant in the younger stockpiles, which may indicate that these guilds are associated with the promotion of the proliferation of invasive plant functional groups in the stockpiled soils. Similarly, the shift from ectomycorrhizal fungi in the

reference soils to saprotrophic fungi in the stockpiles may have serious implications for the carbon degradation and release of greenhouse gasses from these soils.

V. The influence of environmental filters in the structuring of the microbial communities of the stockpiles is relatively low indicating that the harsh or extreme conditions in stockpiles may not be as influential as expected for the variability and assembly of the microbial communities in the stockpiles. Conversely, factors related to population size, functional redundancy, or physical and physiological barriers may be responsible for the variability in the microbial communities of the stockpiled soil. My results thus reveal a higher load of the variability on the disturbance generated by the soil management (i.e., vegetation removal, stripping, mixing, compacting), than in the conditions generated as a result of stockpiling.

5.3 Technology transfer

I. To reduce the impact of stockpile depth on microbial communities, I suggest:

1). For new stockpiles, limit the height of the stockpiled soil to 80 cm.

2). If stockpiles higher than 80 cm are generated, then I recommend the re-spreading and mixing of the piles to avoid compaction and improve aeration in the deeper stockpile layers.

3). If existing stockpiled soil will be used in reclamation, then I suggest not employing the soil below the ~80 cm depth.

II. My results indicate that prokaryotic microbial communities of stockpiles recover to resemble the communities found in undisturbed reference soils after a “rest period” of 5-7 years of topsoil storage. Therefore, we suggest that stockpiled soil should not be used in reclamation during the early storage period (~2 years old), instead, the soil should remain stored and be used once the rest period (~ 5-7 years old) has been achieved. In doing so,

the results of reclamation should not be different (in terms of prokaryotic communities) than those obtained when reclamation is done using fresh soil.

III. My results show that stochastic factors likely associated with soil management and stockpiling practices are more influential in the shift of microbial communities and their functions. Therefore, we suggest modifying the stockpiling practices to reduce the disturbance generated by the mechanized handling and stripping, to mitigate the drastic shifts suffered by the microbial communities of the soil.

IV. Microbial diversity and the microbial taxa that were significantly higher in stockpiled soils were correlated with the conditions that characterized the stockpiling disturbance. Therefore these taxa and diversity metrics should be integrated into soil integrity indexes as efficient indicators of soil health. This may serve to determine the condition of a determined stockpiled soil to be applied in reclamation or to shed light on the outcomes of reclamation using stockpiled soils as a substrate.

V. Vegetation was found to be influential for the structure of the microbial groups profiled as indicators of disturbed and non-disturbed conditions. Therefore we suggest reclamation companies promote the growth of native vegetation on the stockpiled soils to mitigate the impact of stockpiling on microbial communities.

VI. The microbial taxa found to be correlated with the stockpiling disturbance were also predicted by the increase in soil nutrients (e.g. carbon and nitrogen), therefore we suggest reclamation specialists be careful at the time of adding nutrient-rich material and fertilizers to stockpiled soils, since doing so may promote the growth of the undesirable microbial taxa that characterized the disturbance in the stockpiled soil.

5.4 Future research

I. I examined the impact of stockpiling on soil bacterial, archaeal, and fungal communities, revealing the influence of environmental factors and assembly processes on the structure and variability of these communities. Few prior studies have addressed the influence of soil stockpiling on microbial communities when these soils have been used in land reclamation of disturbed ecosystems like the post-mining sites. Further studies should assess if the patterns described here are maintained after the use of stockpiled soils in reclamation and how these patterns affect the trajectories of reclamation in the disturbed ecosystem. Considering that soils are stockpiled to be used in reclamation, the insights generated would serve to evaluate the relevance of my findings relative to reclamation practices.

II. My results indicate that prokaryotic microbial communities in stockpiles require a "rest period", after which bacterial communities in the stockpile recover to resemble the communities found in undisturbed reference soils. This finding led me to suggest the retention of stockpiled soil until this "rest period" is achieved. However, I did not find a similar recovery pattern of the fungal communities, or the fungal functional groups assessed. Previous studies showed the recovery of bacterial communities in the stockpiles within 2-3 years of storage (Gorzelak et al., 2020) and the fungal communities and some of their functional groups within 5-10 years (Birnbaum et al., 2017; Gorzelak et al., 2020; Jasper et al., 1987). Therefore, the time required to achieve the recovery of the microbial communities may depend on the stockpiling practices and environmental or geographic conditions or could vary depending on the microbial group accessed. Future studies should assess the universality of the rest period for stockpiled soil microbial communities and

determine the predictors behind it. Also, prior studies showed a stockpile plant community more closely resembling native plant communities from undisturbed sites following the "rest period". Thus, the correlation between the plant and microbial communities' recovery when the rest period has been achieved should be analyzed by further studies.

III. Stochastic factors were primarily responsible for the structuring of microbial communities and the most important assembly process for prokaryotes is dispersal and for fungi is drift. In the case of the fungal communities, the results reveal that even when their assembly is mainly influenced by drift, the mycorrhizal communities are largely driven by dispersal. These findings indicate that assembly factors influencing the structure of the communities based on their taxonomy are different from those driving the community in terms of their function. Further research should assess the processes driving the prokaryotic communities based on the roles they play in the soil ecosystem, like those involved in nitrogen, phosphorus, and sulfur cycles, as well as litter decomposition. The knowledge generated from the proposed research would help to understand the factors involved in soil functional turnover and their consequences for ecosystem services and multi-functionality.

IV. My study has shown that the most influential taxa in the structure of the microbial communities in undisturbed soils were fungi, but some of these key taxa were displaced by bacterial taxa after soils had been stockpiled. Further studies should be conducted to generate insights into the factors and assembly processes behind the shift in the key microbial taxa and to determine if the shift is maintained with increasing stockpile depth and storage time.

V. Compared to the deep soil layers (80-90 cm and >300 cm), the surface stockpile layers (0-10 cm, 10-20 cm, and 20-30 cm) seem to be less affected by the stockpiling-associated

disturbances in terms of composition, diversity and fungal putative functions. These surface soils should then be experimentally tested as inoculant of blended reclamation soil, to improve the outcomes of land reclamation.

5.5 Potential limitations

a) Compared to Bacteria and fungi, the relative abundance of the archaeal ASV was extremely low, which could have decreased the robustness of some of the analyses regarding the impact of stockpiling on archaeal communities.

b) My inferences on fungal guilds and trophic modes were not based on analysis of functions determined or observed by each of the groups studied, these functions were instead inferred based on the guilds to which fungal communities were assigned by the Funguild annotation database. Even when the precision of the database is generally acknowledged and validated (Nguyen et al., 2016), we lack precise information about the functions played by the communities at the time of sampling.

c) The results regarding the >300 cm stockpiles should be interpreted with caution, since samples for these soil layers (and for the 10-20 cm), were not sampled in triplicate, unlike for the other sample depths, which could reduce the statistical power of the tests performed or led to incorrect inferences regarding the communities in these soil layers.

d) My analyses are based on the amplified marker gene sequences (16s rRNA and ITS) classified into ASV (amplicon sequence variants), due to the higher resolution and sensitivity to errors of the ASV approach, compared to the OTU (operational taxonomic unit) clustering (Callahan et al., 2016). However, recent studies have indicated that the ASV approach may classify marker gene sequences from the same genome as different taxa (Schloss, 2021), or remove rare taxa (Deng et al., 2024). I experimentally tested the

alpha and beta diversity metrics for the prokaryotic communities using the OTU clustering approach, and the patterns obtained did not differ from those presented here. However, I acknowledge that the issues reported above for the ASV approach could have affected the alpha diversity metrics (diversity, evenness, and richness) of the communities or potentially inflated the relative abundance of the ASV.

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Appendices

Appendix A

Supplementary Information for Chapter 2

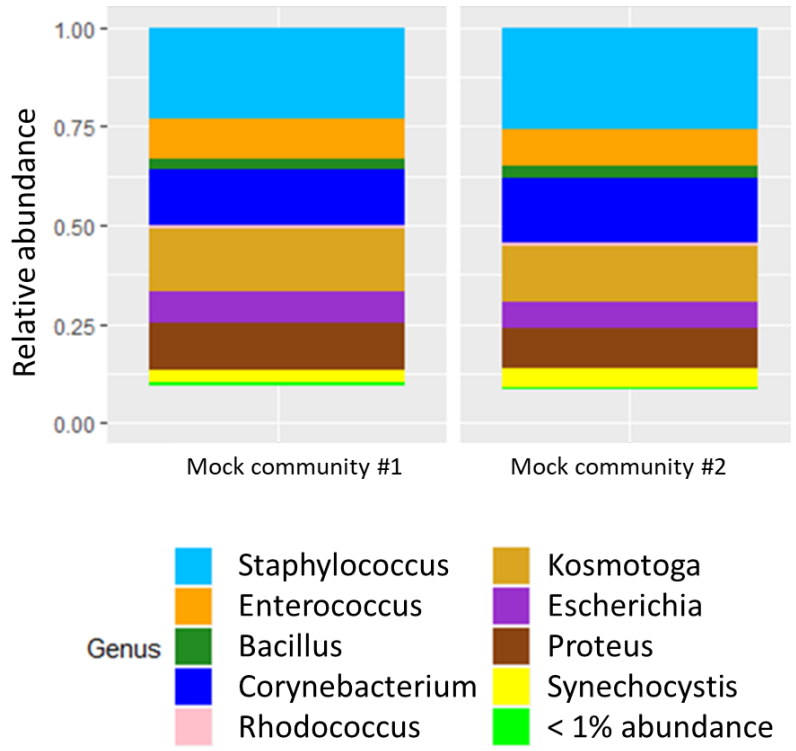

Figure S2-1. Relative abundance of the ten bacterial genera in the lab-constructed mock community. The proportions and identities of the bacterial groups detected by the analyses in the mock community met the expectations in terms of the amount of DNA plated of each bacterium.

Table S2- 1. List of the microbial taxa detected in the negative controls

Lactobacillus	Methylomicrobium	Methylobacter
SUP05_cluster	Staphylococcus	Ralstonia
Faecalibaculm	Escherichia/Shigella	Enterococcus
Pseudolabrys	Candidatus_Udaeobacter	Bacteroides
Faecalibacterium	Ruminococcaceae	Rhodoferax
Akkermansia	Bradyrhizobium	Corynebacterium_1
Mesorhizobium	Cloacibacterium	Sphingomonas
Candidatus_Arthromitus	Alistipes	Kosmotoga
Flavobacterium	Butyricicoccus	Massilia
Erysipelatoclostridium	Hydrogenophilus	Blautia
Pseudarthrobacter	Klebsiella	Hyphomicrobium
Rikenellaceae	Clostridium	Proteus
Romboutsia	Pseudoxanthomonas	Ellin6067
Bifidobacterium	Terrimonas	Galbitalea
Caulobacter	Paeniclostridium	Granulicella
Odoribacter	Agathobacter	Tyzzerella_3
Arenimonas	Pedobacter	Lachnoclostridium
Candidatus_Solibacter	Bryobacter	Anaerotruncus
Methanobacterium	GCA-900066575	GOUTB8
Sneathia	Aquicella	Roseobacter
Pseudomonas	Rhodanobacter	Muribaculum
BSV13	Elev-16S-1166	43911
Gaiella	Bauldia	Xylophilus
Parasutterella	Prevotella	Gemmatimonas
Ruminiclostridium_5	SWB02	Pelosinus
Bilophila	Arcobacter	Acidothermus
Pedosphaera	Subgroup_10	Prevotella
Geobacter	Desulfomicrobium	Weissella
Bdellovibrio	RB41	
Brevundimonas	Vibrio	

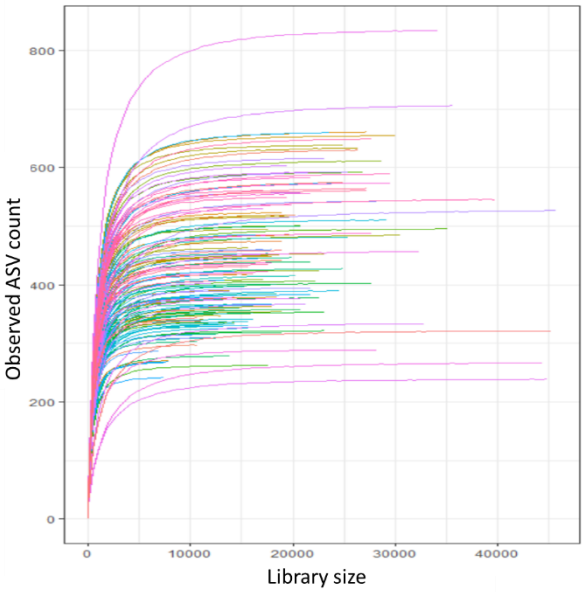

Figure S2-2. Rarefaction curve of the samples analyzed in the study. After the rarefaction, the size of the libraries was set to 7420 reads per sample.

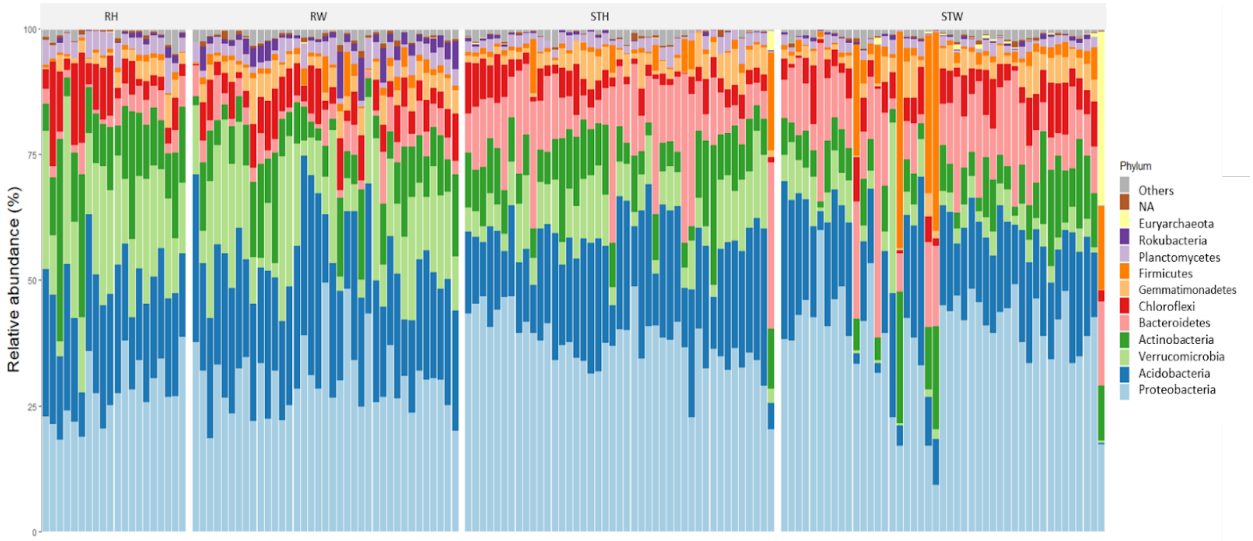

Figure S2- 3. Microbial community composition of the stockpiles and the reference soils. The bars indicate the relative abundance of the most representative phyla in the studied systems. RH: reference site Horizon, RW: reference site Wolf Lake, STH: Stockpile Horizon, STW: Stockpile Wolf Lake.

Table S2-2. Analysis of dissimilarities (ANOSIM), comparing microbial communities of the stockpiles and reference soils according to the soil storage time. Younger (0.5, 1.5, and 2 years), Intermediate (5 and 7 years), Old (11 and 28 years), Rf = Reference (undisturbed soils).

Soil type	R-score	P-value
Reference vs Younger	0.437	<0.01
Reference vs Intermediate	0.087	<0.01
Reference vs Old	0.524	<0.01

Table S2-3. Analysis of dissimilarities between the surface (0-10 cm and 10-20 cm) and depth layers (80-90 cm and >300 cm) of the stockpiles according to their age. The p-values for both ANOSIM and PERMANOVA are shown.

Stockpile ID	Age	R-score	p (Anosim)	p (Permanova)
4J	0.5	-0.126	0.716	0.655
5J	1.5	0.220	0.026	0.015
PS4	2.0	0.780	0.001	0.001
4S	5.0	-0.153	0.838	0.880
5S	7.0	-0.043	0.620	0.705
PE	11.0	0.233	0.07	0.141
WL	28.0	0.413	0.12	0.002

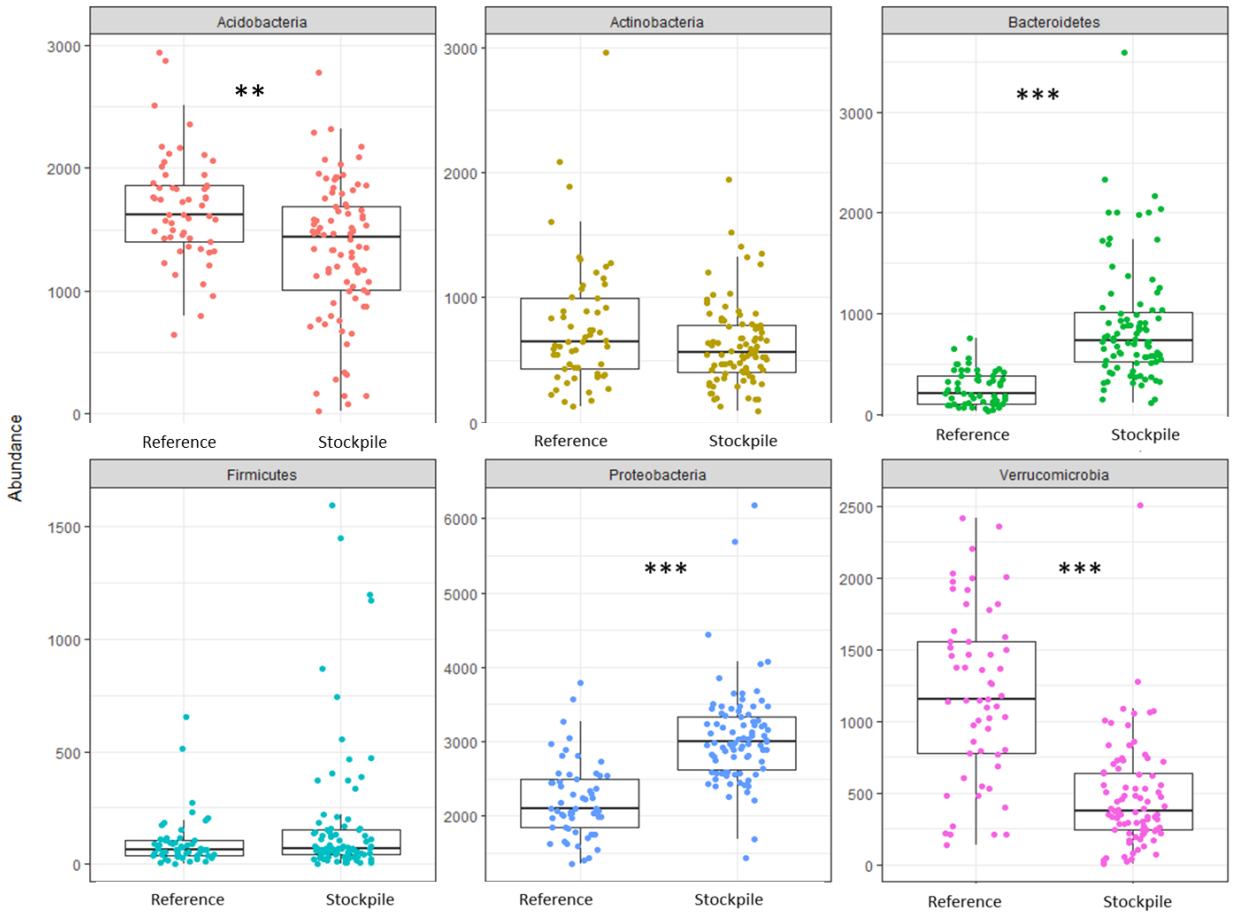

Figure S2- 4. Abundance of the 6 dominant bacterial phyla in the stockpiles and the reference soils. The asterisks indicate the significance of the difference between the two groups: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

Table S2- 4. Analysis of dissimilarities (ANOSIM), comparing the microbial communities in the equivalent depth layers of stockpiles and reference soils.

Stockpile vs Reference	R-score	p-value
Surface stockpile vs surface reference	0.337	<0.001
Deep stockpile vs Deep reference	0.340	<0.001

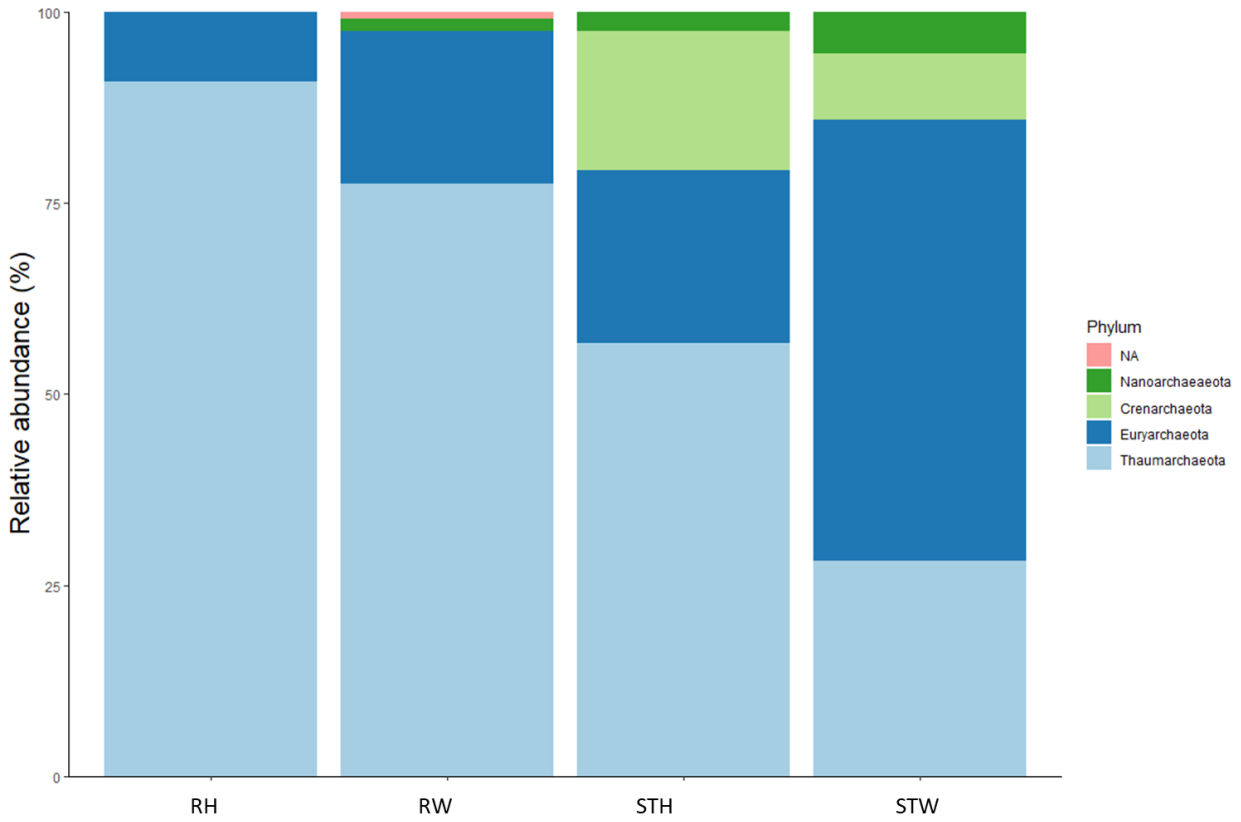

Figure S2- 5. Relative abundance of the archaeal phyla in the stockpiles (STW = Wolf Lake and STH = Horizon) and reference soils (RW = Wolf Lake and RH = Horizon).

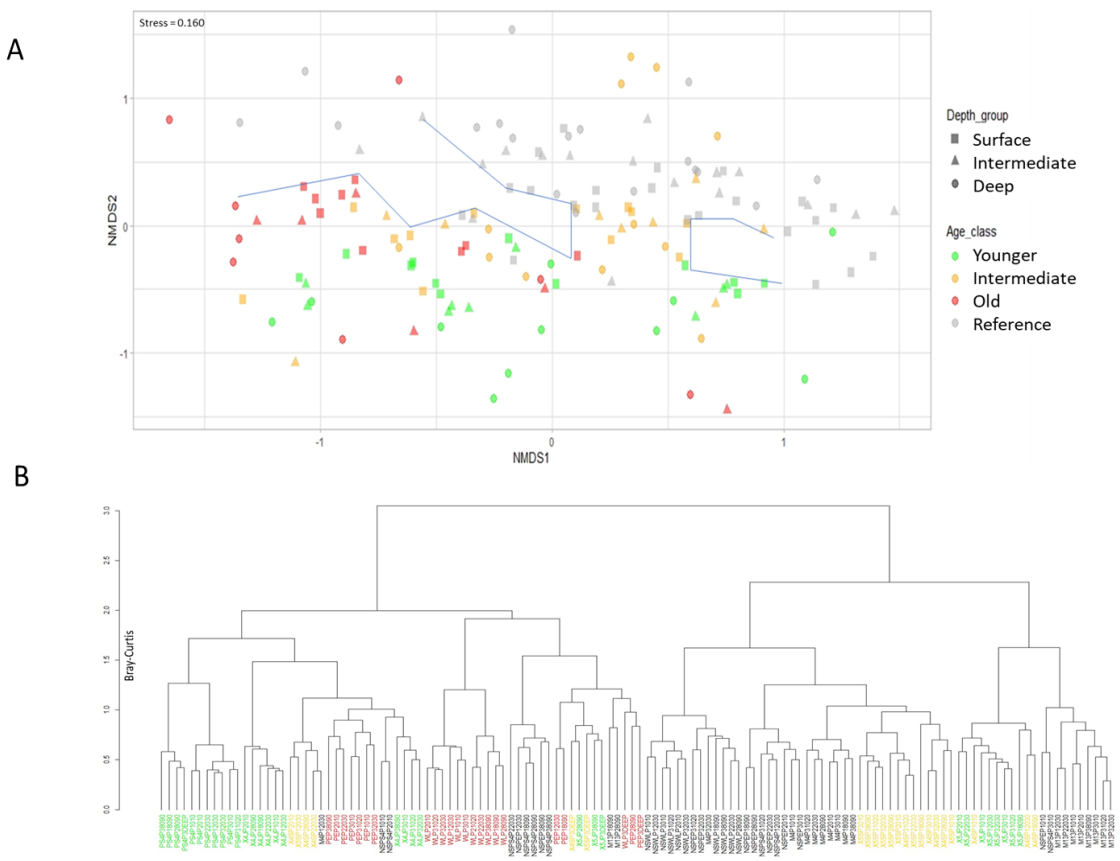

Figure S2- 6. Comparison of microbial communities of the stockpiles and the reference soils. A: NMDS ordination showing the dissimilarities (Bray Curtis) between the microbial communities of the stockpiles and reference soils, considering the effect of three defined age categories and five depth groups. B: Cluster analysis comparing the microbial communities of the stockpiles according to their age and the reference soils. For both figures (i.e., A and B), the contrasted age categories are: Younger (0.5, 1.5, and 2 years), Intermediate (5 and 7 years), and Old (11 and 28 years). The depth groups contrasted for figure A, are: Surface (0 -10 cm, 10- 20 cm), Intermediate (20 - 30 cm), and Deep (80-90 cm and > 300 cm).

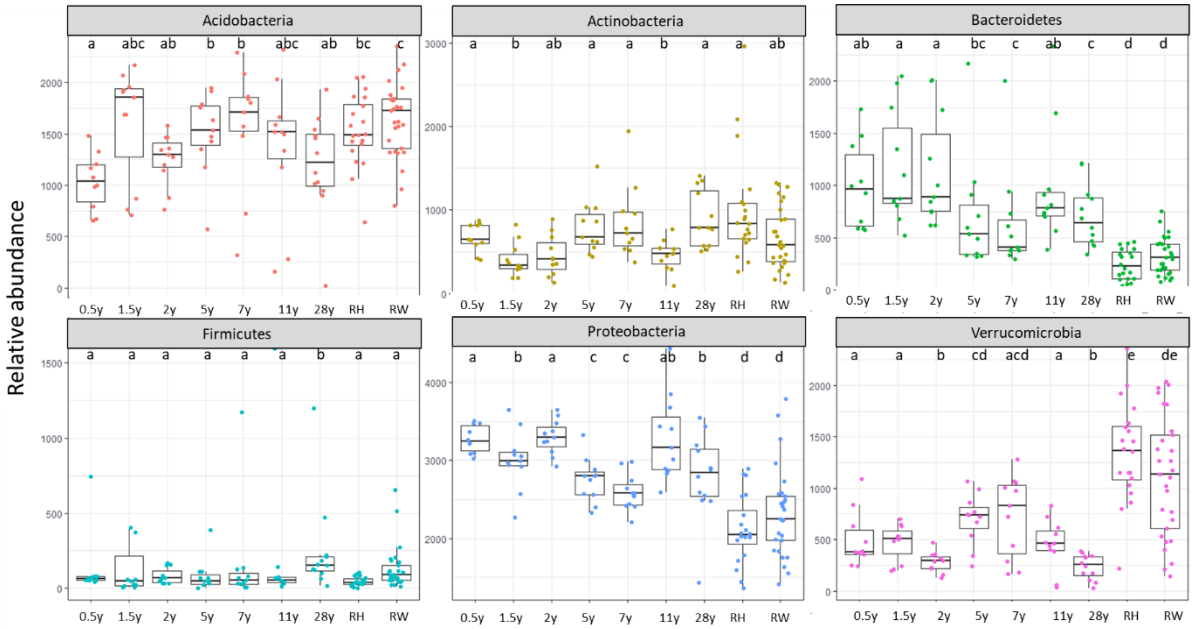

Figure S2- 7. Abundance of the 6 dominant bacterial phyla according to stockpile age. Different letters at the top of the boxplots indicate the significance of the difference ($p \leq 0.05$) between the age groups. The reference soils are represented by RH (reference Horizon) and RW (reference Wolf Lake).

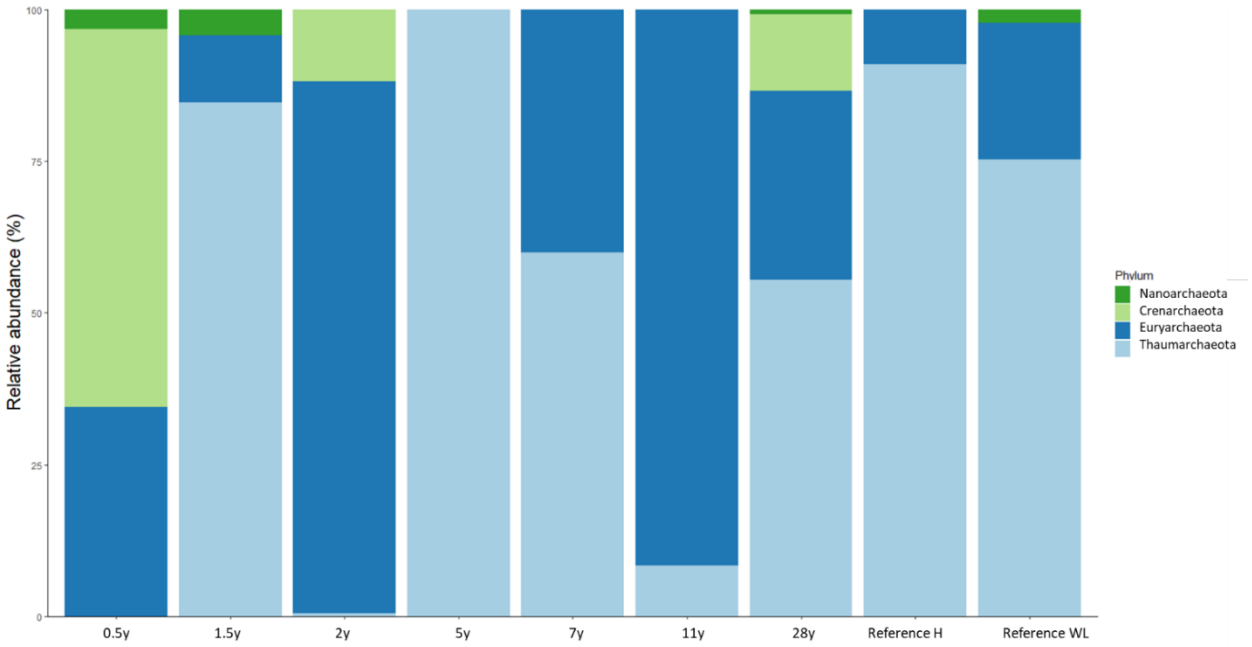

Figure S2- 8. Relative abundance of the archaeal phyla according to the age of the stockpiles and reference soils (RW = Wolf Lake and RH = Horizon).

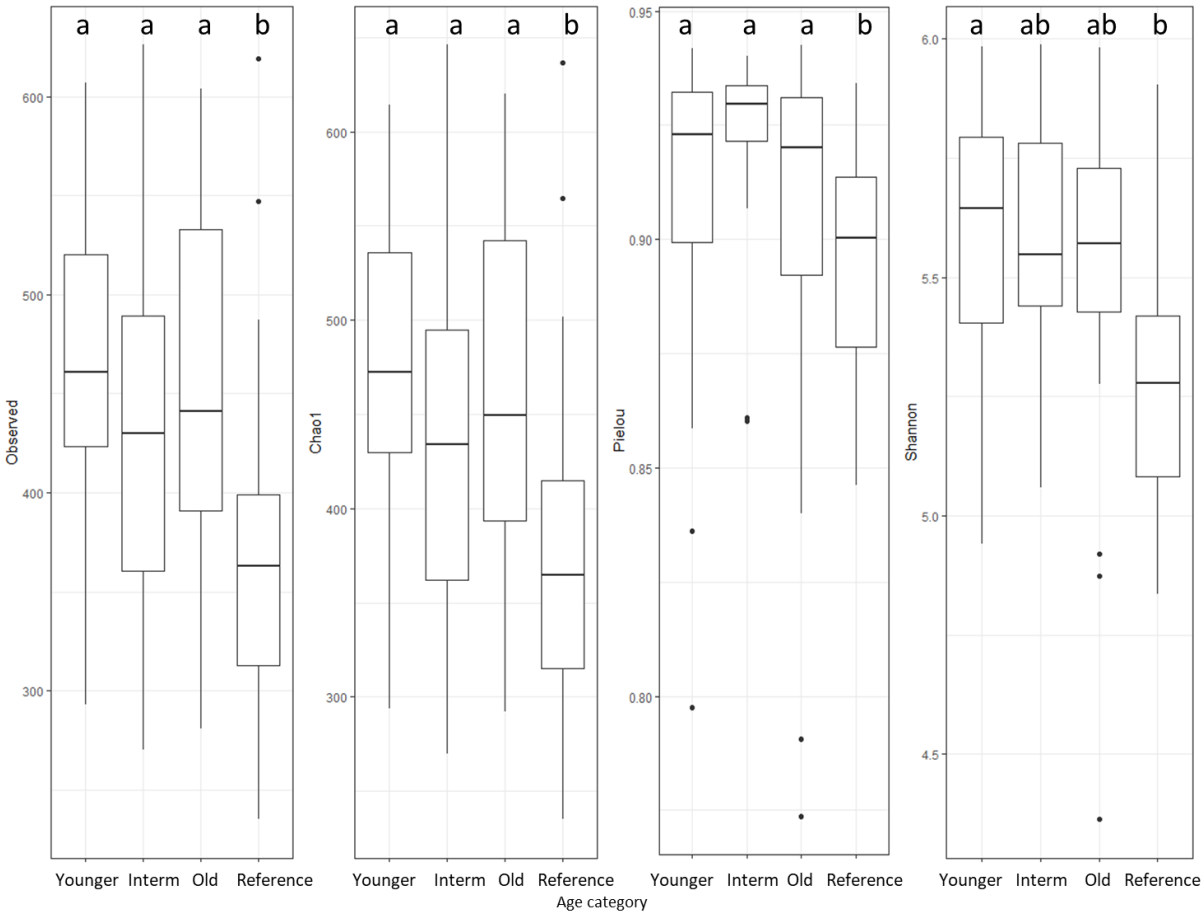

Figure S2- 9. Alpha diversity metrics of stockpiled soils according to their storage time. The groups are Younger (0.5, 1.5, and 2 years), Intermediate (5 and 7 years), and Old (11 and 28 years). The letters at the top of the boxplots refer to the significance ($p \leq 0.05$) of the dissimilarities among the diversity indexes in soil age groups.

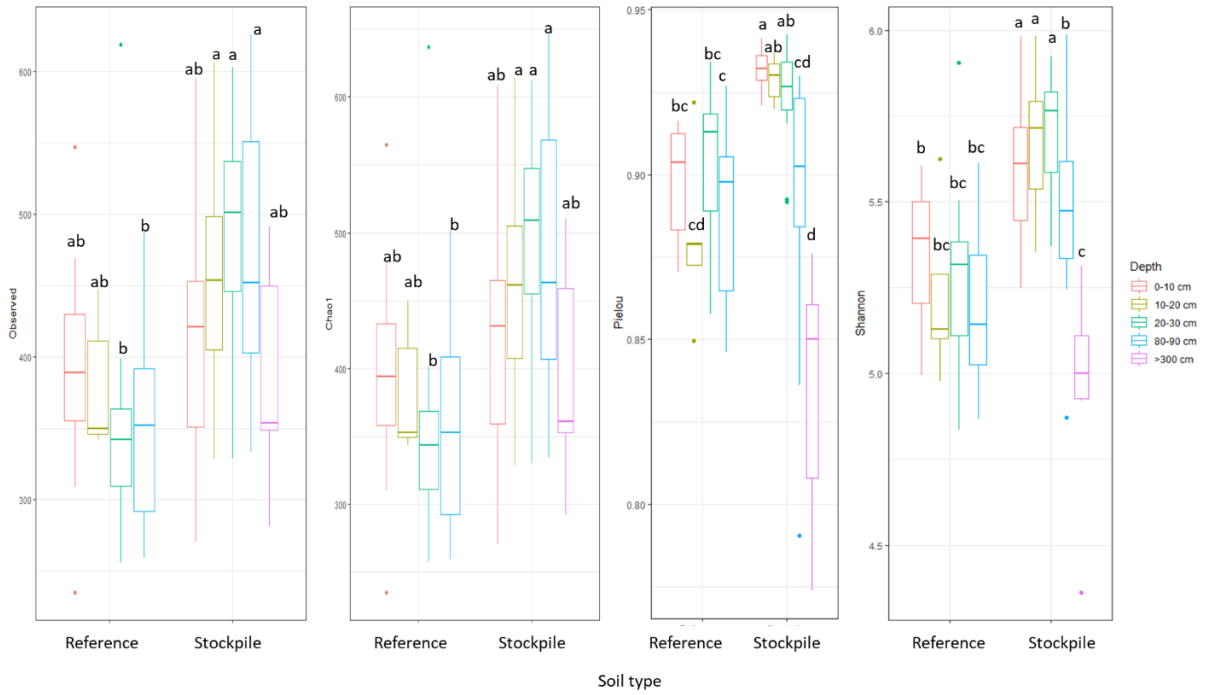

Figure S2- 10. Alpha diversity metrics for the different depths of stockpiles and reference soils. The significance ($p \leq 0.05$) of the differences in each of the metrics (ANOVA with Tukey's test), is indicated by the letters at top of the bars.

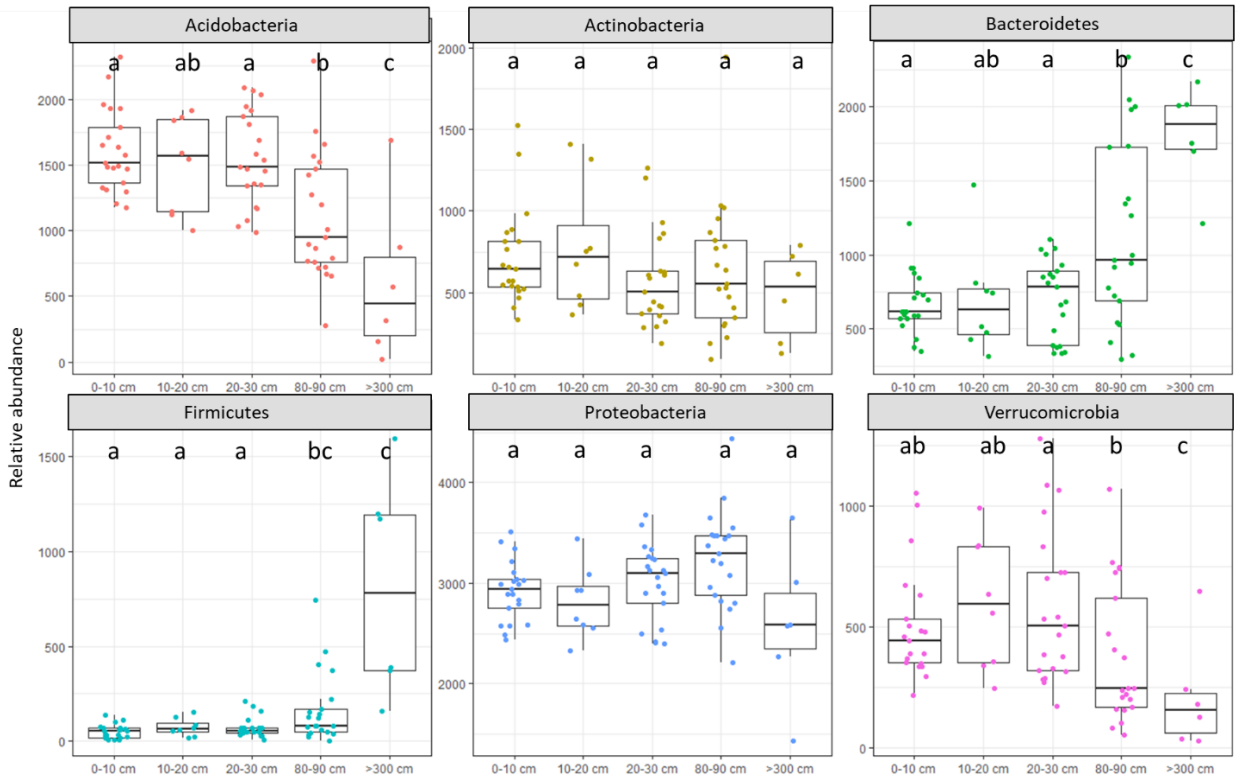

Figure S2-11. Relative abundance of microbial groups according to the stockpile depth. Significant differences ($p<0.05$) among soil depth layers are represented by different letters on top of the boxplots.

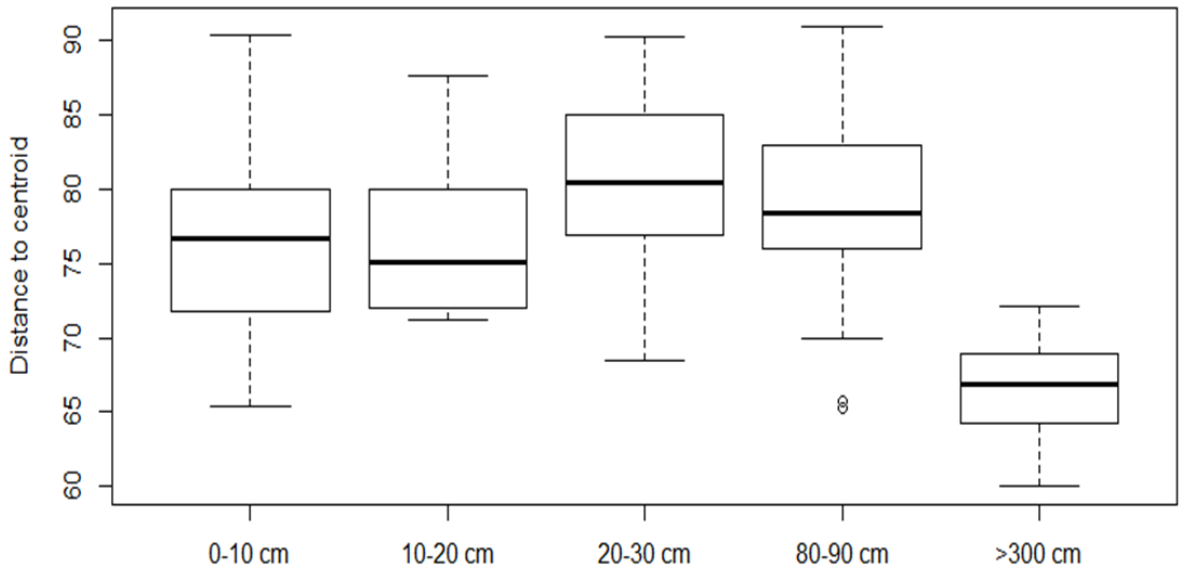

Figure S2-12. Dispersion of microbial communities across different stockpiled soil depths. The variability among microbial communities is represented as larger distances to the centroid on the y-axis.

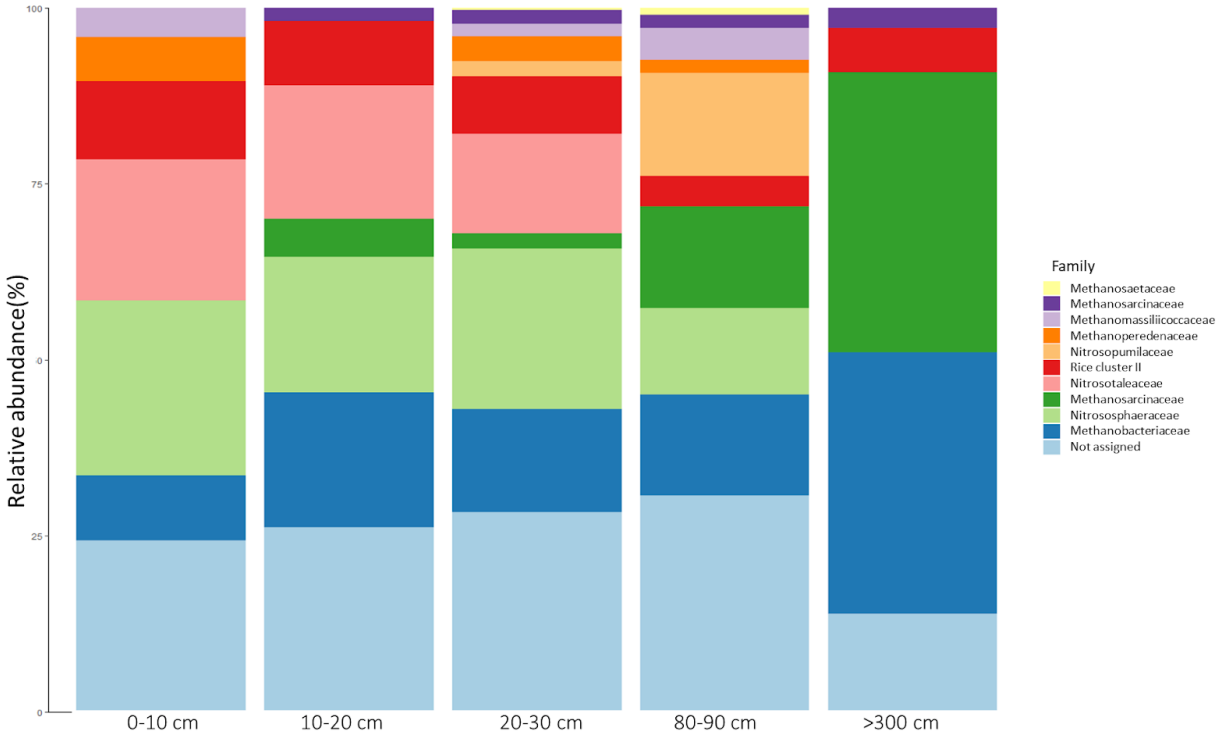

Figure S2-13. Relative abundance of the main archaeal families according to the depth layers of the stockpiled soils.

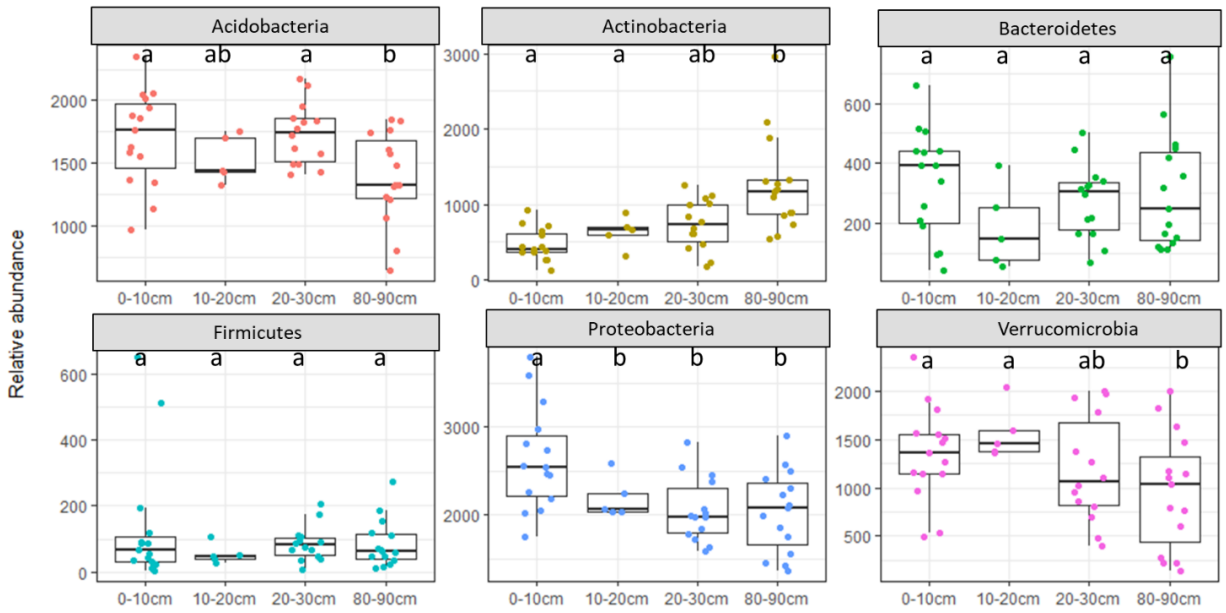

Figure S2-14. Relative abundance of microbial groups in the depth layers of the reference soils. Significant differences ($p<0.05$) among soil depth layers are represented by different letters on top of the boxplots.

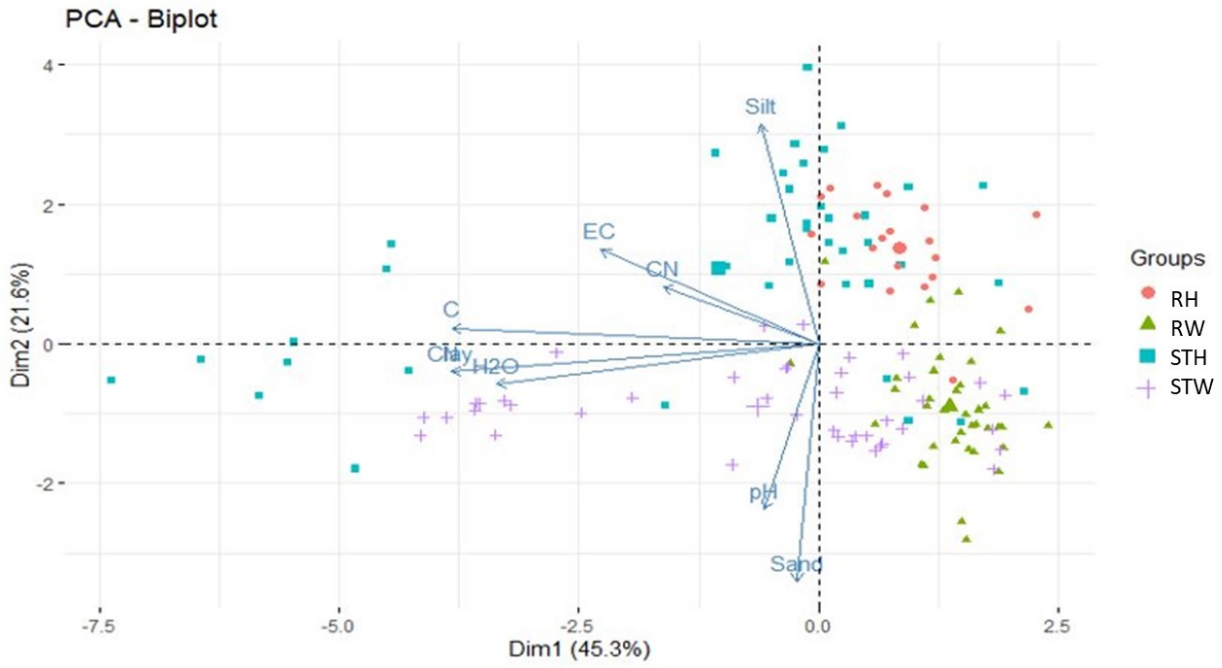

Figure S2- 15. PCA ordination plot showing the dissimilarities of soil samples according to their chemical and physical parameters. RH: reference site Horizon (Red circles), RW: reference site Wolf Lake (Green triangles), STH: Stockpile Horizon (Blue squares), STW: Stockpile Wolf Lake(Purple crosses).

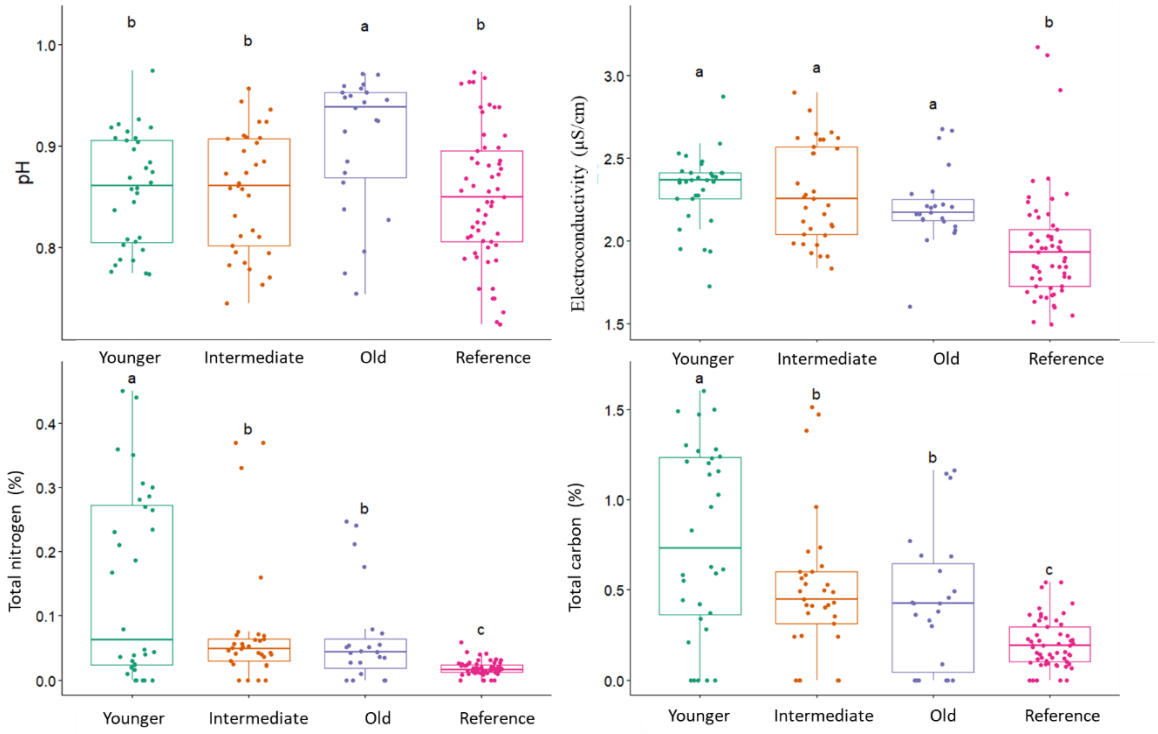

Figure S2- 16. Soil abiotic factors measured in stockpiled soils of different ages and their reference counterparts. The stockpiled soils were grouped in the following categories Younger = 0.5, 1.5- and 2.0-years old stockpiles, Intermediate = 5.0- and 7.0-years old stockpiles, and Old = 11- and 28-years old stockpiles. The letters at the top of the boxplots refer to the significance ($p \leq 0.05$) of the dissimilarities among the soil age groups.

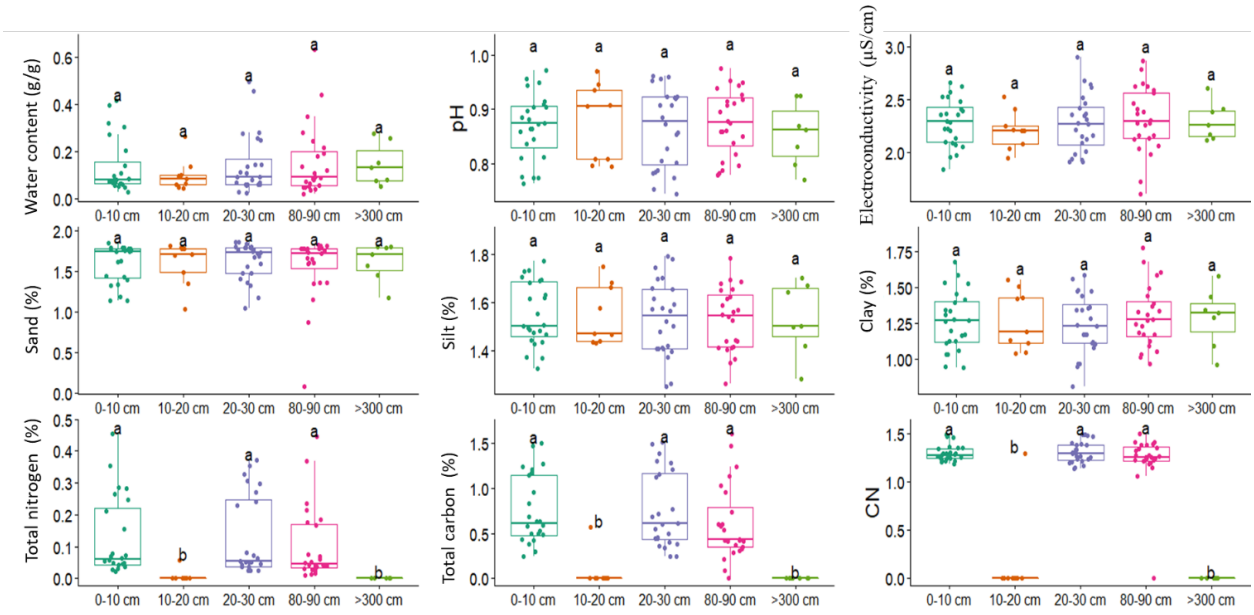

Figure S2-17. Soil abiotic factors measured at different depth layers of the stockpiled soils. The letters at the top of the boxplots refer to the significance ($p \leq 0.05$) of the dissimilarities among the soil age groups**.**

Appendix B

Supplementary information for Chapter 3

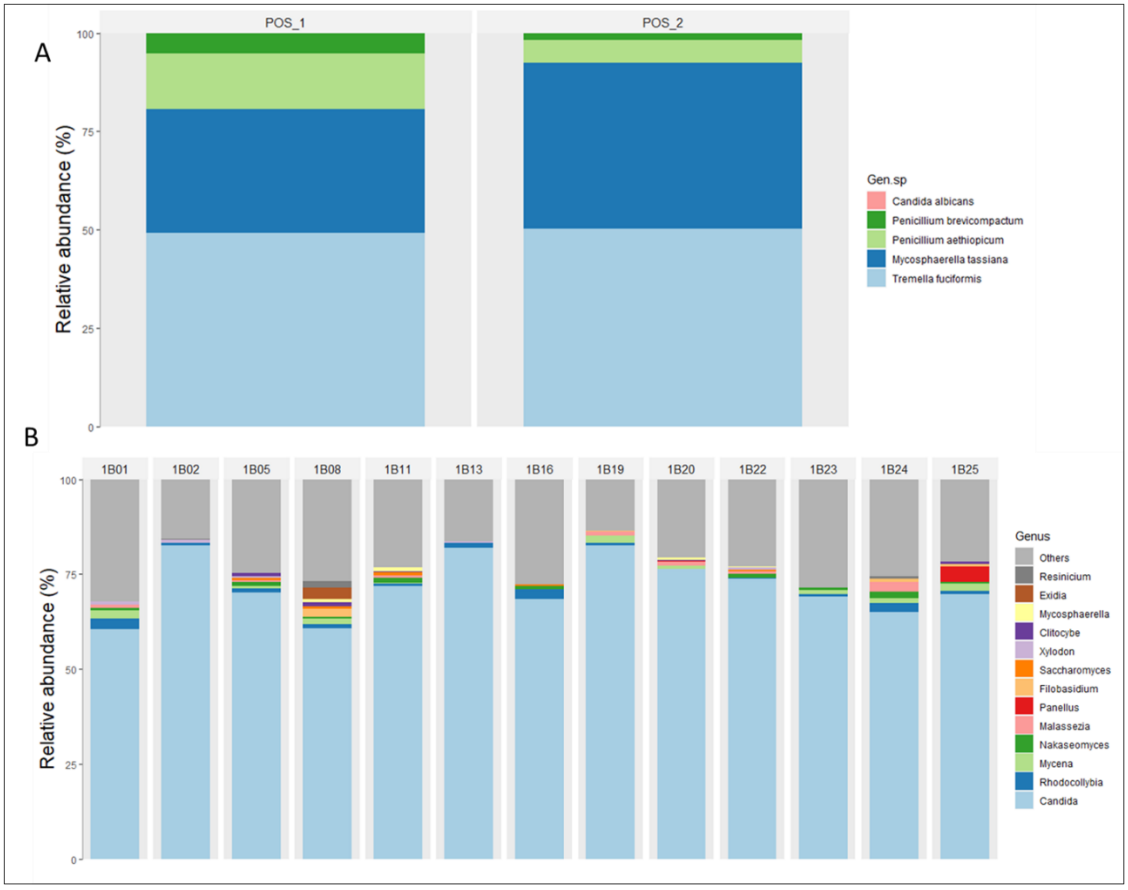

Figure S3-1. Relative abundance of fungi in (A)lab-constructed mock community, and (B)DNA extraction blanks. The fungal groups identified in the mock communities and their proportion corresponded with the quantity of DNA of each fungal group plated.

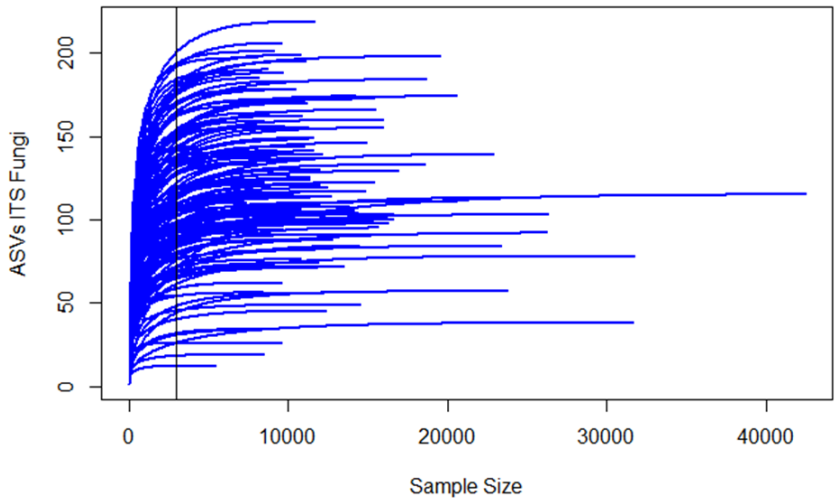

Figure S3-2. Rarefaction curve. Libraries were rarefied to 6,569 reads per sample (the lowest read count), using random subsampling.

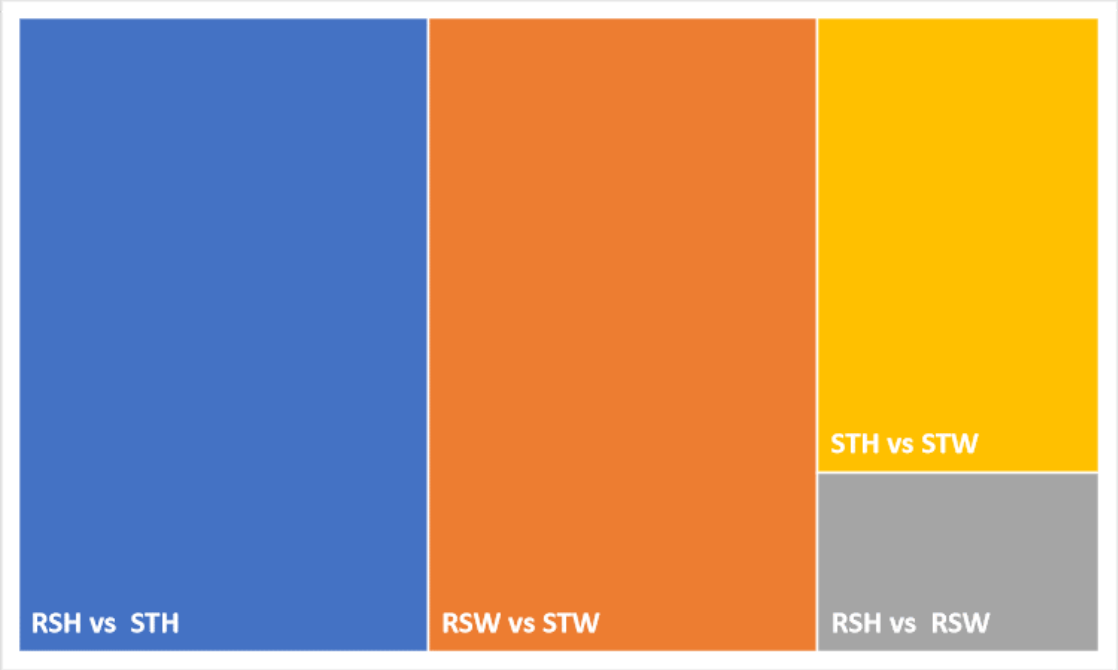

Figure S3-3. Results of the analysis of similarities (ANOSIM), contrasting the fungal communities of the abundance of the stockpiling sites (STH and STW) and the two sites used as reference (RSH and RSW). The size of the boxes indicates the degree of the dissimilarity (ANOSIM R-value) of the pairwise comparison.

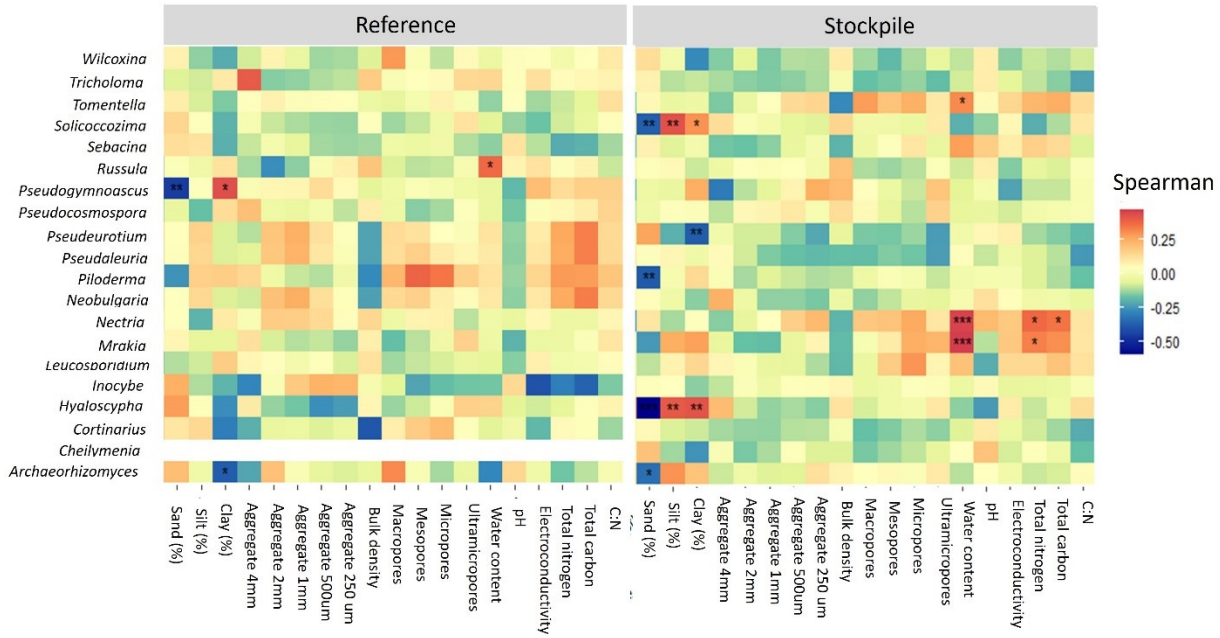

Figure S3- 4. Correlogram based on the Pearson correlation coefficient showing the edaphic factors and stockpile conditions associated with the indicator species that account for the differences between the communities of the stockpiles and the reference soils. The significance of the correlation between edaphic parameters and fungal groups is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

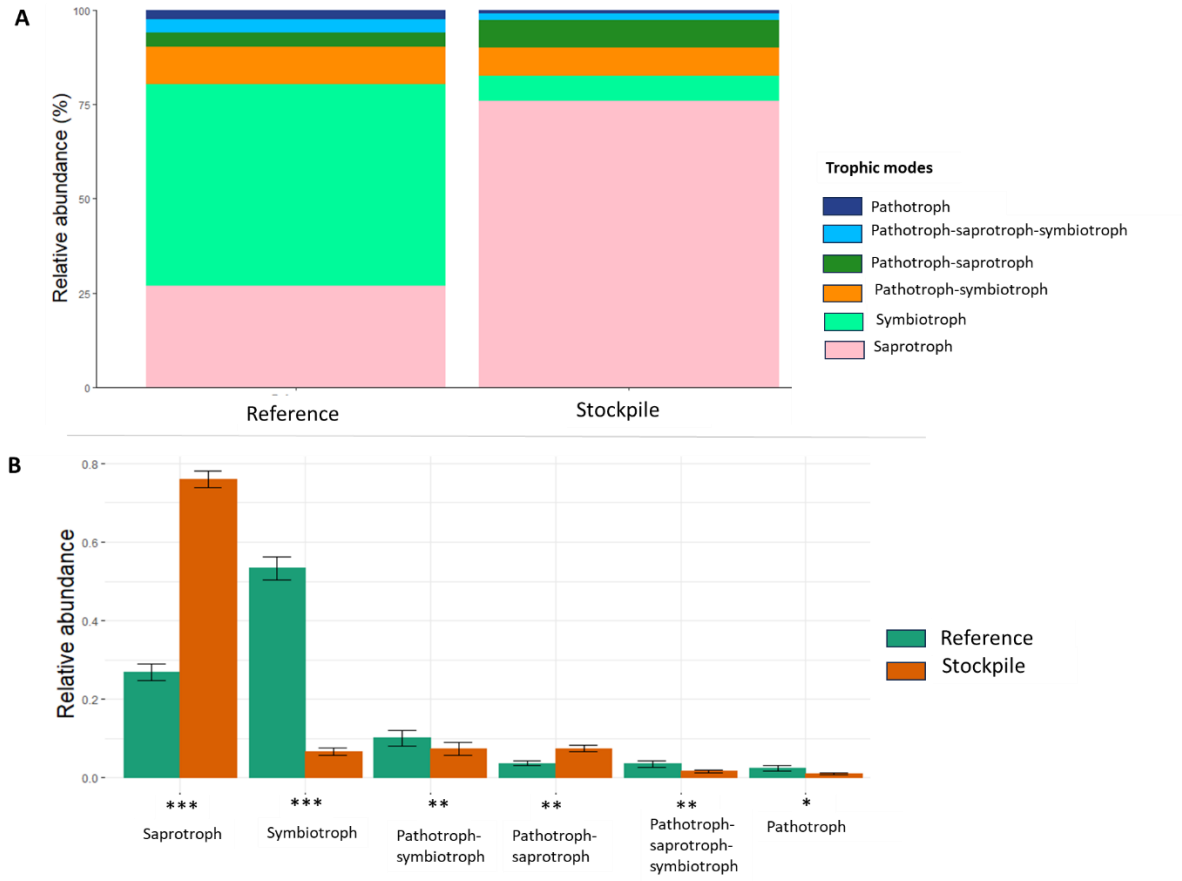

Figure S3-5. Fungal trophic modes in stockpiles and reference soils. A) Relative abundance of the main fungal trophic modes in the two soil types studied, and B) Significance of their abundance in stockpiles and reference soil. $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

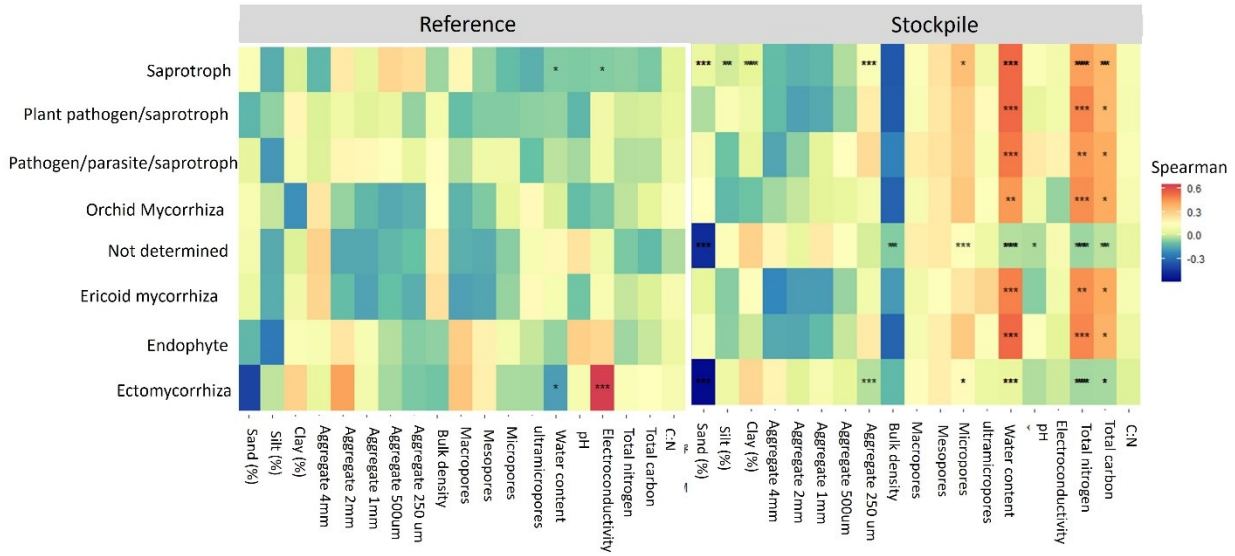

Figure S3-6. Correlogram representing the fungal guilds that were significantly correlated (Pearson) with the edaphic factors or stockpiling conditions. The significance of the correlation between edaphic parameters and fungal groups is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

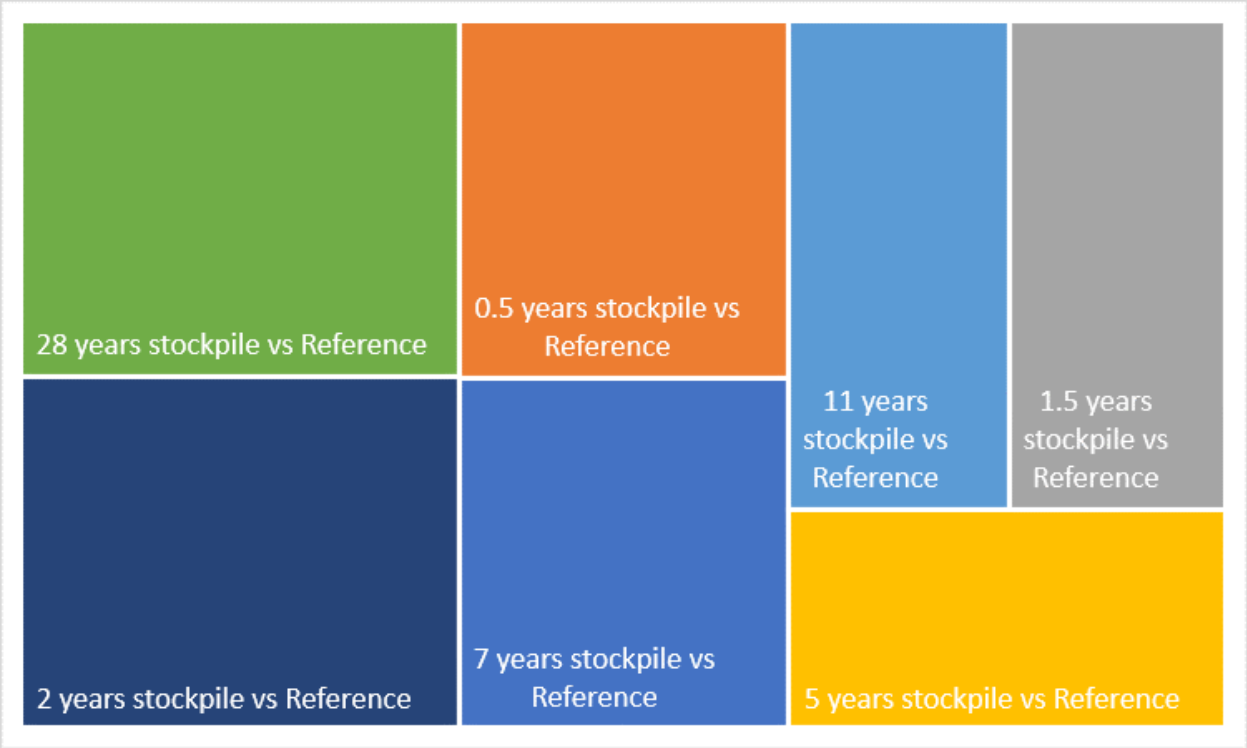

Figure S3-7. Results of the analysis of similarities (ANOSIM), contrasting the fungal communities of the stockpiles according to their age and their respective reference soil. The size of the boxes indicates the degree of the dissimilarity (ANOSIM R-value) of the pairwise comparison.

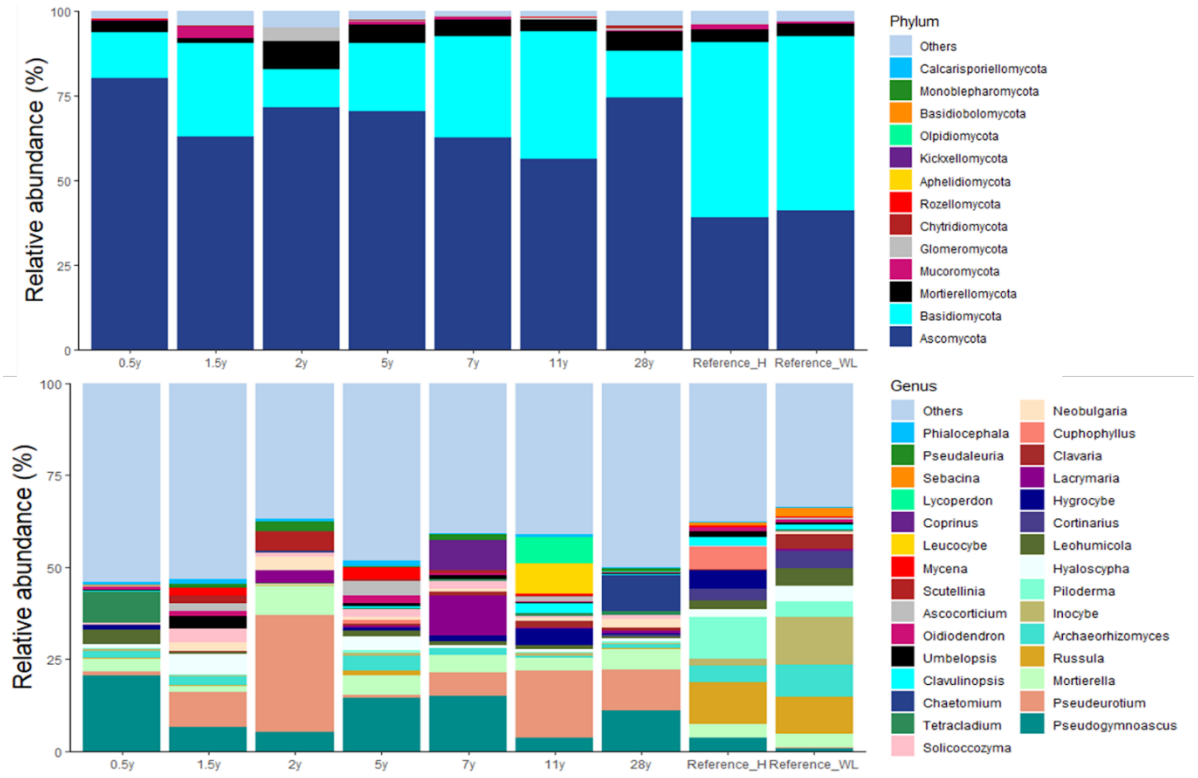

Figure S3-8. Relative abundance of the main fungal taxa according to the age of the stockpiles and contrasted with the reference soils (Reference WL= Reference Wolf Lake, Reference H= Reference Horizon).

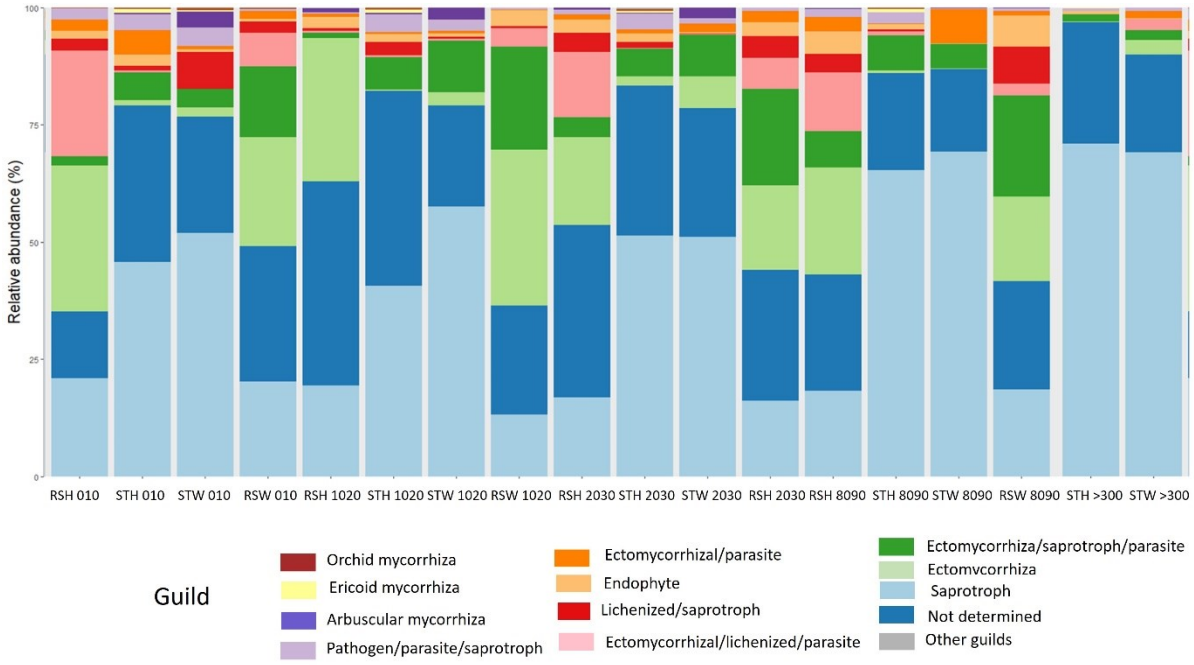

Figure S3-9. Relative abundance of the main fungal guilds according to the depth of the stockpiles and contrasted with the reference soils (STH = stockpile Horizon, STW = stockpile Wolf Lake, RSH = reference soils Horizon, RSW= reference soils Wolf Lake). The numbers at the right of the acronyms are the soil depth (in centimeters) sampled.

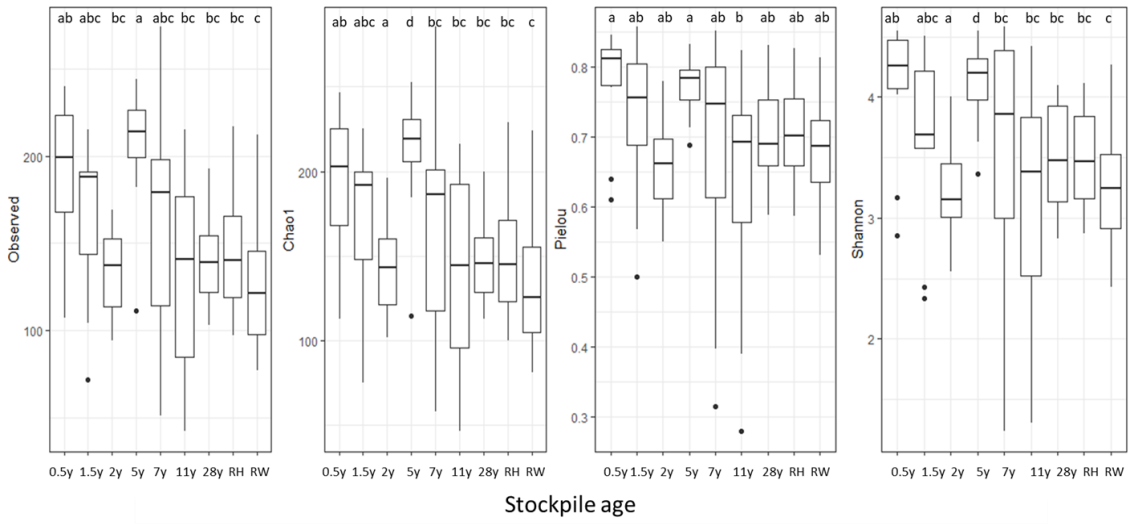

Figure S3-10. Alpha diversity indexes for the stockpiles according to their age. The alpha diversity indexes of the reference soils are also shown (RH=Reference Horizon, RW= Reference Wolf Lake). The letters atop the boxplots refer to the significance ($p \leq 0.05$) of the dissimilarities among the fungal diversity indexes according to soil age.

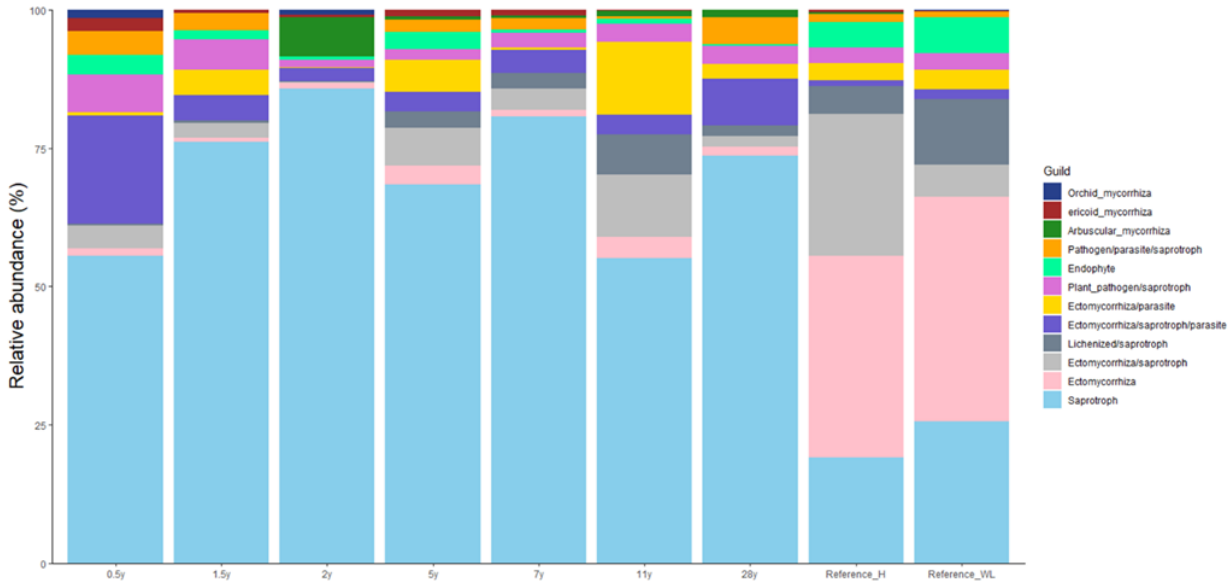

Figure S3-11. Relative abundance of the fungal guilds according to the age of the stockpiles and contrasted with the guilds found in the reference soils.

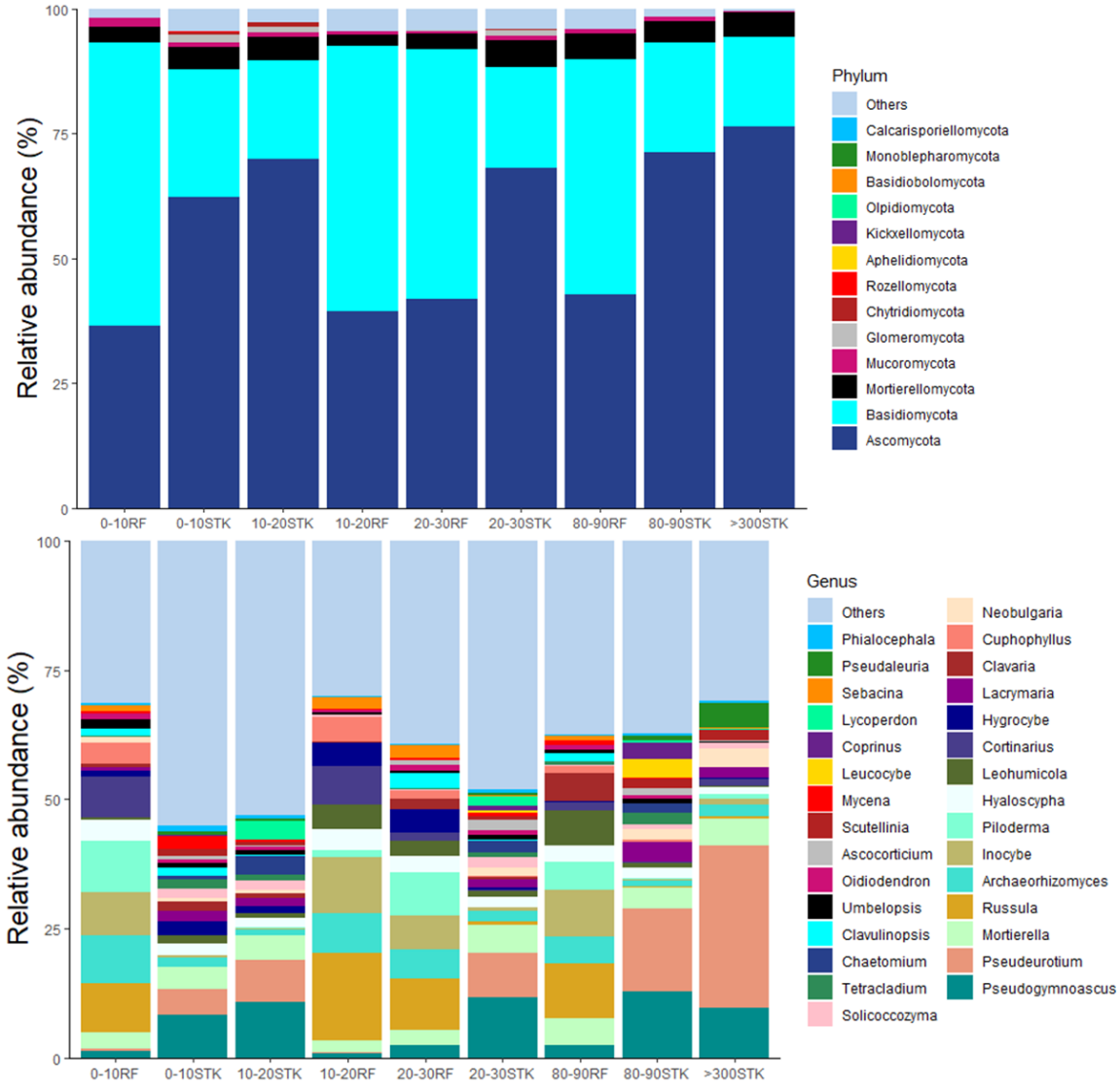

Figure S3-12. Relative abundance of the main fungal taxa according to stockpile (STK) and reference soil (REF) depth layers.

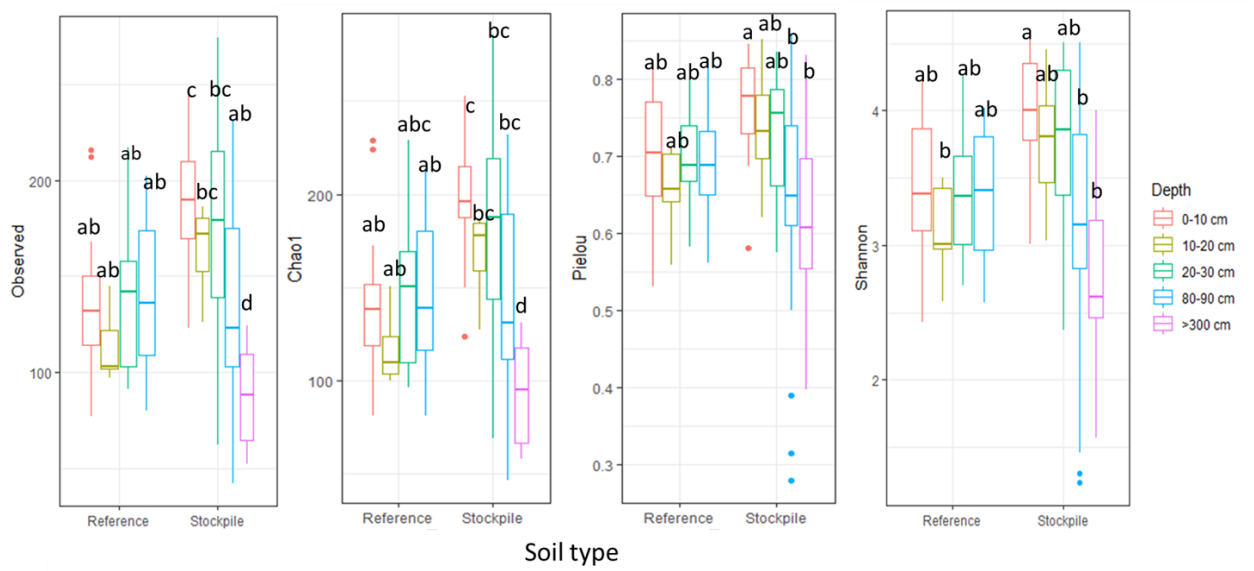

Figure S3-13. Alpha diversity metrics of the communities in the stockpiles and reference soil depth layers. The letters at the top of the boxplots refer to the significance ($p \leq 0.05$) of the dissimilarities among the fungal diversity indexes according to soil depth.

Appendix C

Supplementary information for Chapter 4

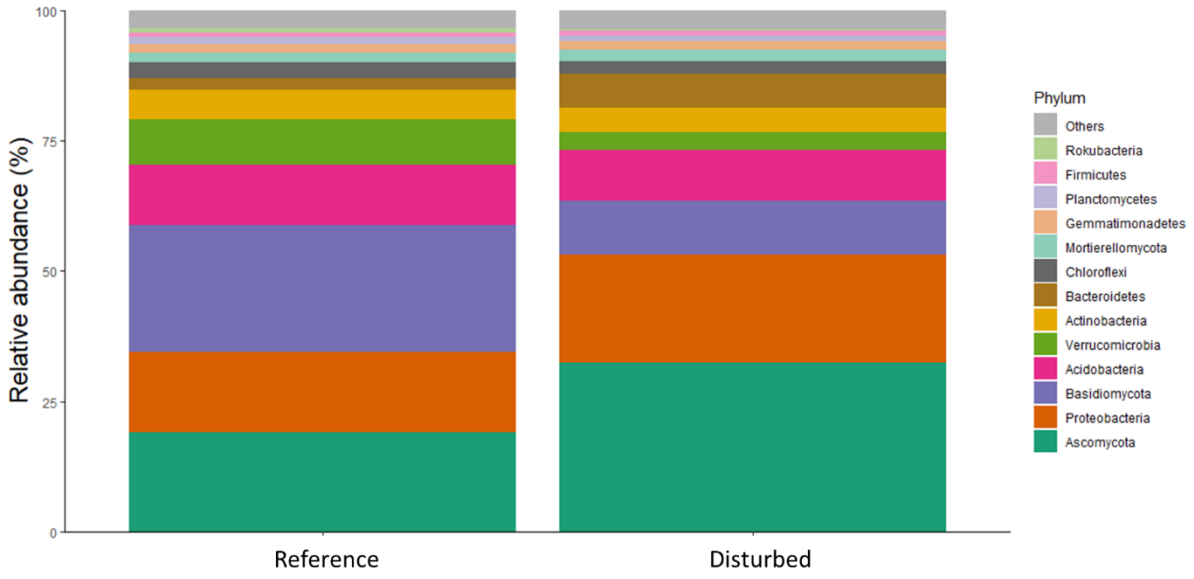

Figure S4-1. Relative abundance of the main microbial groups in the disturbed(stckpiles) and reference soils.

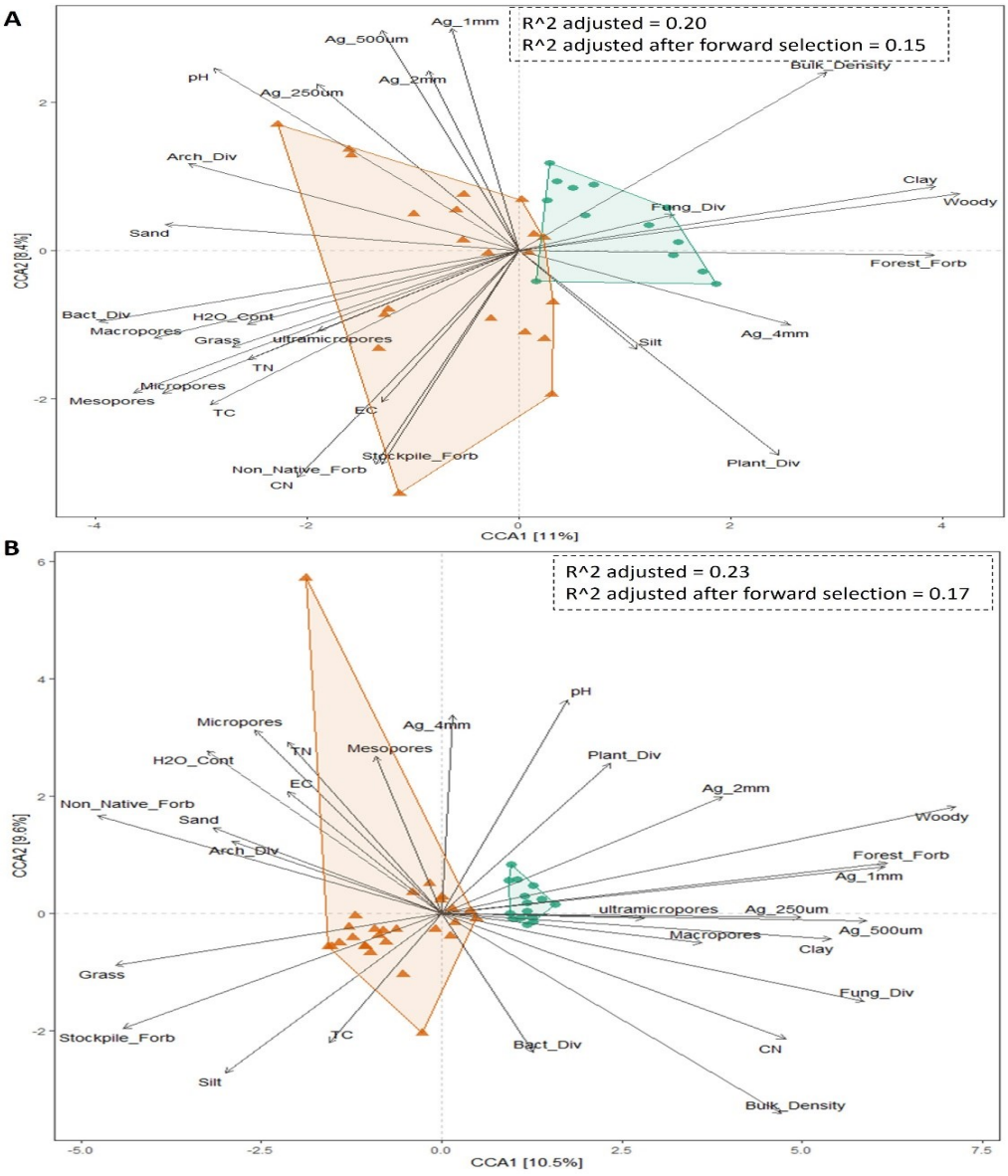

Figure S4-2. CCA ordination of factors explaining the variability of the microbial communities of the reference (green) and disturbed soils (orange). A, CCA ordination on communities in the 20-30 cm depth layer. B, CCA ordination of communities in the 80-90 and >300 cm depth. Abbreviations of some parameters included are: Arch_Div = archaeal diversity, Fung_Div = Fungal diversity, Bact_Div = bacterial diversity, EC= electroconductivity, TC = total carbon, TN = Total nitrogen, H20_cont = water content, Ag = Aggregate size, CN = carbon to nitrogen ratio.

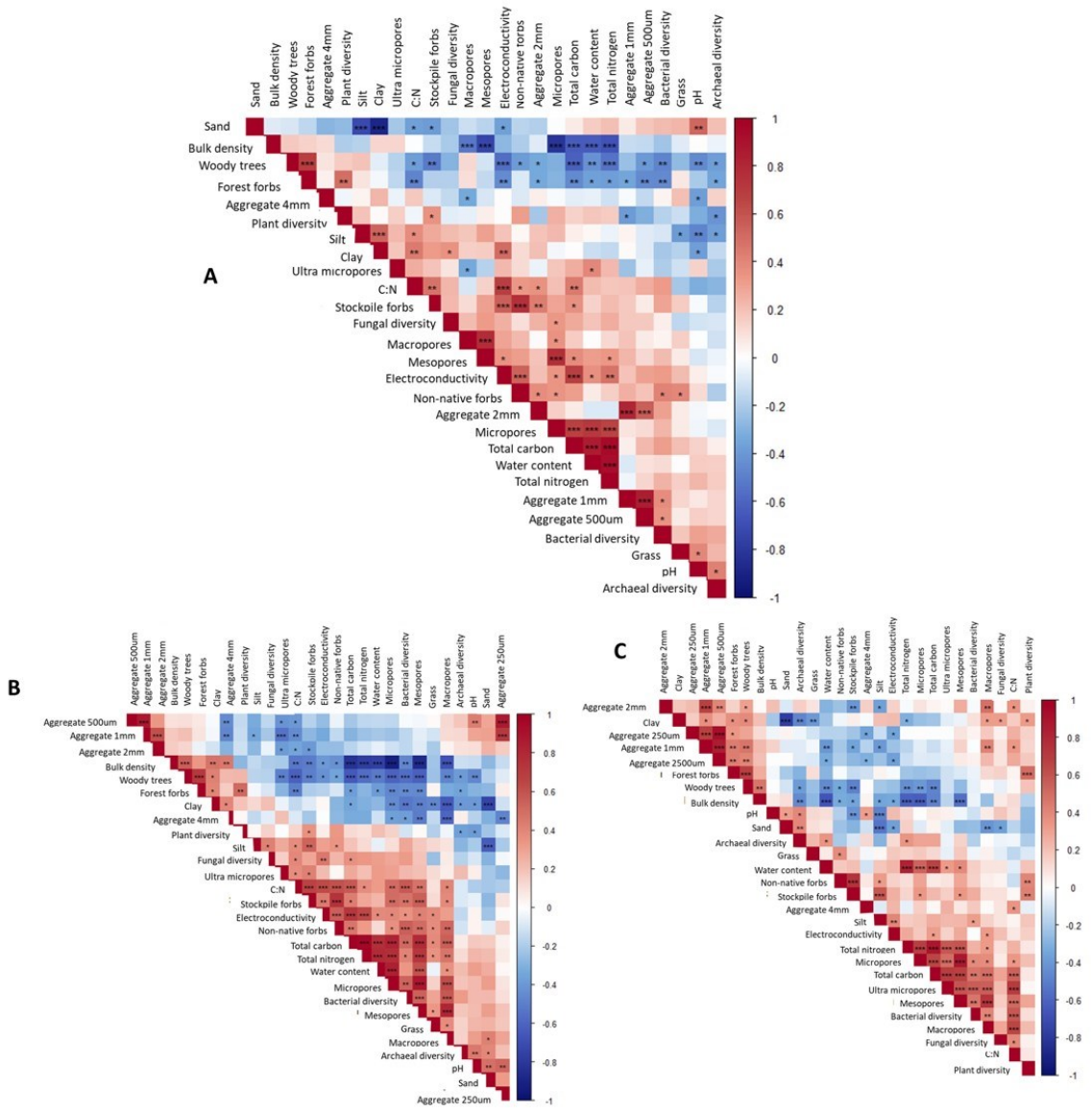

Figure S4-3. Spearman correlation of environmental factors and diversity. The predictors in three soil depth layers were assessed A= 0-10 cm, B = 20-30 cm, and C = 80 to >300 cm. The red color indicates a positive correlation, whereas the blue color indicates a negative correlation. The statistical significance of the correlation is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***.

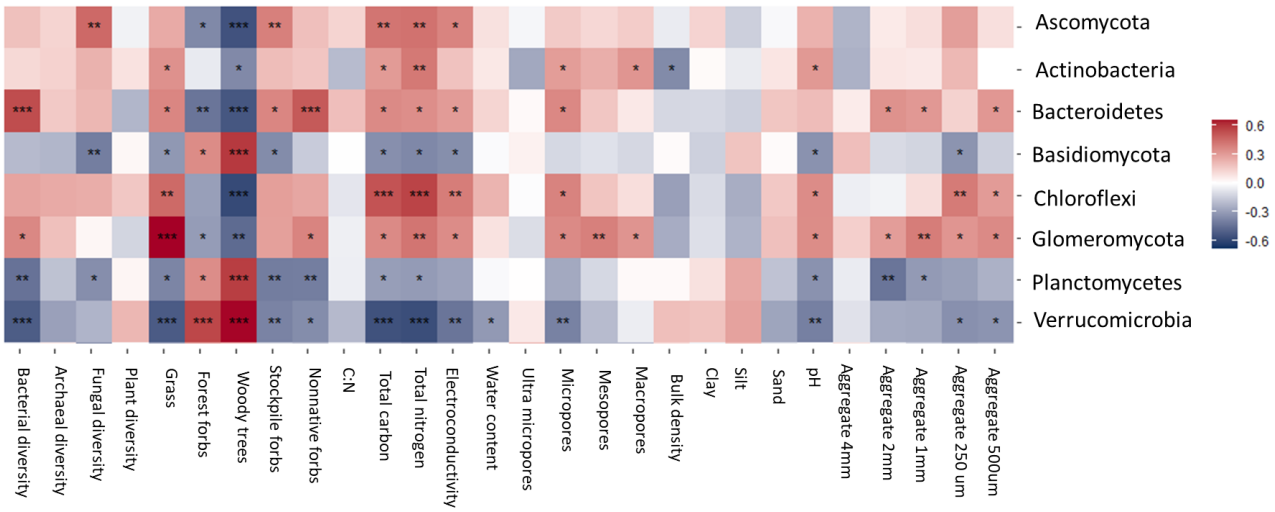

Figure S4-4. Mantel test correlation of environmental factors and the main microbial phyla found in the 0-10 cm depth of the disturbed soils.The red color indicates a positive correlation (Spearman), whereas the blue color indicates a negative correlation. Only the phyla that showed a significant correlation with the predictors were retained in the analysis. The statistical significance of the correlation between the microbial taxa and predictors is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***. Only taxa that show significant correlation (*) with at least one of the predictors were retained in the analysis.

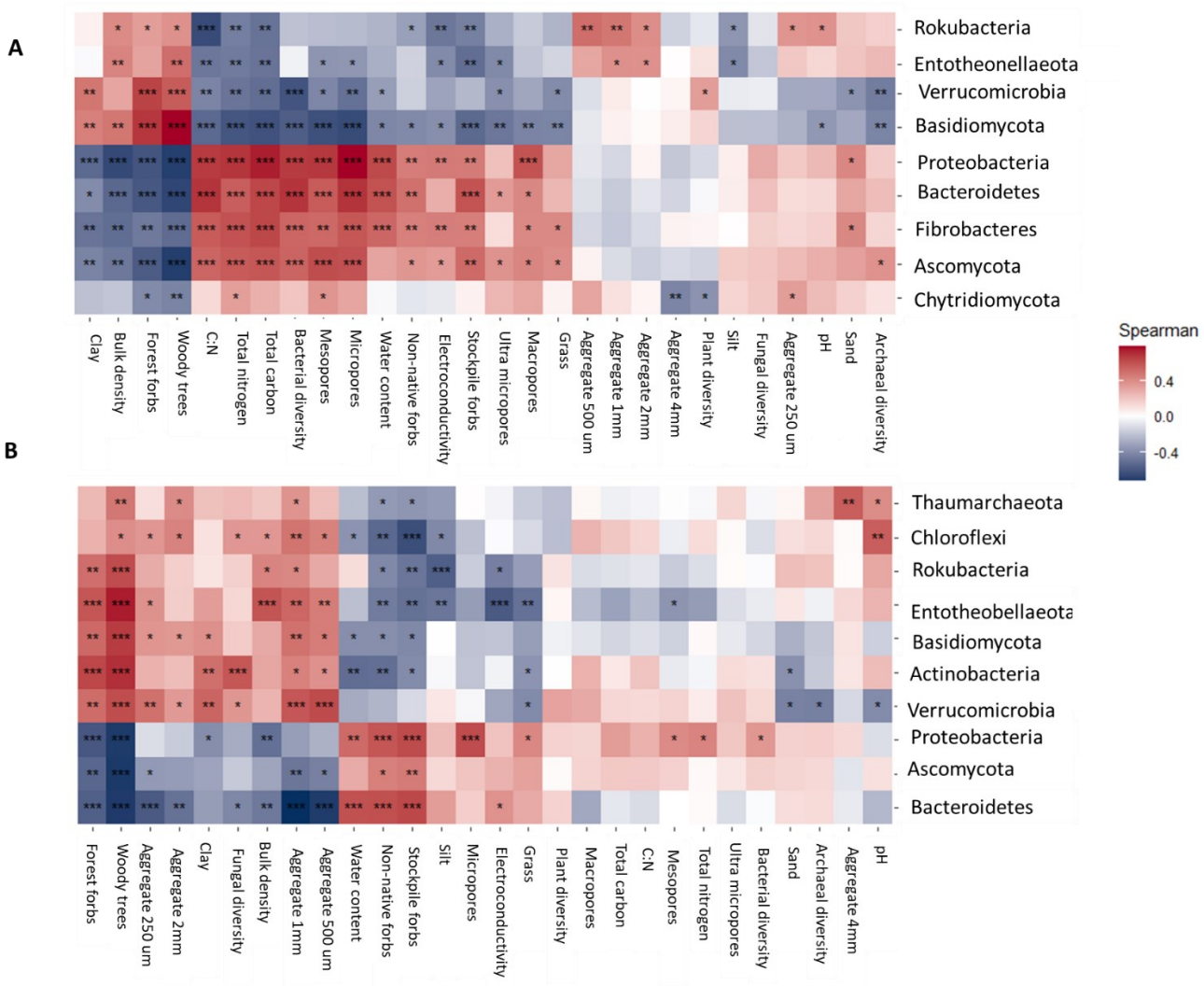

Figure S4-5. Mantel test correlation of environmental factors and the main microbial phyla found in the (A) 20-30 cm, and B, 80-90 and >300 cm depth of the disturbed soils. The red color indicates a positive correlation (Spearman), whereas the blue color indicates a negative correlation. Only the phyla that showed a significant correlation with the predictors were retained in the analysis. The statistical significance of the correlation between the microbial taxa and predictors is indicated by asterisks: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***. Only taxa that show significant correlation (*) with at least one of the predictors were retained in the analysis.

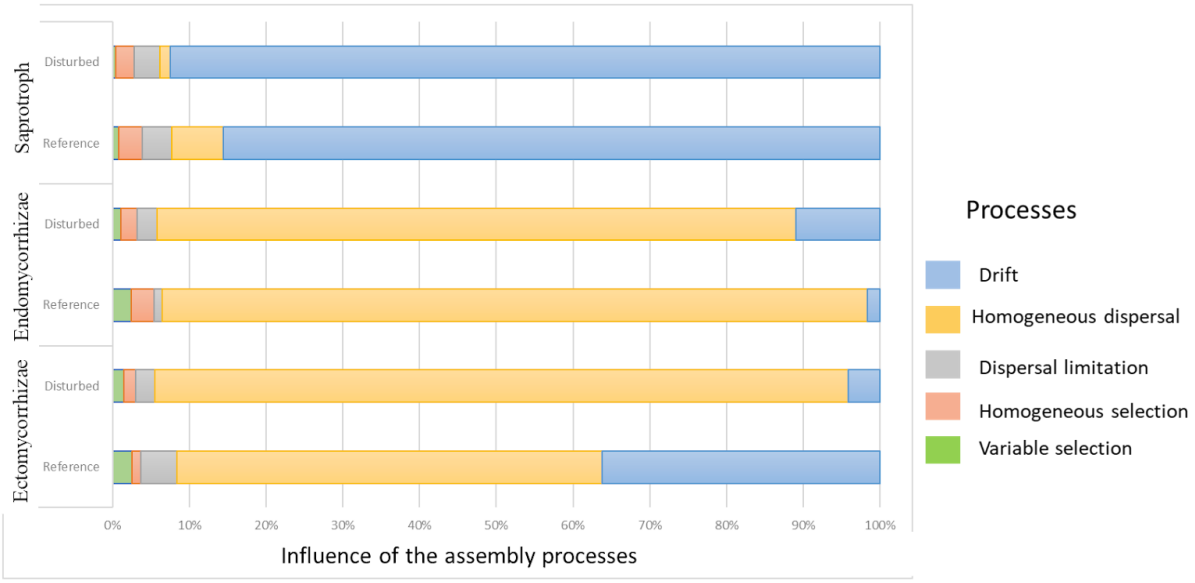

Figure S4-6. Assembly processes responsible for the distribution of the main fungal guilds and trophic modes. The endomycorrhiza includes fungi classified as arbuscular mycorrhizae, orchid mycorrhizae, and ericoid mycorrhizae. The deterministic assembly processes considered are: variable selection and homogeneous selection. The stochastic processes are homogeneous dispersal, dispersal limitation, and drift.

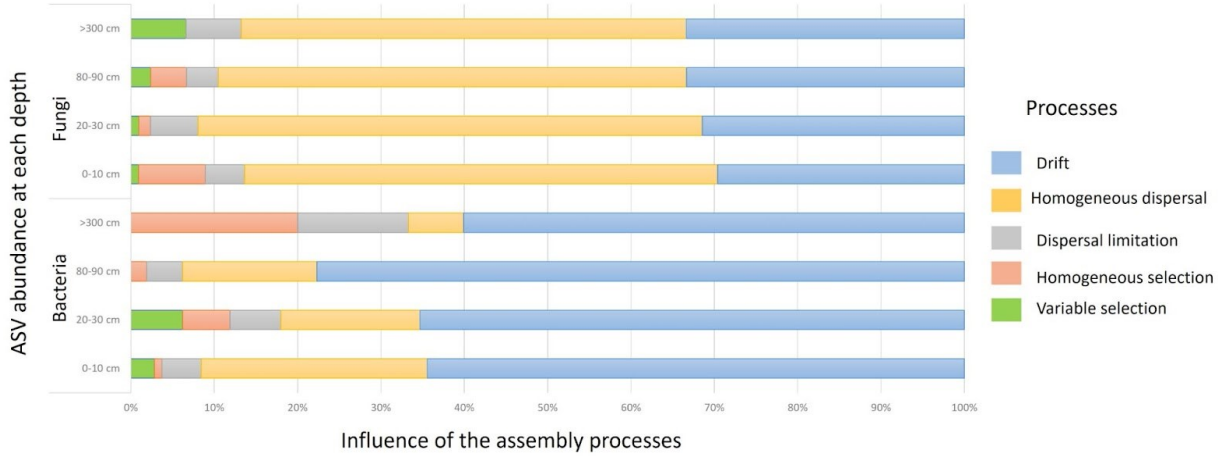

Figure S4-7. The community assembly processes that are most influential for the less abundant ASVs, defined as those ASVs with a mean relative abundance of <0.1% in all samples. The deterministic assembly processes considered are: variable selection and homogeneous selection. The stochastic processes are homogeneous dispersal, dispersal limitation, and drift.

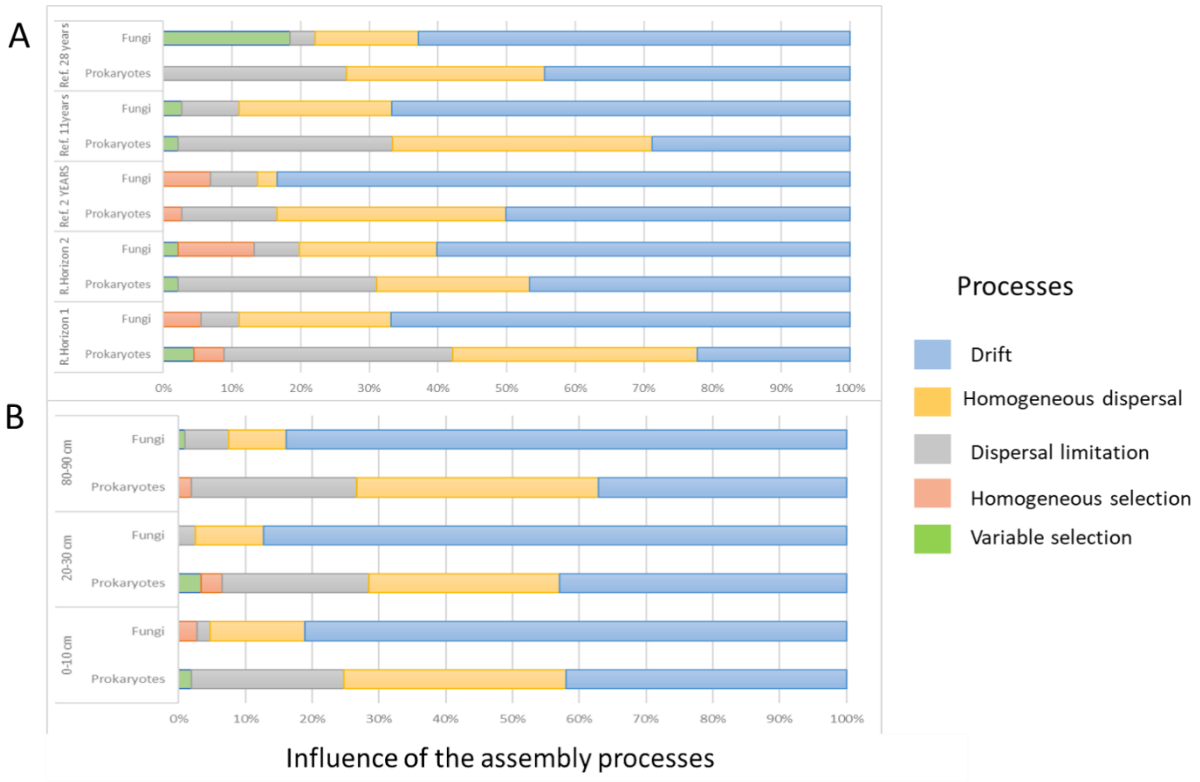

Figure S4-8. Relative importance of assembly processes shaping the communities in the reference soils. A = assembly processes in reference soils are shown as follows, R. Horizon 1 and R. Horizon 2, are the two reference soils for the Horizon site. Ref. 2 years, Ref. 11 years, and Ref. 28 years, are the reference soils for the three Wolf Lake sites. B= Processes shaping the microbial communities in the depth layers of the reference soils.

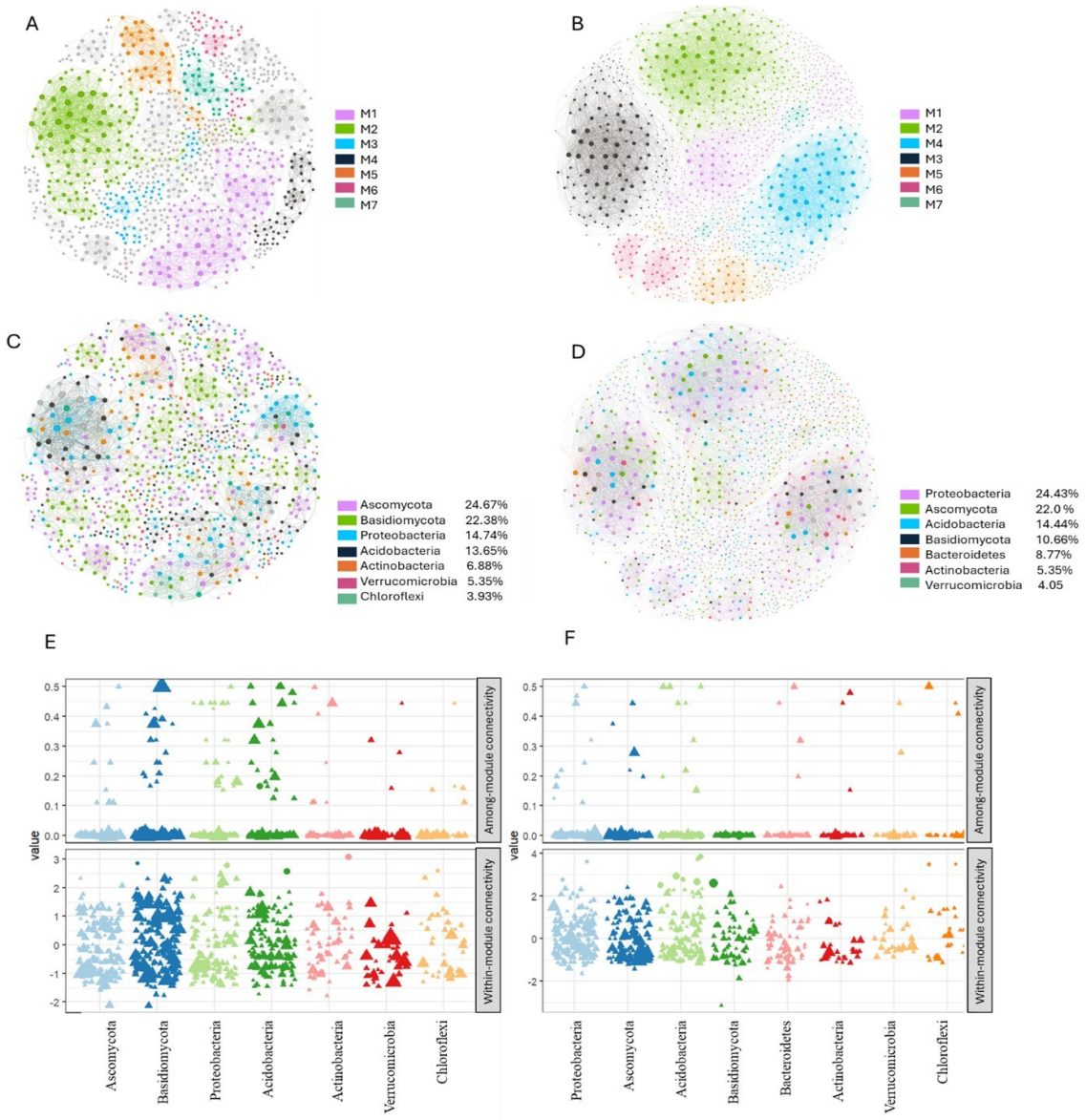

Figure S4-9. Co-occurrence network of microbial communities in the stockpiles (A, C, E), and the reference soils (B, D, F), based on pairwise Spearman correlations. The size of the nodes corresponds to their degree (i.e. number of connections). A connection is a significant (P-value < 0.01) and strong (Spearman $r > 0.6$ or $r < -0.6$) correlation between taxa or nodes. A and B show the main modules (M), and C and D the main phyla in stockpiles and reference soils respectively, and their degree (% of connections in the commun*i*ty). E and F list the keystone phyla (i.e. highly connected taxa with *a* paramount role in the maintenance of the community), for stockpiles and reference soils respectively. Module hubs and nodes are represented by circles and triangles respectively. The size of the figures (triangles and circles) corresponds to the abundance of the taxa in hubs and modules.

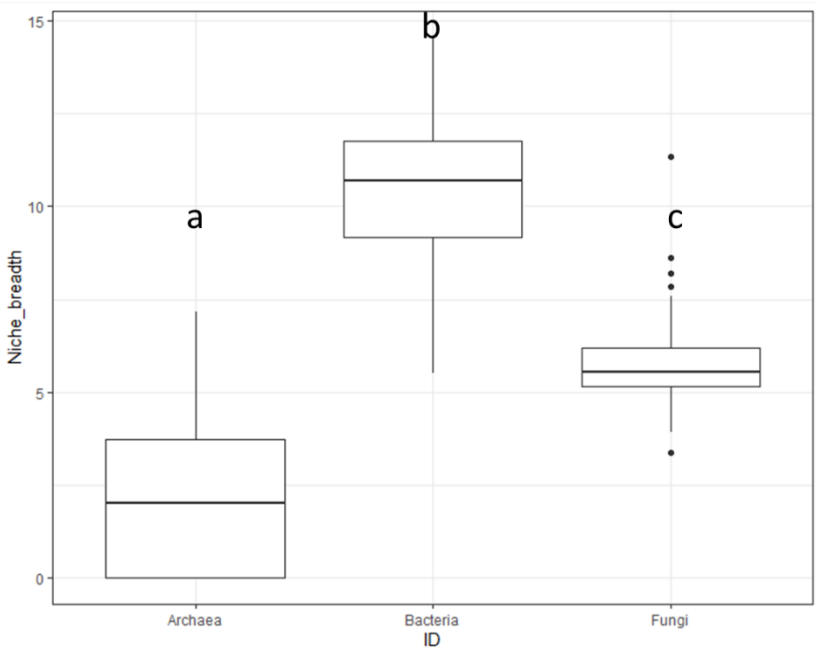

Figure S4-10. Niche breadth (Levins'), estimated for the microbial groups found in the disturbed soil ecosystems. The letters at the top of the boxplots indicate the significance ($p \leq 0.05$) of the dissimilarities among the niche breadth indexes in soil.

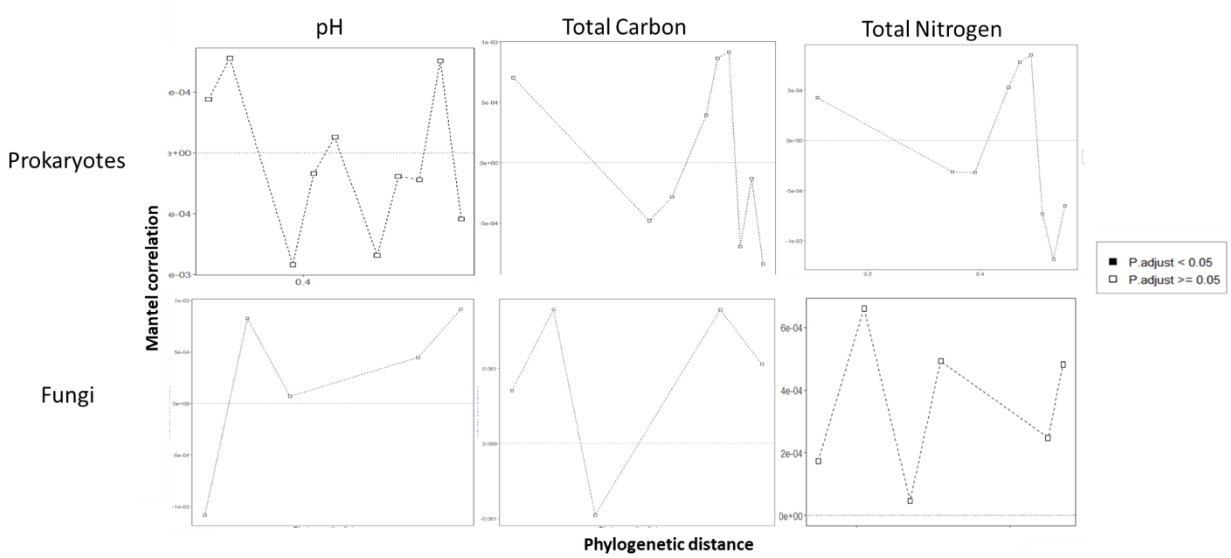

Figure S4-11. Mantel test correlogram. The analysis indicates the correlation (Spearman) between the niche preferences (C, CN, and pH) and the phylogenetic distance between related taxa (i.e. phylogenetic signal). Significant and non-significant correlations are denoted by filled and empty squares, respectively.

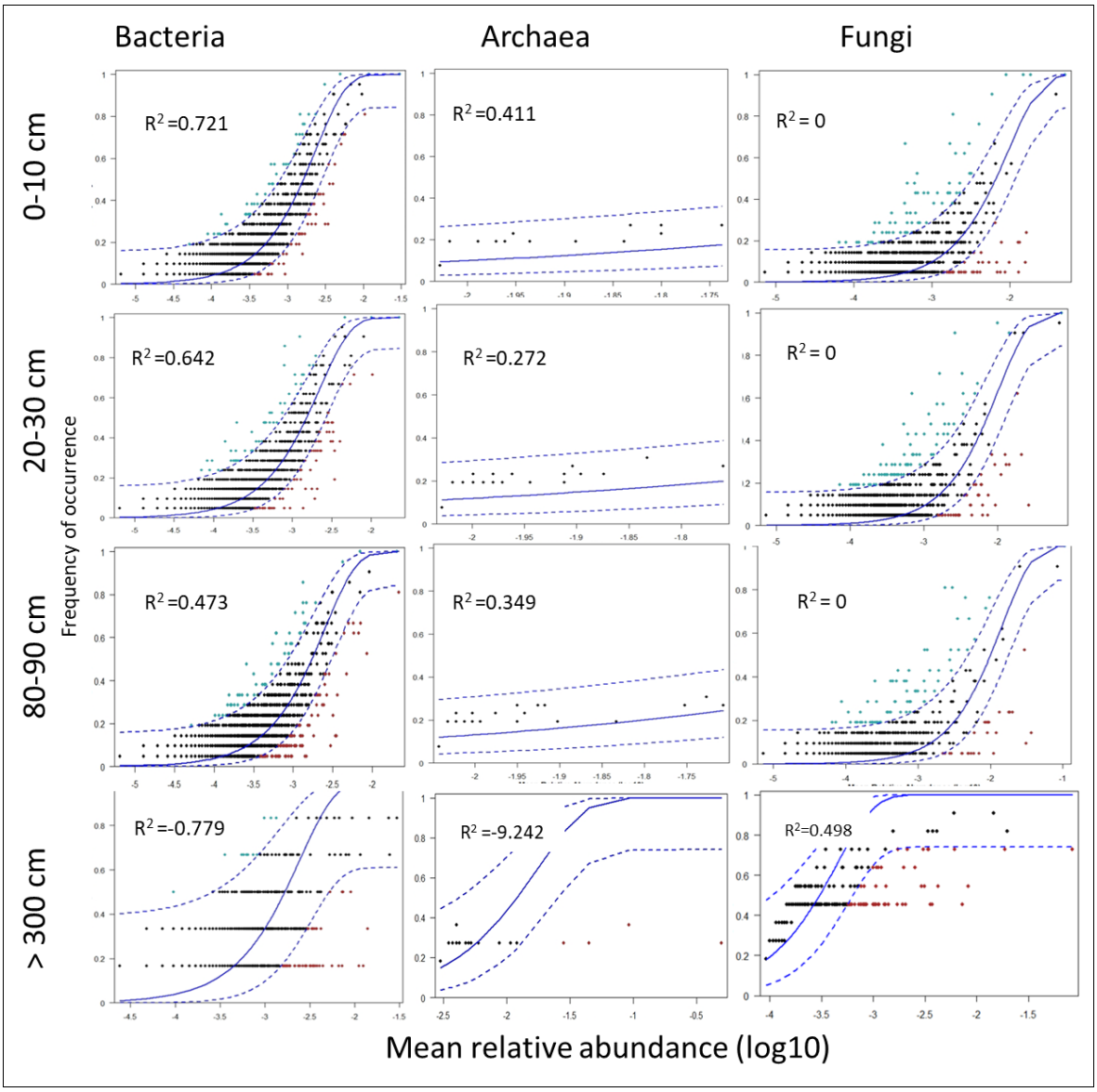

Figure S4-12. Fitness of the microbial communities to the neutral model of community assembly (NCM). The frequency of occurrence indicates how often an ASV is found in a range of communities (samples). Mean relative abundance shows the ASV abundance across the metacommunity. The best fit to the neutral community model is indicated by blue solid lines. 95% confidence intervals around the model predictions are represented by the area within the discontinuous lines, so ASVs found in this area follow the NCM. R^2 indicates the best fit for the NCM. Green dots represent the ASVs that occurred above the model prediction, whereas the red dots represent the ASVs occurring more frequently below the model prediction.

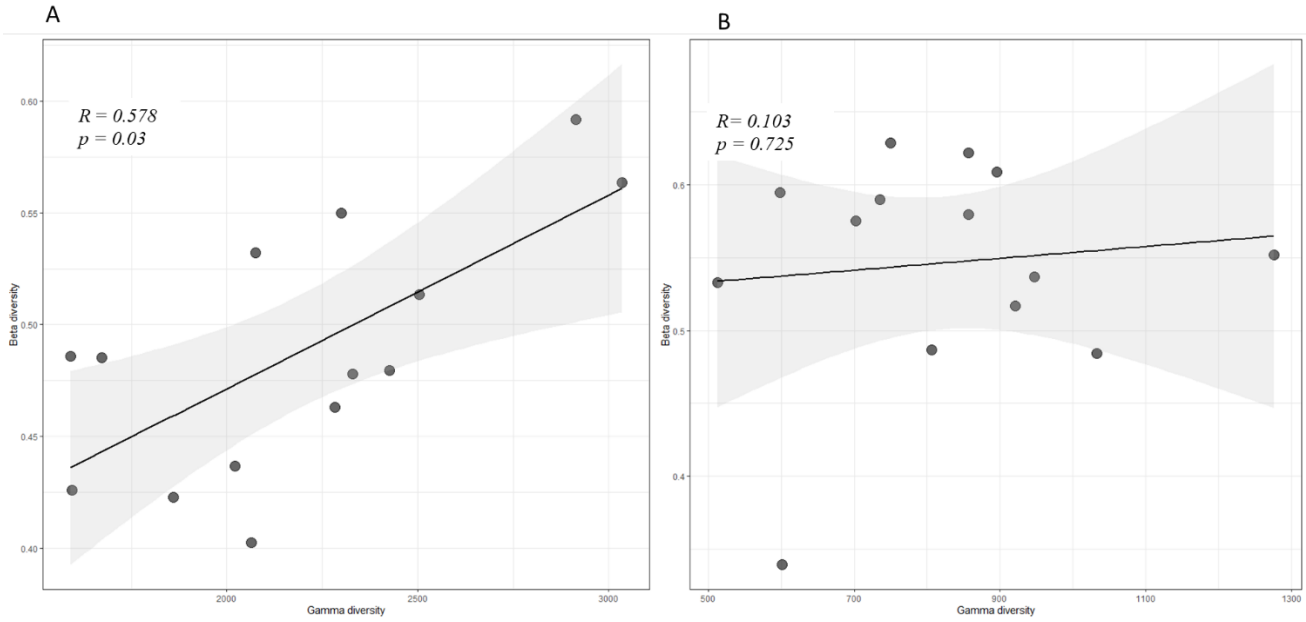

Figure S4-13. Relationship between gamma diversity and beta diversity of prokaryotic communities (A), and fungal communities (B). A correlation (Spearman), between the distances (Bray Curtis), was applied. Gray dots are the samples. Gray areas indicate the confidence intervals (95%). R denotes the correlation coefficient, and p represents the significance of the correlation.