SOIL BIOLOGICAL FUNCTIONING AND RECOVERY IN SOILS DISTURBED FROM BAKKEN OIL AND GAS ACTIVITIES

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MASTER OF SCIENCE

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ABSTRACT

Oil and gas activity-related soil disturbances can alter soil properties and reduce microbial function, which is crucial for nutrient cycling and proper soil function. The goal of this research was to investigate effects of remediation techniques on soil biological property recovery following various types of oil and gas disturbances. A series of studies evaluating microbial abundance, crop growth, and biological properties were conducted on crude oil-contaminated subsoils remediated either with thermal desorption or land farming. Thermally desorbed subsoil achieved similar yields to non-contaminated topsoil after four years. Additionally, biological properties in blended soil (1:1 topsoil to subsoil) were studied using two biostimulant inoculant products. Overall, biostimulant amendment improved microbial responses and plant growth over the control. While blended subsoil and/or remediated subsoil could replace topsoil during instances of topsoil scarcity and can facilitate soil biological property recovery similar to topsoil, biostimulants improved biological metrics regardless of soil blend.

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GENERAL INTRODUCTION

Soil is an invaluable resource that provides many services to the ecosystem and humanity but is experiencing oil and gas (O&G) related disturbances for extraction and transportation infrastructure development. Commonly, O&G disturbances involve topsoil stripping, stockpiling, and accidental contamination of soil which disrupt soil aggregates, reduce soil organic matter, and mix topsoil and subsoil, lowering biological activity and soil health, necessitating contaminant removal and/or reclamation to ensure ecosystem health. In the Great Plains of the United States O&G resources are abundant but often reside beneath the soil surface where crops are being grown (Conant et al., 2018), and present potential issues for landowners that depend on soil productivity for their livelihood such as crop or livestock production.

Because the greatest inhibitor of soil reclamation success is soil microbial community recovery, due to microbial driven nutrient cycling and plant growth promotion, more holistic reclamation methods must be chosen or developed (Croat et al., 2020), and may or may not require adding or altering current industry practices. Biological recovery may be achieved by conserving impacts to native topsoil, introducing sources of organic matter, and minimizing abiotic stress and may also include the use of a range of novel living and non-living organic materials made into soil amendments called “biostimulants.” However, some reclamation and remediation methods’ effects on long-term soil biological recovery are not yet thoroughly studied. For example, thermal desorption allows for crude oil contaminants to be neutralized while allowing soil materials to have reduced concentrations of hydrocarbons, but alters soil properties and microbial communities.

Additionally, soil-mixing is an inevitable outcome of soil handling processes which introduces subsoil to topsoil and disrupts soil aggregation (Wick et al., 2009). Notably, subsoils can contain different chemical levels compared to topsoil (i.e. Na, $CaCO_3$) that diminish topsoil quality and/or chemical properties, potentially affecting future plant productivities. Although chemical amendments have been observed to combat these changes to an extent, such as gypsum used to displace Na, or inorganic fertilizers to boost crop yield, mixed soils generally continue to display reduced organic matter concentrations, and lower microbial diversity and abundance decades following reclamation (Taylor et al.,

2002; Viall et al., 2014). Therefore, further studies on novel methods to enhance biological property recovery following soil disturbances and reclamation are needed.

THESIS ORGANIZATION

This thesis contains three chapters organized in manuscript format. Chapter 1 is a literature review of biostimulants and their utilization in soil reclamation focusing on the main ingredient types, and their observed effects on plant growth, soil nutrients, and soil microbiological communities. Due to the new attention on biostimulants, the majority of peer-reviewed papers available that have investigated biostimulants are in strictly agricultural or horticultural settings. Therefore, Chapter 1 utilizes some agricultural research and connects findings to its potential in soil reclamation. The second chapter is a field and lab study titled “Plant Growth, Soil Properties, and Microbial Community Four Years After Thermal Desorption” and investigates crop growth and soil microbial communities four years after crude oil contaminated soil was thermally desorbed. This chapter has been accepted within *Agronomy Journal*. Chapter 3, “Microbial Activity and Hard Red Spring Wheat Growth Improvement Following Biostimulant Application,” is an exploratory study on using two different types of biostimulant products to improve plant growth and microbial activity in blended soils in a greenhouse. The totality of this research provides information that is primarily focused on recovery, functionality, and improvement of soil biological metrics following O&G-related disturbances.

CHAPTER 1. CREATIVE USE OF BIOSTIMULANTS TO IMPROVE SOIL BIOLOGICAL PROPERTY RECOVERY IN RECLAIMED SOILS: A REVIEW

Abstract

Dating back to ancient civilizations, organically-derived amendments, or “biostimulants”, have been used to improve crop growth and yields, and are widely studied in agricultural and horticultural settings. Following soil disturbances, overall soil function and productivity is lowered, and challenges reclamation success and biological recovery. As a result, there has been growing interest and marketing for using biostimulants in reclamation to improve reclamation success. However, there have not been adequate peer reviewed studies documenting their use or benefits in reclamation. Thus, studies describing their influence on soil reclamation is needed to determine if biostimulant products and/or application methods could be efficiently implemented in the field. This review details available research on biostimulant products and their ingredients’ uses for improving soil biological property metrics in soil reclamation settings. In some instances, inadequate literature was available for a given biostimulant ingredient, and was supplemented by papers with a setting in degraded soils. Broadly, all potential ingredients contained positive effects on biological metrics such as crop growth, abiotic stress tolerance, and increased microbial abundance, but the reported responses heavily depended on the type of biostimulant applied. Overall, the literature reviewed suggest biostimulants can be tools for enhancing reclamation and could be considered for use by reclamation professionals with further studies.

Introduction

Disturbance of soil resources for oil and gas infrastructure development is necessary for safe and economical extraction and transportation of target fossil fuels (Martínez-Palou et al., 2011; Fernando and Stika, 2021). The disturbances may include well pad and access road construction, pipeline installation, or soil contamination from accidental releases of crude oil or produced water, and will all require remediation or reclamation at some point to ensure future land productivity and public health and safety (O’Brien et al., 2017). For example, over 23,000 acres of land in western North Dakota were converted from farmland to well pad and access roads and had 200 m^3 of uncontained crude oil spills on soil from June 2018 through June 2019 (Croat et al., 2020; Fernando and Stika, 2021). Regardless of the disturbance type or subsequent soil remediation, reclamation, or restoration method, these disturbances

disrupt soil microaggregation and pore networks, reduce microbial activity, and risk soil organic carbon (SOC) loss via mineralization or erosion (Ingram et al., 2005; Anderson et al., 2008; Wick et al., 2009, Gasch et al., 2014). Ultimately, this degraded soil can inhibit soil biological recovery and future plant yields without chemical fertilizer or additional SOC inputs (Croat et al., 2020), further threatening soil salinization (Shrivastava and Kumar, 2015), soil erosion (Brindle, 2003), or reduced SOC accumulation. Because soil biological properties participate in soil physical and chemical property recovery, identifying novel reclamation strategies that mitigate impacts to soil biological properties is imperative.

Although different processes, oil and gas disturbances can degrade soil in a similar fashion to conventional agricultural practices, such as how tillage disrupts porosity, reduces microbial activity and nutrient availability, and potentially salinizes soil (Caolderón et al., 2001; Grandy and Robertson, 2007). Due to the increasing interest in alleviating the effects of conventional agricultural practices on soil and surrounding environments (tillage, chemical fertilizers, and herbicides), biostimulant products have held increased relevance in the ag sector and in scientific literature (Calvo et al, 2014; Rouphael and Colla, 2018; Karapouloutidou and Gasparatos, 2019). Biostimulants are products applied in agricultural or horticultural settings with the purpose to enhance SOC accumulation, improve vegetative function and stress tolerance, or to promote microbiological nutrient cycling (du Jardin, 2015). While the effects of biostimulants on soil biological function in agricultural settings are well-reported (du Jardin, 2015; Tejada et al., 2011; Desoky et al., 2018), literature examining the potential of biostimulants in enhancing recovery of biological properties in disturbed/degraded soils is lacking (Tuttobene et al., 2009). Due to the vast number of products and brands available, further studies are needed.

Despite the number of products on the market being advertised to reclamation professionals, and a projected global market of value of $4.5 billion by 2027 (Business Wire, 2020), biostimulants are not actively regulated by the United States government, with only draft guidance available (US EPA, 2020). Thus, independent studies reporting if (or how) different biostimulants can enhance reclamation in disturbed soil is vital. Biostimulant ingredient sources range from various organic materials such as, humic substances (HS), seaweed extracts, manure, industrial wastes, compost, and living microbial cultures (Tejada et al., 2006; Brown and Sea, 2015). However, each ingredient or specific sources of

materials may influence plants and the soil ecosystem differently, and numerous questions remain pertaining to ideal application rates, timing, method, or plant growth stages (Rouphael and Colla, 2018).

Some HS have been reported to benefit soil aggregation and potential accumulation of recalcitrant SOC (Piccolo, 2002), while improving microbiological activity and plant growth (Rose et al., 2014). On the other hand, seaweed products used as foliar sprays or soil amendments have been reported to increase plant stress tolerance through various avenues, but were also applied with HS (Rayirath et al., 2009; Battacharyya et al., 2015), and may confound exact interpretations. Other seaweeds can contain the major growth regulating hormones, and have doubled root or shoot growth (Zhang et al., 2003; Neily et al., 2010; Zhang et all, 2010; Spann and Little, 2011; Lötze and Hoffman, 2016; Lötze and Hoffman, 2017). Additionally, microbial communities are necessary for soil functioning and organic matter decomposition, but provide different benefits to soil biological properties if symbiotes are applied to host plants (Kuimei et al., 2012; Zhang et al., 2019), and may display more positive results with specific host species than what is actually grown (Janoušková et al., 2013; Gu et al., 2020). In the end, comparing products to other products is complicated due the variety of sources for formulation, but also due to private companies keeping ingredients as proprietary information to protect their business. Therefore, without continued or consistent synthesis of data on biostimulants in reclamation settings, it may be difficult to determine what products, if any, are more valuable in recovering soil biological function after disturbances.

Thus, this review aims to identify the broad sources and ingredients commonly used as or used in biostimulants. A single ingredient that is commonly used as a biostimulant amendment will be introduced and discussed before introducing a new ingredient type. Discussion will focus on an ingredient's reported effects on plant or soil properties, beneficial or not. However, determining specific physiological mechanisms is beyond the scope of this review, as this topic has been discussed in preceding studies and is naturally highly spatially variable. Studies describing their use in agriculture is plentiful, but the use of biologicals in reclamation is not. Therefore, when studies in reclamation were not available, studies on highly degraded or marginal soil were included, and are directed to potential uses in reclamation. Finally, the general conclusions will include practical implications pertaining to using biostimulants as a

reclamation tool, and will outline precautions for potential hurdles for utilizing biostimulants on a large-scale.

Biostimulants Types and Effects on Soil Biological Properties

Humic Substances

Humic substances are partially decomposed and disordered organic compounds (mostly carboxyls, phenolic and alcoholic hydroxils, and more) derived from plant tissues and microbial biomass, and can constitute >50% of soil organic matter (Stevenson, 1994; Brookes et al., 2008; Park et al., 2015). Humic substances are synthesized during humification, but are considered more labile than fully humified soil (Tate, 1992). Thus, its natural production and value as a commercial product is entirely dependent on soil properties. Due to the natural heterogeneity and scale of soil ecosystems (Zanin et al., 2019), exact structures or concentrations in HS for comparison are difficult to determine (Table 1; Dell'Abate et al., 2002; Fong and Mohamed, 2007). For example, areas with high C plant residue addition and anaerobic conditions can increase the C content of the humic fraction, whereas HS in upland aerobic areas can contain different components from different decomposition or nutrient cycles (Olk et al., 2006). Generally, HS are extracted from the soil using alkaline solutions in order to produce a solution of humic material, and are known commercially as humic and fulvic acids.

Numerous studies suggest that humic and fulvic acids positively influence plant and root growth in reclaimed, disturbed, or saline soil, and can improve some contaminant degradation rates (Canellas and Olvares, 2014; Kandil et al., 2016). Application of these biostimulants is achieved via dispersal into soil in a solution, or as a foliar spray (Kandil et al., 2016). Generally, HS are paired with gypsum or Ca, or other biostimulants when applied to saline soil (Türkmen et al., 2007; Nan et al., 2016; Liu et al., 2019; Abourayya et al., 2020; Liu et al., 2020). In other instances, HS may be applied as solids, such as leonardite, where application in a crude-oil-contaminated soil enhanced degradation rates due to their sorptive nature as well as stimulating soil enzymatic activity compared to the control (Turgay et al., 2009). In addition, Rose et al. (2014) conducted a meta-analysis showing a 22% increase in root and shoot dry weights in response to HS applications. Similarly, field-grown potato yields increased 13 to 17% following HS application compared to the control treatments (Verlinden et al., 2009).

Table 1. Effect (positive "+", negative "-", or not significant "ns") of humic substance amendment on selected soil biological metric relative to the control treatment; studies are grouped according to study setting, author, humic ingredient (and source), and the metric.

Author	Ingredient (Source)	Metric	Effect (+/-)
Saline Soils			
Türkmen et al., 2007	Humic acid (polymeric polyhydroxy acid)	-Seed germination	+
		-Root biomass	+
		-Shoot biomass	+
Nan et al., 2016	Humic acid (Lignite coal)	-Bulk density	+
		-SOM	+
		-Soil aggregation	+
Liu et al., 2019	Humic acid (unidentified)	-Grain yield	+
		-Root biomass	+
		-Shoot biomass	+
		-Soil aggregation	+
		-SOM	+
		-Bacterial diversity	ns
		-Fungal diversity	+
Liu et al., 2020	Humic acid (unidentified)	-SOM	+
		-Soil aggregation	+
		-EC	-
		-N	+
Agricultural Soils			
Haider et al., 2015	Humic acid (commercial product)	-Total plant biomass	ns
Waqas et al., 2014	Humic acid (Lignite coal)	-Grain yield	+
Dinçsoy and Sönmez, 2019	Humic acid (unidentified)	-Grain yield	+
Soil Contamination			
Turgay et al., 2010	Humic substance (leonardite coal)	-Semivolative hydrocarbon degradation	+
		-Heavy petroleum hydrocarbon degradation	+
		-Urease activity	+

However, some specific modes of action are still not fully understood for plant enhancement and contaminant degradation (Shah et al., 2018). Due to different charges and random molecular structures resulted from the disordered humic material, HS can form supramolecules via hydrogen bonds, and chelate nutrients, digestive enzymes, sugars, or plant hormones (Tahir et al., 2012). For example, auxin is a plant hormone that stimulants plant cell elongation and growth (Canellas et al., 2015). Due to the

abundance of auxin and auxin-like molecules (Ferro et al., 2006) that exist in soil, they may be present in humic acids used as biostimulants.

While microbial activity responds positively with HS addition (Tejada et al., 2011), and may aid in soil aggregation (Zanin et al., 2019; Mosa et al, 2020;), the supramolecular humic networks can also help build aggregation (Piccolo, 2002) through binding to soil particles and prevent of soil organic matter leaching through the soil profile. Over time, the recovery of soil aggregates improves moisture retention, and erosion resistance (Wick et al., 2009; Costa et al., 2018). Additionally, chelated nutrients and phenolic compounds that occur in HS are important sources of C, N, and other nutrients necessary for microbial activity, nutrient cycling, and soil fertility (Canellas et al., 2015; Vaccaro et al., 2015; Zanin et al., 2019). Naturally, HS can be solubilized from SOM and transported through soil to root zones, depositing nutrients for both microbial activity and plant uptake (Vaccaro et al., 2015). Solubilization of O-, N-, and S-containing functional groups can also help form stable complexes with metal micronutrients, such as Fe. This relationship suggests that HS can help hold micronutrients in solution, reducing leaching and increasing bioavailability (Senesi, 1992; Tipping, 2002). Although biostimulant amendments are intended for terrestrial ecosystems, their sources may include marine ecosystems.

Seaweed Extracts

Seaweed has been utilized in agriculture for centuries (Khan et al., 2009), and may be undervalued as a biostimulant amendment for reclamation. These multicellular macroalgae are essential pieces in marine ecosystems, and may contain over 10,000 different species (Battacharyya et al., 2015). The three main types of seaweed used are brown, green, and red, which contain unique substances valuable in regards to plant physiology (Khan et al., 2009; Matysiak et al., 2011; Battacharyya, et al., 2015). Specifically, the natural biologically active compounds are thought to influence numerous plant properties such as abiotic stress tolerance, plant growth and yield, and increased root/shoot ratio (Battacharyya et al., 2015). Common constituents in extracts are shown in Table 2.

Table 2. Common compounds or chemicals in seaweed extracts used as biostimulant products.

Extract	Ingredient (Source)	Author
Inorganic nutrients (Such as N, P, K, Ca^{2+}, Fe^{3+}, Zn, Na^{+}, and S)	Brown seaweed powder (*Ascophyllum nodosum*)	Rayirath et sl., 2009
		Abdel-Fattah and Merwad, 2016
Lipophilic components	Brown seaweed powder (*Ascophyllum nodosum*)	Abdel-Fattah and Merwad, 2016
Polysaccharides	Brown seaweed powder (*Ascophyllum nodosum*)	Abdel-Fattah and Merwad, 2016
	Brown seaweed (*Laminaria digitata)*	Mercier et al., 2001
Amino Acids	Green seaweed (*Ulva armoricanai*)	Fleurence,1999
	Green seaweed (*Ulva Pertusa*)	Fuhiwara-Arasaki et al., 1984
	Red seaweed (*Palmaria palmata*)	Galland-Irmouli et al., 1999
	Red seaweed (*Porphyra tenra*)	Fowden et al., 1954
Cytokinin	Brown algae powder (*Ascophyllum nodosum*)	Abdel-Fattah and Merwad, 2016
Indoleacetic acid	Unknown (Extract spray)	Roshdy, 2014
Abscisic acid	Unknown (Extract spray)	Roshdy, 2014
Phytin	Brown algae powder (*Ascophyllum nodosum*)	Abdel-Fattah and Merwad, 2016

Similar to HS, seaweed biostimulants may be applied in different forms, and may be processed into biostimulant products using a wide range of proprietary extraction processes. However, the method of application and species of seaweed affects the effectiveness, or plant response (Matysiak et al., 2011). For example, green seaweed (*U.* ohnoi) mixed with compost was applied to the soil for sugarcane production and resulted in a sevenfold biomass increase from the low-seaweed treatment (3 g dry weight) and high-seaweed treatment (20.8 g dry weight; Cole et al., 2016). Additionally, the two composts with the greatest proportion of green seaweed were most effective at lowering C:N ratios below 20:1.

Importantly, soil application of seaweed can enhance soil bacterial diversity and activity of enzymes involved with C and N cycling. Wang et al. (2018) reported fermented seaweed application increased dehydrogenase activity, an indicator of SOM decomposition, by an average of 120%. Meanwhile, enzymes crucial to the N cycle, protease (r = 0.328) and urease (r = 0.374), were positively associated with total bacterial community composition. In another study, brown algae species *Ecklonia maxima* and *Saragassum* spp. improved seed germination 16.3-18.8% relative to the control and humic acid treatments via soaking, but only the *Saragassum* sp. significantly increased maize shoot biomass. When the same treatments were used for foliar application, plant growth was only improved by the seaweed by approximately 29.7% (Matysiak et al., 2011). Therefore, investigating biostimulant products

for redundancies in plant effects may be beneficial for improving the cost-efficiency of soil reclamation professionals by reducing the need for additional fertilizer inputs.

Soil disturbances in drought-prone or salt-stressed areas can further complicate reclamation activities whether the goal of reclamation is to revegetate the area with perennial vegetation or produce agricultural crops (O'Brien, 2017). During a drought-stress study, seaweed extracts increased water content in soybeans (*Glycine max*) by 50% (Shukla et al., 2017). In citrus trees, Neily et al. (2010) reported that a foliar-applied brown seaweed (*A. nodosum*) treatment was also successful in maintaining tree root and shoot growth rates under 50% deficit irrigation, possibly due to the product containing plant growth hormones, and an enhanced stress response from phenolics or betaines, which can be found in seaweeds. Similarly, various seaweeds can contain high levels of the plant growth-stimulating primary metabolite- auxin (Tarakhovskaya et al., 2007; Zhao, 2010; Lötze and Hoffman, 2016; Lötze and Hoffman, 2017). Although there have been few studies until recently on using seaweed extracts strictly in reclamation settings, these findings suggest utilization of processed seaweeds could improve reclamation success through enhancing plant growth. In turn, if plant biomass is improved with seaweed extract, plant residues can then enter the soil system, and be decomposed by soil microbes helping to restore SOC and sustainable soil functioning.

Organic and Inorganic Wastes

Manure, or animal waste, is a well-documented organic fertilizer and nutrient source for plants and microbial communities. However, the opposite is true about the advantage of using waste materials like wood pulps, industrial or sewage sludges, or food processing wastes obtained from industrial processes for use as biostimulants (Charmley et al., 2006). Some materials, such as sewages, require microbial-driven anaerobic digestion prior to use in order to reduce odors, pest attraction, waste volume, pathogens, or unintended secondary environmental impacts (US EPA, 2015). For more benign materials, little-to-no pre-application treatment is required. An example is orange peel waste that was used as organic fertilizer in the Mediterranean, which had been experiencing losses of soil fertility from intensive agriculture (Tuttobene et al., 2009). In a two-year study, different rates of orange waste (4 and 8 kg m^{-2}) high in organic C was applied as fertilizer; the authors reported a 400% increase in plant biomass compared to mineral fertilizer while producing similar grain yields (Tuttobene et al., 2009). The greatest

rate of orange waste was observed to decrease wheat yields in that same timeframe, and ultimately suggests some lower rates of waste can supplement or replace mineral fertilizers for two-years while increasing biomass growth and potential SOC formation via plant litter before a potential new waste source is needed.

In addition to containing high amounts of organic C, industry wastes have been observed to act as sources of other nutrients for microorganisms and could provide additional reclamation benefits in disturbed soil. According to d'Errico et al., (2013), composted vegetable waste encouraged soil bioremediation of eutrophic waste lagoon sediments via a soil priming effect that increased degradation of organic-rich molecules, thereby potentially increasing organic matter turnover (Christofoletti et al., 2013). Though some industrial wastes are approved to be land applied, their intentional use as biostimulants for reclaiming disturbed soils would provide greater benefits to the overall recovery of the soil ecosystem than on agricultural soils with adequate topsoil amounts but may require creative problem solving (Larney and Angers, 2012). One such breakthrough was the use of green compost, paper mill sludge, and thermally treated soil contaminated by polycyclic aromatic hydrocarbons used to grow rye and alfalfa (Séré et al., 2008). Plots were constructed in layers mimicking soil horizons with the mentioned components and produced greater total biomass (0.92 kg m^{-2}) relative to the control (0.59 kg m^{-2}; Séré et al., 2008).

Often, reclamation may require contaminated soil to be deposited at landfills and for foreign replacement soil to be brought in. Likewise, many food (and industrial) wastes are lost as a resource to landfills (US EPA, 2015). Another approach to utilize otherwise discarded industrial byproducts is spent lime (calcium carbonate; $CaCO_3$) used to refine sugar beets (*Beta vulgaris* L.) in the Red River Valley of North Dakota and Minnesota (DeSutter and Godsey, 2010), where its use as a soil treatment spent lime was reported to lower %Na by 35% (Breker et al., 2018), and increase microbial activity >50% in saline soil, and >430% in thermally desorbed soil (Kruger et al., 2020). Additionally, spent beet lime was observed to achieve 33% and 46% greater root and aboveground biomass, respectively, than other available commercial lime products while increasing soil pH (DeSutter and Godsey, 2010). These observations demonstrating spent lime's potential to facilitate microbial recovery, and ameliorating disagreeable soil properties. Together, Séré et al. (2008), and the other authors cited above provide a

possible resolution to disposing of soils and byproducts, where industrial waste and remediated soil are utilized together to conserve soil resources and deserves greater consideration when designing reclamation and waste-stream studies.

Microbial Inoculants

Soil microbiology is often undervalued during reclamation processes, but is important to consider for their role in nutrient cycling, soil formation, and plant interactions, and are potentially the largest determiner of reclamation success (Vaill et al., 2014; Sheoran et al., 2015; Thavamani et al., 2017). Using beneficial microbes for agricultural or horticultural purposes is a long-standing practice, such as inoculating legume seeds with *Rhizobia* spp. (Deaker et al., 2004; O'Callaghan, 2016), or to improve pest resistance (Valenzuela-Soto et al., 2009). Despite the documented studies of beneficial microbes and crop growth improvement, there has been comparably fewer studies on the potential of microbial biostimulants on enhancing reclamation, especially in regions with adverse or challenging climates. Under standard reclamation practices soil microbial biomass and microbial-derived SOC levels decrease and may require >30 yrs to return to pre-disturbance levels (Mummey et al., 2002; Dangi et al., 2014; Vaill et al., 2014). Thus, biostimulants containing live microbial inoculants may be a tool that shortens the reclamation timeline and recovery of microbial biomass.

Understandably, inoculating reclaimed soils is expected to improve reclamation by encouraging microbial decomposition of organic matter, and plant-microbe mutualistic symbiosis through increasing the abundance of symbiotic soil microbes (Bago et al., 2000). Non-symbiotic bacteria and fungi are decomposers, breaking down organic matter into bioavailable compounds that can increase plant function and nutrition without directly associating with them (Sahain et al., 2007). Oppositely, symbiotic microorganisms such as rhizobacteria or mycorrhizal fungi help plants obtain nutrients (Rouphael and Colla, 2018) by forming mutualistic relationships, where microbes access nutrients, or by producing digestive enzymes to breakdown organic matter into plant available forms. By introducing symbiotes or reclaiming soil with microbial properties in mind, revegetation success may be enhanced through improving plant nutrition (Ingram et al., 2005; Boldt-Burisch, 2018). As an example, Sahain et al. (2007) determined that a commercial biostimulant with 60 soil microbial species improved leaf N, P, and K, increasing fruit weight by 7.5%, while total bacteria, fungal, and actinomyces abundance increased 1-2-

fold each, relative to the control following amendment in reclaimed calcareous soil in Egypt. At the same time, if N-fixing bacteria *Rhizobacteria* spp. are present in the inoculation, there may be an increase in the rate of atmospheric N fixation and conversion to ammonia, supporting plant growth and SOC (Hayat et al., 2010; Pagano and Miransari, 2016).

In addition to N, P uptake in most terrestrial plant roots can be increased with symbiotic relationships to arbuscular mycorrhizal fungi (AMF; Schüßler et al., 2001). Arbuscular mycorrhizal fungi form arbuscules (nutrient exchange sites) and vesicles (nutrient storage site) in host plant roots. The hyphal network produced by AMF in and around the rhizosphere act as an extension of the plant roots, increasing the total surface area for P acquisition through enzymatic activity. The N and C pools in reclaimed soil can also be positively influenced with AMF (Kuimei et al., 2012; Zhang et al., 2019). In the presence of AMF, SOC and ammonium (NH_4^+) was significantly increased, while also reducing soil salinity levels, known to affect plant growth (Zhang et al., 2019). Similarly, Kuimei et al. (2012) observed that AMF were positively associated with SOC increases in reclaimed mining soil versus soils without AMF, indicating AMF's role in potential SOC accumulation in disturbed soil. However, AMF alone may not be enough to significantly improve reclamation in severely disturbed landscapes, such as mine tailings (Boldt-Burisch, 2017), as there is evidence microbially diverse soils are more successful in improving plant growth and microbial recovery (Colla et al., 2015; Rouphael and Colla, 2018; Gu et al., 2020), suggesting that the greater diversity of species in products will allow for greater microbial resilience.

As climatic regimes are expected to change globally, abiotic stressors may become more prevalent and extreme in coming years (Begum et al., 2019). Water is essential for microbial activity and for plant function to produce biomass, however, reclaimed soil in semi-arid regions experience frequent water shortages that threaten biomass production and microbial populations. Fortunately, studies have reported plant-AMF associations can increase water-use efficiency and grain yield significantly greater than non-inoculated soil, due to the increased soil exploratory capabilities of hyphal networks (Al-Karaki et al., 1998; Yildirim et al., 2006; Afshar et al., 2014), improved stomatal conductance (Augé et al., 2015), or manipulating enzymatic antioxidant levels (Li et al., 2019). The association between AMF and plants can also significantly reduce plant stress resulting from salinity (Sylvia et al., 1993; Begum et al., 2019; El-Shazly, 2020). Although salt accumulation in soil can occur naturally, it may transpire unnaturally in

irrigated croplands (Shehzad et al., 2020), reclaimed areas where subsoils with salts are mixed with topsoil (Wick et al., 2009), or when deep-rooted native vegetation is cleared and replaced with shallow-rooted annual vegetation which mobilizes salts upwards with the water table (Hatton et al., 2003; Wong et al., 2008). In induced drought stress, Yildirim et al. (2006) compared six different microbial plant biostimulants to observe salinity tolerance in squash, and reported all treatments containing either bacteria or fungi produced significantly greater growth than the control, and helped reduce Na uptake by the plant while maintaining adequate K:Na ratios within plants. By reducing stress, plant growth can be improved and provide greater root and shoot biomass that can return to the soil for microbial decomposition. This relationship can be explored easily by reclamation professionals as numerous companies have inoculation products in mulches, sprays, or other materials, that may improve reclamation success in regions with environmental conditions that challenge soil reclamation.

Conclusion

Ultimately, soil biological properties are necessary to restore to rebuild nutrient pools, SOC, and soil functioning. Biostimulants offer promises for the agricultural sector, but could be more studied in largescale reclamation studies. From the available research, it seems hopeful that the organic amendments outlined can enhance soil reclamation from myriad disturbances, and may display synergistic properties when applied together. Specifically, foliar application of biostimulants may be more successful in quickening reclamation timelines when applied with microbial inoculants and industrial waste. However, this literature review process demonstrated some biostimulant products are difficult to transparently report and compare evenly due to technological copyrights, or incomplete ingredient lists, and rarely were more than one product examined at a time. Therefore, there is a need for further studying the effect of these products when- two or three products are used together, which may help determine efficient waste streams and partners for reclamation in the regions the disturbances are occurring.

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CHAPTER 2. PLANT GROWTH, SOIL PROPERTIES, AND MICROBIAL COMMUNITY FOUR YEARS AFTER THERMAL DESORPTION[1] [1]

Abstract

The effects of thermal desorption (TD) on soil physical and chemical properties after crude oil contamination are recently well-studied. However, there is limited field-scale studies on long-term soil biological property recovery such as microbial communities and plant growth, which are vital for meeting global agro-system demands and restoring ecosystem health. This study describes the status of soil biological properties after four years of crop production on oil-contaminated cropland remediated via TD and a modified land farming technique. Plots were constructed in 2015 with native, uncontaminated topsoil (A), TD-treated subsoil (TDU), untreated land-farmed subsoil (SP), TDU+A (TDA) and SP+A (SPA) where soil ratios were 1:1 by volume, and composted manure was applied at 40 Mg ha^{-1}. After three years of crop production (2019) grain sorghum (*Sorghum bicolor* L.) was planted. Soil microbial community characteristics were assessed through phospholipid fatty acid analysis and by estimating mycorrhizal root colonization. Notably, inherent soil chemical and physical properties influenced the recovery of microbial communities in remediated soils. However, sorghum biomass production in TDU was 50 $\pm$ 9% greater than SP, while the microbial abundance in these treatments remained similar. Mycorrhizal colonization variation likely reflected rhizosphere nutrient scarcity and not the interactions of either remediation strategy. Based on these results after four-years of cropping, TDU does not diminish soil microbial recovery and when possible, blending TDU materials with topsoil provides the greatest level of recovery relative to topsoil only.

Introduction

An increase in oil infrastructure development within agricultural lands has presented accidental crude oil releases that diminish soil function (i.e., physical, chemical, and biological properties). Crude oil

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contains petroleum hydrocarbons (PHC's) that when exposed to soil or water, pose health risks to humans and livestock, alter soil chemical properties, reduce biological activity (Rowe et al., 1973; Townsend et al., 2003; Lopez et al., 2007; Klamerus-Iwan et al., 2015; O'Brien et al 2018; Croat et al., 2020b), and necessitate soil remediation in order to return the land back to productivity. In this paper, soil remediation describes the process of removing or stabilizing contaminants from soil, while reclamation involves the process of replacing remediated soil back and returning the land to natural or economical productivity. Numerous soil remediation strategies exist to reclaim soil after contamination, such as dig and hauling to a landfill, landfarming, TD, and others (Bekele et al., 2015; O'Brien et al. 2016; O'Brien et al., 2017b; Green et al., 2020), all which risk or exacerbate topsoil loss. Overall, the remediation and reclamation method chosen, and inherent soil characteristics will influence the time and cost to recover soil ecosystem function and land productivity.

Ex-situ TD is an effective method to remediate PHC contamination in a short amount of time (Khan et al., 2004), and is achieved by excavating and heating contaminated soils to temperatures ranging from 100 to >600°C, depending on organic contaminant type. Thermal desorption facilitates quicker remediation times than the land-farming or dig-and-haul methods while avoiding putting soil into landfills, but is more expensive due to the required machinery and energy inputs. Additionally, TD can drastically alter soil physical, chemical, and biological properties (Khan et al., 2004; Vidonish, et al., 2016; O'Brien et al., 2018). Changes in aggregation and porosity from excavation and soil handling have been reported (Guebert and Gardner, 2001; Wick et al., 2009; Gasch et al., 2014). Piña et al. (2002), Khan et al. (2004), and O'Brien et al. (2016) also observed significant reductions in soil organic carbon (SOC) and biological enzymatic activity, but some low-level TD treatments (<300°C) can be less harmful to soil properties (O'Brien et al., 2018). Regardless, the overall long-term effects of TD on soil function should not be ignored (Yi et al., 2016).

Despite recent progress in connecting the effects of TD to soil function (Cébron et al., 2011; O'Brien et al., 2016; Croat et al., 2020; Croat et al., 2020b), there has been limited field-scale opportunities to assess long-term plant and soil microbiological recovery from TD. Due to the growing pressure on food-agrosystems and the numerous soil properties benefitted by microbial processes, soil microbial recovery in disturbed lands is gaining interest worldwide. As one of the largest participants of

soil ecological processes, soil microbial communities are dynamic and change in response to weather, soil water content, vegetation community or crop, or the season of the year, and may be especially more variable in semiarid climates (Collins et al., 2005; Frank et al., 2006; Viall et al., 2014). Likewise, microbiota are sensitive indicators for environmental stressors (Schloter et al., 2018). For example, decomposers such as saprotrophic fungi are integral for carbon (C) cycling via decomposition of a variety of organic materials (cellulose, lignin; Deacon et al., 2006), but are sensitive to changes in environmental conditions such as pollutants, temperature regime, and physical disturbance (Cho et al., 2017). Other fungi such as arbuscular mycorrhizal fungi (AMF) are crucial for providing phosphorus (P), water, and pathogen resistance to symbiotic plants, while also improving soil structure and soil organic matter retention (Ingram et al., 2005; Thavamani et al., 2017), and are still sensitive to environmental changes. Soil bacteria can fix nitrogen, improve plant health and stress resistance, and restore organic carbon (OC) into the soil system (Hayat et al., 2010), improving nutrient availability, and thus crop production (Pinto et al., 2004; Xu et al., 2019). Presently, reclamation success is generally measured relative to crop yield or vegetative cover, which ignores soil microbial recovery (Mummey et al., 2002; Dangi et al., 2012).

Because the goal of reclamation is to repair disturbed ecosystems (including plants, soil, and topography) to sustainable, healthy conditions, the long-term soil biological recovery after PHC-remediation should be incorporated into the determination of reclamation success. Accordingly, using three treatments this study describes the recovery of various soil biological properties after crude-oil remediation and four years of crop rotation. The assessed treatments were a modified land farming technique, TD, and mixing remediated soils with native, non-contaminated topsoil (O'Brien et al., 2016; O'Brien et al., 2017b). The objectives of this study were to 1) evaluate treatment effects on grain sorghum (*Sorghum bicolor*) growth and associated soil properties across treatments; 2) quantify AMF colonization in grain sorghum roots and AMF abundance in soil among treatments, and 3) determine the abundances of soil microbial groups. Results from this study may assist in determining successful reclamation strategies that balance time, cost, and energy in order to enhance the physical, chemical, and biological properties of reclaimed or highly disturbed soils.

Materials and Methods

Study Site Description

In 2013, a pipeline released 21,000 barrels of Bakken crude oil (API gravity 42; sulfur < 0.2%) onto farmland surface and subsurface soils in Mountrail Co., ND (48.523804, -102.859061; O'Brien et al., 2018; Croat et al., 2020). The native topsoil was mapped as a Williams-Zahl loam complex (Williams: Fine-loamy, mixed, superactive, frigid Typic Argiustolls; Zahl: Fine-loamy, mixed, superactive, frigid Typic Calciustolls) (Soil Survey Staff, 2020). Williams series are economically important soils for crop production and is the representative state soil (Daigh et al., 2016). According to the Köppen-Geiger climate classification system, the study region is BSk cold semi-arid (Peel et al., 2007). Total growing season (May-October) precipitation received in 2019 was 655 mm where potential evapotranspiration (PET) was 836 mm during the same time period (NDAWN, 2020). Deviations from normal of air temperature, rainfall, and PET can be found in Table 3.

In 2015 contaminated non-contaminated topsoils were excavated and stockpiled to represent the expected handling of topsoil during oil and gas activity, and is assumed to be the control condition of disturbed soils used for crop production. Contaminated subsoil was excavated up to 15 m deep, homogenized, and treated using TD to decrease the total petroleum hydrocarbon (TPH) levels to 500 mg kg^{-1} or less (Croat et al., 2020). Petroleum-contaminated subsoils were stockpiled and continuously mixed via agronomic field disk while awaiting thermal treatment for several months. The continuous mixing was done to homogenize the TPH levels prior to TD, which typically ranged, post-disking, to between 2,000 and 5,000 mg kg^{-1}, and was similar to land farming except for water or nutrient additions. While remediation was occurring, research plots were constructed in fall, 2015. Soil treatments were: non-contaminated topsoil (A; control); PHC-contaminated and stockpiled subsoil that was thermally desorbed (TDU); untreated, PHC-contaminated stockpiled and land farmed subsoil (SP), and added composted livestock bedding manure (m) applied at 40 Mg ha^{-1} to the 0-15 cm depth to create the following paired treatments: A, A+m, SP, SP+m, TDU, TDU+m, SP+A, SPA+m, TDA, and TDA+m, where soil ratios were 1:1 by volume. Each plot was 17 m x 15 m x 0.9 m, with a surface area of 765 m^2 in a randomized complete block design and replicated three times (O'Brien et al., 2017a). Table 4 provides a description of soil and composted manure chemical data.

Table 3. Growing season air temperature and rainfall 30-yr averages (National Oceanic and Atmospheric Administration Regional Climate Centers xmACIS, 2020). The 18-yr potential evapotranspiration (PET) and 2019 rainfall was collected from the Ross, ND reporting station within the North Dakota Agricultural Weather Network (NDAWN, 2020).

Month	30-yr average	2019 Growing Season	Departure from 30-yr average
	Air Temperature (°C)		
May	12.8	9.44	-3.36
June	17.8	16.7	-1.10
July	21.1	18.9	-2.20
August	20.6	17.8	-2.80
Sept.	13.9	13.3	-0.60
Oct.	6.67	2.22	-4.45
	Rainfall (mm)		
May	53	21	-32
June	71	208	137
July	63	136	72.8
August	42	73	31.2
Sept.	31	203	172
Oct.	26	15	11.0
	PET (18-yr; mm)		
May	6.8	7.0	0.2
June	6.9	7.4	0.5
July	7.7	7.1	-0.6
August	6.9	6.0	-0.9
Sept.	5.0	3.2	-1.8
Oct.	2.9	2.3	-0.6

Crop Production and Sorghum Sampling

Starting in 2016, the four years of crop production were hard red spring wheat (*Triticum aestivum* L.) variety Barlow, field pea (*Pisum sativum* L., variety Birdsall) in 2017, hard red spring wheat in 2018 variety Glenn, and for this study, grain sorghum variety 419x124 in 2019. Crop rotations, and specifically grain sorghum, was chosen to match the surrounding crops being produced by the landowner. Additionally, there has been recent interest in growing grain sorghum in western ND due to its characteristically drier and warmer climate than other portions of the state. The plots were always maintained under no tillage. Agronomic details from 2016-2018 can be found in Croat et al. (2020), and in 2019 glyphosate herbicide was applied at a rate of 0.84 kg a.e. ha^{-1} to control any volunteer vegetation in the spring before planting. On day of year (DOY) 188 granular urea (46-0-0) and mono-ammonium phosphate (11-52-0) were hand-broadcasted onto plots at normalized rates to reach 101 kg N ha^{-1} and 90

kg P ha^{-1} from soil samples from the 0-15 cm depth, and was incorporated less than 24 hours later from seeding. On day of year 189 an 85-day grain sorghum variety 419x124 was drilled to 25-cm row spacing at 9 kg seed ha^{-1}, a recommended seeding rate (Graham and Beck, 2019). On DOY 199 dicamba (3,6-dichloro-2-methoxybenzoic acid) and Starane [(4-Amino-3,5 dichloro-6-flouropyridin-2-yl)oxy]acetic acid was tank mixed and hand broadcast applied at concentrations of 2.5 mL L^{-1} and 1.6 mL L^{-1}, respectively, and did not exceed 1.16 L a.i. ha^{-1} and 0.47 L a.i. ha^{-1}. These mixing concentrations were recommended for control of kochia (*Kochia scorpia*), and other broadleaves, but most importantly, safe for grain sorghum at its present growth stage (Personal communication, K. Howatt, 2019). This was the second and final herbicide application of 2019.

On DOY 213, at the 3rd vegetative leaf stage, roots and approximately 1000 cm^3 rhizospheric soil (10 cm x 10 cm x 10 cm) of three random plants from each plot, were collected with a shovel, sealed in plastic bags, and transported the same day in coolers with ice to NDSU where each sample was moistened to field capacity using DI water and then placed into a refrigerator. To ensure roots contained observable AMF colonization, roots were randomly sampled in plots and analyzed on DOY 193. Additionally, sorghum roots have been observed to be colonized by AMF within 40 days after planting (de Oliveira et al., 2020). Following a 24-hr refrigeration period, each subsample had soil 10 g of soil collected and combined according to plot ID (30 total soil samples), and frozen at -15 °C. Then, roots were carefully washed over a 2-mm screen until all visible residual soil was removed. Clean roots were cut from the plant and preserved in centrifuge tubes with formaldehyde-acetic-acid (FAA; by volume: 2-parts formaldehyde, 1-part glacial acetic acid, 10-parts 95% ethanol, and 7-parts deionized water) until ready to stain and examine under a microscope (Phillips and Hayman, 1970).

Aboveground plant material was hand harvesting from 1 m^2 quadrats from each plot occurred on DOY 290. Grain heads were first cut and placed into paper bags. Biomass samples were cut at the ground, kept in closed cloth bags, and weights recorded immediately after sampling. All samples were transported to NDSU on the same day and placed in a forced-air drying oven (60 °C, 168 hr), after which dry biomass was recorded. Grain heads were dried to below 12% moisture content, and were threshed (Agriculex SPT-1, Canada) to remove non-spike plant material. Finally, the grain was cleaned to remove chaff and immature seeds and final mass was recorded and adjusted for 12% moisture content.

Soil Sampling

Soil samples (0–15 cm) were taken following spring wheat harvest in fall 2018 using hand probes and are discussed in Croat et al. (2020). Samples were air-dried and ground to pass through a 2-mm sieve. Total C and total inorganic C were determined using a Primacs TOC Analyzer (Skalar Analytical B.V., Breda, Netherlands). Soil organic carbon was then calculated as the difference between total C and inorganic C. Soil nutrients were analyzed by a third-party laboratory (Agvise Laboratories, Northwood, ND) where soil N was quantified as the sum of NO_3-N and NH_4-N determined by KCl extraction (Mulvaney, 1996) and soil P was quantified by the Olsen method (Frank et al., 1998).

Table 4. Characteristics of the soil treatments and composted manure used in this study. Total carbon (TC), Total nitrogen (IN), phosphorus (Olsen-P), potassium (K_2O), Calcium (Ca), Magnesium (Mg), Zinc (Zn), Sulfur (S), and Sodium (Na).

Treatment†	TC	TN	P_2O_5	K_2O	Ca	Mg	Zn	S	Na
	%	mg kg^{-1}							
A	2.14	1820	16.0	282	9190	561.0	0.70	13.3	19.8
SP	1.94	635.0	7.83	161	4750	860.0	0.30	48.5	65.3
TDU	1.94	538.0	7.50	220	4720	607.0	0.70	38.5	76.8
SPA	2.13	1390	17.8	213	3970	640.0	0.60	23.8	32.8
TDA	1.99	1180	13.5	259	4260	565.0	0.70	17.0	43.5
Composted Manure	6.8	7000	2700	6000	11500	4400	45.0	1000	325

† Treatments are: native, non-contaminated topsoil (A); stockpiled contaminated, untreated subsoil (SP); stockpiled, contaminated subsoil and topsoil mixed 1:1 by volume (SPA); thermally desorbed contaminated subsoil (TDU); thermally desorbed subsoil and topsoil mixed 1:1 by volume (TDA).

Quantification of Mycorrhizal Colonization

A modified staining procedure was adopted from Koske and Gemma (1989) and Phillips and Hayman (1970) in order to estimate the percentage of microscope views of root segments containing AMF structures. Upon sampling, root color and texture were used to distinguish any dead roots from the live roots, as dark colors and rigidity may indicate dead roots (Bernaola et al., 2018). Live adventitious roots were cut from the aboveground plant mass with forceps, and preserved in FAA until they were ready to be processed. FAA contains formalin and is a compound commonly used to preserve cellular structure, and was chosen to retain root sample integrity over a certain period of time. After preservation, the roots were rinsed in deionized (DI) water to remove FAA, and roots $\leq$ 1-mm were carefully removed from larger fibrous roots with forceps, and cut into 1-cm segments. Commonly, the primary, older roots

are larger and contain more pigmentation, potentially requiring greater clearing time than smaller, younger roots (Phillip and Hayman, 1970). Roots greater than 1 mm in diameter were found to be too large to place on microscope slides with slide covers, and therefore cannot be examined for mycorrhiza correctly.

Segments were then placed in 40 mL test tubes with 10% potassium hydroxide (KOH; 10% weight/volume) aqueous solution and submerged in 90 °C water bath for 15 min for clearing. In this process, the pigmentation is removed from roots to allow for increased contrast between the plant root and fungal biomass (Phillips and Hayman, 1970). Next, segments were rinsed thoroughly with DI water and stained in lacto-glycerol trypan blue (by volume: 1-part lactic acid, 1-part glycerol, 1-part water, 0.00066-part trypan blue) for 15 min. Finally, the stained root segments were rinsed and suspended with DI water in a watch glass beaker cover. Random selection of root segments was accomplished by mixing the roots evenly, and allowing them to settle. After which one 1-cm root segment was extracted with tweezers. One root was randomly chosen after each subsequent mixing and settlement. The ten selected segments were mounted on 22x40 mm glass slides with glycerol for viewing.

The procedure for estimating percent root colonization was modified from Allen and Allen (1980). Under 40x magnification, five passes were taken across all ten root segments for a total of 50 observation points per slide, and 150 observation points for one treatment of a replicated block. In accordance to the procedure, each observation point was marked for the presence or absence of AMF structures including AMF hyphae, vesicles, or arbuscules. Finally, percent root colonization was determined as the total observation points with AMF structures present over the number of total observed views. No AMF species-specific structures or characteristics were recorded.

Microbial Biomarker Analysis

Phospholipid- and neutral lipid fatty acid (PLFA and NLFA) analysis offers a snapshot of the microbial community at the time of sampling, and was used to assess microbial group abundances of the remediated soils. The PLFA method of analysis reports data as estimates of broad taxonomic groups (actinomycetes, AMF, bacteria, fungi, and protists/eukaryotes), and is considered a useful method for microbial community analysis (Ramsey et al., 2006). Soil samples were prepared by freeze drying 10 g of the composite samples. After, samples were shipped and processed for phospholipid and neutral lipid

abundances by Microbial Identification Labs, Inc. (MIDI; Newark, DL). The MIDI lab follows the lipid extraction procedures following Buyer and Sasser (2012), and subsequent analysis using gas chromatography. The neutral lipid fatty acid (NFLA) analysis was included to provide a measure of AMF in the field soils to complement the PLFA biomarker associated with AMF (16:1w5c) (Sharma and Buyer, 2015). The NFLA extraction and quantification follow the PLFA methodology with the addition of an internal standard of 19:0 trinonadecanoin glyceride and collection of the neutral lipid fraction in chloroform elution (Sharma and Buyer, 2015). Peaks were identified by MIDI and the Sherlock Chromatographic Analysis System software and the PLFAD2 peak naming table. The abundance of each microbial group is considered in terms of absolute abundance (nmol fatty acid g^{-1} soil) as opposed to relative terms.

Gram+ and Gram- Bacteria

Gram+ to Gram- soil bacterial ratios were reported by MIDI labs using the respective biomarkers, included in Appendix A. These ratios have been found to exhibit dynamic population levels that fluctuate with SOC concentrations, and studies analyzing Gram+/Gram- ratios have reported such ratios can be used as indicators of SOC input quality and quantity entering the soil system (Kourtev et al., 2002; Zhang et al., 2013). Because of the reduction of SOC that often occurs during remediation, scarce SOC may cause stress to microbial processes by limiting biomass accumulation, available water, and nutrient availability until adequate levels are restored. Therefore, analyzing Gram bacterial abundances may be a potential tool for suggesting stress in the reclaimed soil system and its recovery after four years of crop production.

Statistical Analyses

To answer the objectives of this study both manure and soil treatments were considered as factors in analysis. All response variable differences in means were calculated with a two-factor analysis of variance (ANOVA), using manure and soil treatment as factors, at $\alpha = 0.05$, with Tukey's Honestly Significant Difference post-hoc test in SAS (SAS version 9.4, SAS Institute, Cary, NC) using Proc GLM. If the combined soil and manure model was not significant for a given variable, it was reported as such. Linear regression was used for observing the relationships between various biological and chemical data collected, and was reported with R^2 and confidence interval value. SAS Proc REG (SAS version 9.4, SAS

Institute, Cary, NC) was run for all regressions. The response variables analyzed were total microbial abundance-aboveground biomass yield.

Multivariate principal components analysis (PCA) was conducted on microbial group abundances and soil chemical data on correlations to explore how our multiple response variables varied across treatments using JMP 14.0.0 (SAS Institute, Inc.). Factor loadings were conducted on the principal components to find correlation coefficients between observed variables and common factors influencing them. Additionally, response variables were assessed for correlation with JMP 14.0.0's multivariate analysis tool, and correlations were recorded.

Results and Discussion

Sorghum Yield and Biomass

Grain yields were not significantly different among soil treatments, between composted manure treatments (with or without), or in the SxC interaction term (Table 5). Since grain sorghum is a new crop to this region no county or regional data was available for comparison. Past studies have reported wheat yields in the A topsoil reached the county average, indicating A is a suitable medium for reclamation and justifies its use for comparison of biological metrics. Lack of significant findings, and relatively large standard deviations were likely driven by the reoccurring cold stress soon after planting and above-average growing season precipitation, which prohibited seed heads from reaching maturity in the 2019 growing season (Table 3), and should not be interpreted as crop yield recovery. Biomass production varied among soil treatments and between the composted manure, but not in the SxC interaction term. Blending remediated subsoil and non-contaminated topsoil 1:1 was successful in reaching the A topsoil, and agrees with past studies conducted on the study site. In the remediated subsoil treatments, our findings diverge from previous studies where TDU and SP produced similar grain and aboveground biomass production over three years (Croat et al., 2020). Biomass in 2019 was significantly least in treatment SP, producing 45% of biomass relative to all other treatments (11.1 Mg ha^{-1}). Soil that received composted manure produced significantly more biomass than nontreated plots. Overall, these findings agree with multiple studies which reported the addition of topsoil to subsoil improved crop yields, and likely reflects the differences in SOC between treatments (Power et al., 1981; Roh et al., 2000; O'Brien et

al., 2017b; and Croat et al., 2020). However, this does not explain the increased biomass in TDU production for 2019.

Plant biomass production is positively associated with soil organic matter (plant material, microbial tissue) due to its associated SOC content, availability of nutrients, and water holding capacity regulation (Anderson et al., 2008). Yet, TDU yielded nearly double SP's biomass production despite similar SOC levels. Importantly, the past PHC contaminants were not likely a factor of biomass production. O'Brien et al. (2019) reported plots SP, SPA, TDU, and TDA contained initial (December 2015) PHC contents of 1394, 678, 229, and 110 mg kg^{-1}, respectively, with half-lives ranging from 455 to 573 days. Thus, for treatment SP, the predicted May 2019 PHC concentration would have been 208-308 mg kg^{-1}. Because PHC contamination has been observed to decrease sorghum growth at concentrations of $\geq$1000 mg kg^{-1} PHC (Banks et al., 2010; Iheme et al., 2017), primary cause of biomass production differences is likely a result of altered soil properties following thermal treatment.

Thermal desorption has been observed to increase soil P availability via conversion of organic P to inorganic forms (Yi et al., 2016; Liu et al., 2019; Croat et al., 2020b). In addition to increasing P availability in the topsoil and subsoil used in this study, TDU increased the amount of P sorption (S_{max}) and the affinity (bonding energy*; k*) P has for a soil surface (Croat et al., 2020b). Other factors affecting P-availability such as pH or clay content were relatively unchanged from TD (O'Brien et al., 2016), but aboveground biomass and P availability were moderately associated in 2019 (r = 0.31). The multivariate analysis in Figure 1 shows TDU samples were slightly more associated with bioavailable P (Olsen-P) and biomass production than treatment SP, and both positively associated with Principal components 1 and 2, which account for >75% of the data's variation. However, the flush of P availability from TDU is expected to happen only once, and the associated increase in k and S_{max} thresholds for TDU may risk the removal of P from plant and microbial pools via sorption to soil surfaces potentially leading to crop production decline without proper nutrient management (Croat et al., 2020b). In our study, Olsen-P values were not significantly different between subsoil treatments. Therefore, these results suggest 1) while the flush of available P has occurred, the legacy k and S_{max} values were not deleterious to crop production with adequate nutrient management/fertilization, and 2) other TD-related changes, such as soil-water relationships, may also be influencing biomass yields.

Table 5. Mean values (with standard deviation) of sorghum grain yield, aboveground biomass (AGB), AMF root colonization percentages, arbuscular mycorrhizal fungi (AMF) abundance, and soil organic carbon (SOC) for each treatment in 2019. Treatments are: native, non-contaminated topsoil (A); stockpiled contaminated, untreated subsoil (SP); stockpiled, contaminated subsoil and topsoil mixed 1:1 by volume (SPA); thermally desorbed contaminated subsoil (TDU); thermally desorbed subsoil and topsoil mixed 1:1 by volume (TDA).

Effects	Level	Grain Yield	AGB	Root Colonization	AMF Abundance	SOC	P
		Mg ha^{-1}		%	nmol g^{-1} soil	g kg soil^{-1}	
Soil (S)	A	0.89 (0.50)	27.0 (7.73) a[b]	50.8 (29.4) bc	3.63 (0.86) a	18.9 (0.28) a	16.0 (4.00) ab
	SP	0.44 (0.57)	11.1 (4.79) b	61.1 (28.4) a	1.86 (0.65) b	5.08 (0.08) c	7.83 (4.67) b
	TDU	1.06 (0.88)	22.1 (2.07) a	44.1 (27.8) c	2.43 (0.65) ab	3.95 (0.08) c	7.50 (2.26) b
	SPA	0.94 (0.42)	21.5 (3.12) a	53.3 (27.6) ab	3.00 (0.73) ab	12.2 (0.16) b	17.8 (7.30) a
	TDA	1.13 (0.57)	27.6 (7.49) a	58.7 (28.1) ab	3.01 (0.73) ab	10.3 (0.09) b	13.5 (8.09) ab
	P-value	ns[a]	***	***	*	***	*
Composted Manure (C)	40 Mg ha^{-1}	1.03 (0.68)	24.0 (8.10) a	57.0 (11.3)	2.88 (0.78)	10.7 a	10.4 (5.37)
	0.0 Mg ha^{-1}	0.76 (0.56)	19.7 (7.47) b	53.1 (7.80)	2.69 (1.05)	9.40 b	14.7 (7.56)
	P-value	ns	*	ns	ns	*	*
S x C		ns	ns	*	ns	ns	ns

*P<0.05

***p<0.001

[a]ns, not significant

[b]Values not followed by a common letter within columns and effect are significantly different at α= 0.05 in a post-hoc Tukey's Honestly Different test.

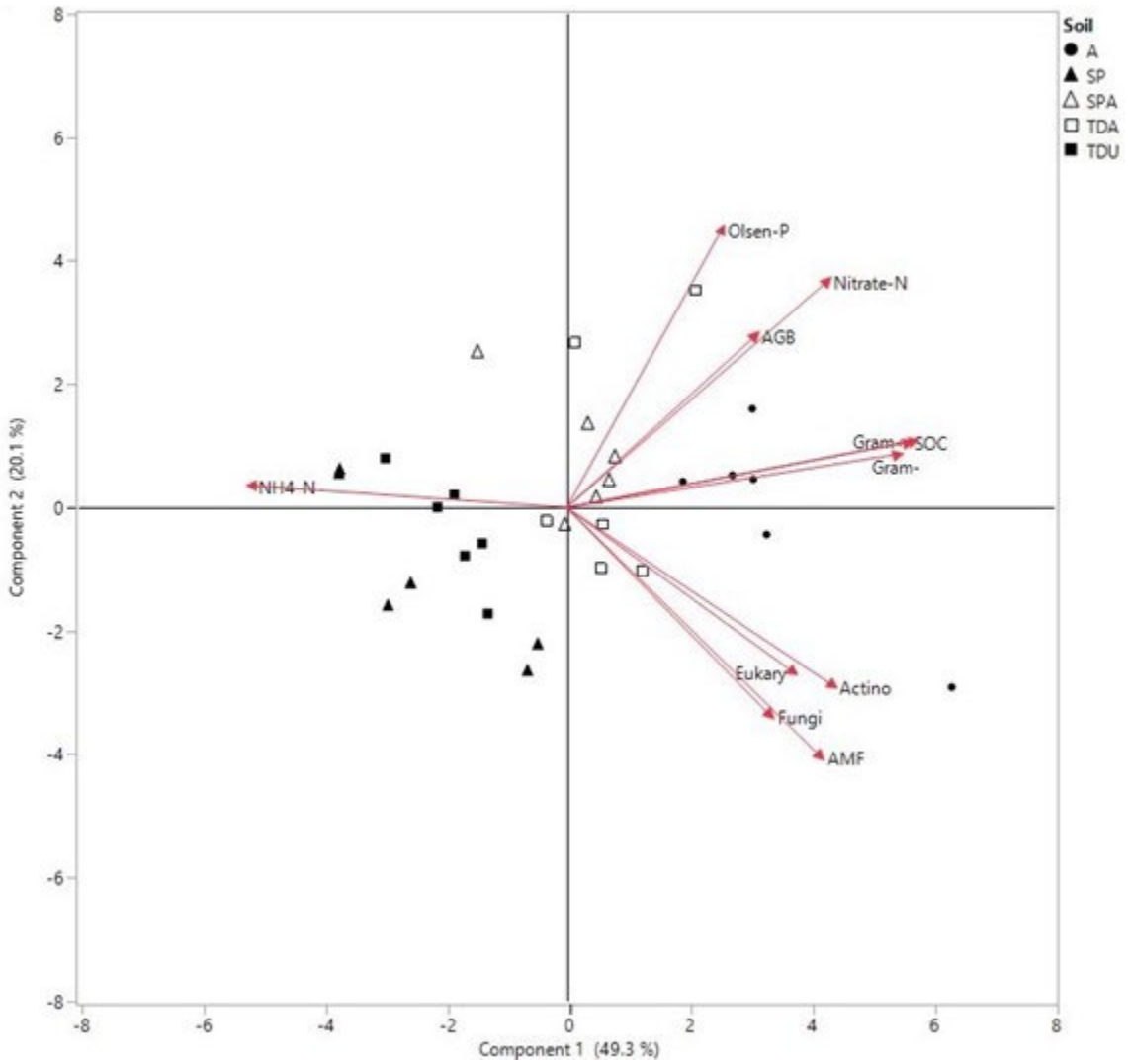

Figure 1. Principle component analysis of biological and soil chemical data with loadings identified by arrow and text annotation. Amount of variance explained is 49.3% (component 1) and 20.1% (component 2). Samples are identified by soil treatments, which are native, non-contaminated topsoil (A); stockpiled contaminated, untreated subsoil (SP); stockpiled, contaminated subsoil and topsoil mixed 1:1 by volume (SPA); thermally desorbed contaminated subsoil (TDU); thermally desorbed subsoil and topsoil mixed 1:1 by volume (TDA). [Note] Actino- Actinomycetes, AMF- Arbuscular mycorrhizal fungi, Eukary- Eukaryotes, Fungi- Saprophytic fungi, Gram- - Gram negative bacteria, Gram+ - Gram positive bacteria, AGB- Aboveground dry biomass, SOC- soil organic carbon.

AMF Root Colonization

Arbuscular mycorrhizal root colonization was similar among the soil treatments except the A and TDU (Table 5). Arbuscular mycorrhizal fungi colonization data in SPA and TDA similar to the A were likely a result of the SOC levels from the A topsoil, which can enhance some AMF activity (Yang et al, 2011). In the subsoil, greater colonization in SP compared to the control disagrees with previous studies where AMF colonization has been observed to be negatively associated with PHC contamination (Cabello,

1997; Franco-Ramirez et al., 2007). Importantly, the past crude oil contamination concentrations in this study were likely not inducing the AMF-sorghum response due to the half-life associated with the hydrocarbons (O'Brien et al., 2019). But, AMF root colonization between SP and TDU did not follow the same trends as SPA and TDA. This suggests the greater colonization percentages observed in SP were likely a result of altered S_{max} and k thresholds (Croat et al., 2020b), and water dynamics following TD.

Notably, O'Brien et al. (2016) reported a 400% increase in K_s values in thermally desorbed topsoil and subsoil, and a change in soil hydraulic properties that resulted in hydraulic characteristics more similar to a sandy loam than a loam soil. Infiltration, which is crucial for providing water to roots for photosynthesis, microbial respiration, and nutrient transport (Olsen and Kemper, 1968; Minasny and McBratney, 2017), is generally related to soil texture, among others (O'Brien et al., 2016). Not only could TDU have achieved greater downward water movement during periods of saturation, such as the intense precipitation in June through September, but TDU could also have had increased infiltration- regardless of the amount soil saturation. If greater quantities of water traveled through the rhizosphere, more P may have been able to reach the root-zone and influence AMF colonization. For example, composted manure applied at 44 Mg ha^{-1} decreased AMF root colonization of wheat and corn by 20% compared to treatments that didn't receive compost and its added nutrients (Tarkalson et al., 1998). From a soil ecological perspective, this suggests translocation of nutrients in TDU may have reduced the competition between microbial species and plants when P was adequately available.

Conversely, SP soils in this experiment displayed lower K_s,values and could be more prone to ponding and runoff during intensive precipitation events, and may have resulted in P transporting from the plots. Lower k values in SP may also further increase the risk of P solubilization and loss via runoff (Croat et al., 2020b). The runoff and transport of soluble P from the SP plots likely reduced the quantity of P fertilizer transported to the rootzone of the subsoils. As a result, AMF root colonization may have been positively influenced in SP compared to TDU to increase rhizosphere competition with other microbes (St. John et al., 1983; Bisht et a., 2015), while TDU experienced less runoff and greater transportation of P and other nutrients, and potentially produced greater sorghum growth in TDU than in SP. This notion is further supported in Nagahashi et al. (1996) and Gadkar et al. (2001) where P amendments applied to soil suppressed AMF hyphal growth and plant exudates which can encourage AMF spore germination,

hyphal growth, and soil exploration. Inside the plant, genes regulating the expression of nutrient transportation from fungus to plant is usually related to plant nutrition, which can be affected by mycorrhizal-colonized roots (Burleigh and Bechmann, 2002). Specifically, gene expression was reported to be down-regulated by P-fertilization (Liu et al., 1998), suggesting that if plant nutrient demand is being met, the symbiosis is not as crucial for plant survival. Similarly, nutrient transfer from fungus to plant can also result in transporter gene expression to be lower in colonized roots.

Additionally, four years of greater infiltration in TDU soil compared to SP could have allowed for a greater reservoir of plant-available water at the start of the growing season (Data not collected). Total growing season precipitation (May–October) received at the study site in 2019 was 656 mm, and accounted for 59% of the previous four year's precipitation levels combined. While semi-arid climates can and do receive smaller, infrequent precipitation events, which may limit plant growth and ecosystem recovery (Miransari, 2010), semi-arid soil ecosystems can rapidly respond to precipitation events. Collins et al. (2005) modeled precipitation cycles in arid lands, reporting that while dry periods limited primary production, it led to a net accumulation of nutrients in the soil, and any precipitation events quickly stimulate biological activity and plant growth (Collins et al., 2005). Revealed is a dynamic biological response to precipitation in semi-arid soils, suggesting AMF colonization may have been influenced by both runoff in SP soil containing P, and altered soil water dynamics in TDU which potentially transported more P to the rootzone following greater-than-normal precipitation. Regardless, it can be concluded grain sorghum and AMF symbiotic relationships were not impacted by either remediation method or soil mixing, but it remains to be answered if AMF abundance played a role in root colonization.

The NFLA biomarker 16:1w5c was considered when assessing AMF biomarker abundance, and this fraction is useful in determining storage lipids, such as carbon transport systems and spores (Olsson and Johansen, 2000; Ngosong et al., 2012). The NLFA analysis did not vary among soil, composted manure, or SxC interaction term. The lack of variation in NFLA AMF biomarker abundance may suggest remediation disturbances and/or grain sorghum did not affect spore counts among treatments. Interaction of grain sorghum on NLFA marker abundance is expected to be relative across all treatments as spore density is generally associated with plant community (i.e. host plant versus non-host plant), and seasonal variation (Ngosong et al., 2012; Silva-Flores et al., 2019). Meanwhile, PLFA AMF biomarker abundance

only varied between treatment A and SP, with SP achieving 39% of treatment A abundance levels (3.63 nmol g^{-1}; $p<0.05$).The A's AMF abundance is similar to reclaimed and undisturbed soil in western North Dakota that was sampled in the same month of the year, and was 3.98 nmol g^{-1} (Viall et al., 2014), suggesting A is an appropriate method to compare recovery for AMF.

Because host plants provide significant amounts of C to AMF for metabolism (Bago et al., 2000), the limited four-year plant growth in treatment SP likely decreased AMF populations compared to treatment A due to reduced photosynthesis. Because SP subsoil is not as suited to produce crops as topsoil, the reduced plant biomass production may have resulted in decreased transport of C to AMF symbiotes, whereas A carbon accumulation and transport was likely greater. Overall, increased AMF abundance could be used as a proxy for predicting crop yield potential in remediated subsoil, or perhaps in disturbed soils, based off the Tukey's post-hoc mean separations (Table 5). However, root colonization did not respond the same across the treatments, which was evident by low biomass production in SP despite greater colonization percentages compared to all other treatments. Ultimately, the findings of our study agree with Tarkalson et al. (1998), where wheat grown in subsoil contained greater AMF colonization but produced significantly less yields. A plant stress response in SP sorghum likely contributed to increased mycorrhizal colonization as well (Medina, et al., 2003).

Total Microbial Abundance

Total microbial abundance (as a sum of all biomarkers) varied among the soil treatments, but not between composted manure treatments or the SxC interaction term (Table 6). Expectedly, the A contained the overall greatest mean total microbial abundance of 74.3 nmol g^{-1}, and is slightly lower than the total microbial abundance reported in Vaill et al., (2014) of 80.6 nmol g^{-1}. Additionally, blending remediated subsoil with topsoil 1:1 produced similar total microbial abundance, and likely reflects the greater quality and quantity of SOC sources available for supporting greater microbial abundance (Kotroczó et al., 2014; O'Brien et al., 2017a). Total microbial abundance in our study was likely slightly lower due to the recent extraction, stockpiling, and replacement processes relative to Viall et al., (2014) or nearby undisturbed topsoil. Total abundance of all the various microbial groups were associated with SOC, as were the TDA and SPA soil treatments (Figure 1). Principal component 1 indicates that SP and TDU were more separated from the microbial groups and SOC than other treatments, which agrees with

Block et al. (2020) where microbial abundance and SOC content was greatest in shallow stockpiled (25-yr) topsoil rather than topsoil from the bottom of a stockpile, which was essentially functioning as subsoil.

Additionally, while SPA and TDA were separated on the biplot, mixing subsoil and topsoil allowed microbial populations to recover to levels comparable to the A alone after four years of crop production. This finding is supported by Dangi et al. (2005), who found that total biomarker abundance in a reclaimed semi-arid grassland soil was similar to undisturbed sites after five to 14 years. The greater SOC in treatments SPA and TDA likely helped biological communities recolonize over the subsoil-only treatments (Larney and Angers, 2012). Mixing A with TDU (1:1 ratio) resulted in a 25% reduction in total microbial abundance compared to the A alone (Table 5). Taylor et al. (2002) reported similar reductions in microbial biomass-C of 35% (silty clay loam), and 70% (sand) of subsoils at the 1.3-meter depth compared to the topsoil and was positively correlated with SOC content ($r > 0.90$). However, TDU, which contained the least microbial abundance (and similar to SP), yielded significantly higher plant production. This finding illuminates possible shortcomings that if reclamation success is assessed only on aboveground characteristics that soil function may not have yet fully recovered to reclaimed topsoil conditions.

With the exception of antagonistic microbial species, there is a positive association between soil microbial abundance and plant growth (Miransari, 2011). Total abundance was analyzed as a predictor in a regression with the harvested aboveground dry biomass as the response variable (Figure 2). Here, aboveground dry biomass production was positively related with total microbial abundance (R^2=0.2609; p<0.01). While plant biomass production in TDU suggests reclamation success, PLFA results of TDU did not differ from SP. Instead, plant production in TDU may have been similar to the A because of appropriate nutrient management, (chemical fertilizer), above-normal precipitation received during the growing season, and altered P-dynamics and K_s values as a result of TD. For example, when precipitation was less than 656 mm year^{-1}, plant productivity in treatment TDU was similar to treatment SP, and statistically less than yields in treatments A, SPA, and TDA (Croat et al., 2020). This suggests inconsistencies in biological property recovery in TDU where microbial abundance might be limited by inherent soil properties, but the soil can support productive crops if the correct environmental conditions are met. Ultimately, the effects of TDU appear to be different for plants and microbes. Regardless, focusing resources on holistic reclamation strategies that consider biological activity recovery, such as

Table 6. Mean values (with standard deviation) of saprophytic fungi, actinomycetes, Gram positive (Gram+) bacteria, Gram negative (Gram-) bacteria, and eukaryotic biomarker abundances for each treatment in 2019. Treatments are: native, non-contaminated topsoil (A); stockpiled contaminated, untreated subsoil (SP); stockpiled, contaminated subsoil and topsoil mixed 1:1 by volume (SPA); thermally desorbed contaminated subsoil (TDU); thermally desorbed subsoil and topsoil mixed 1:1 by volume (TDA).

Effects	Levels	Total Microbial Abundance	Saprophytic Fungi	Soil Bacteria	Gram+ to Gram- ratio	Eukaryotes	Actinomycetes
			----------nmol g soil^{-1}----------				
Soil (S)	A	74.3 (14.1) a[a]	2.69 (0.89)	55.0 (8.74) a	0.99 (0.09) a	2.63 (3.47)	10.4 (0.73) a
	SP	34.1 (11.2) b	2.18 (1.27)	26.0 (8.13) b	0.76 (0.04) c	0.78 (0.54)	5.74 (2.99) b
	TDU	34.2 (9.44) b	1.75 (0.35)	26.0 (6.88) b	0.80 (0.05) bc	0.81 (0.47)	4.96 (3.10) b
	SPA	55.9 (12.5) a	2.11 (1.02)	42.2 (9.56) a	0.92 (0.05) a	0.75 (0.14)	5.38 (1.65) b
	TDA	55.8 (10.3) a	2.42 (0.82)	42.1 (8.24) a	0.90 (0.05) ab	0.84 (0.19)	5.75 (2.17) b
	P-value	***	ns	***	***	ns	***
Composted Manure (M)	40 Mg ha^{-1}	51.4 (22.9)	2.7 (1.00)	40.3 (11.1)	0.88 (0.11)	0.90 (0.43)	6.94 (2.56) a
	0.0 Mg ha^{-1}	50.3 (14.7)	2.09 (0.82)	36.2 (16.0)	0.87 (0.09)	1.42 (2.31)	5.93 (3.27) b
	P-value	ns[b]	ns	ns	ns	ns	*
S x M		ns	ns	ns	ns	ns	***

*P<0.05

***p<0.001

[a]Values not followed by a common letter within columns and effect are significantly different at α= 0.05 in a post-hoc Tukey's Honestly Different test.

[b]ns, not significant

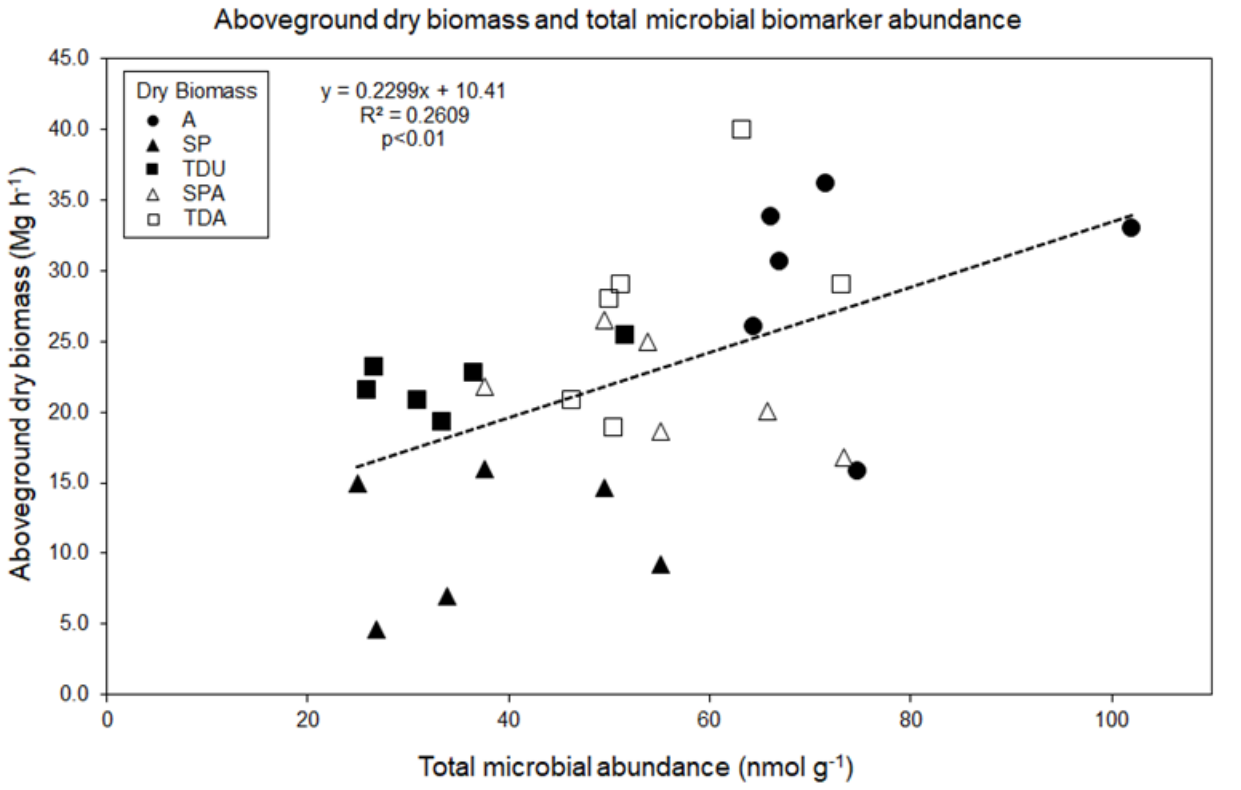

Figure 2. Total microbial PLFA biomarker abundance (microbial abundance; x-axis) plotted against grain sorghum aboveground dry biomass yields from 2019. Data is from one growing season. Difference symbols indicate different soil treatments. The linear regression line (dashed black line) is displayed along with R^2 value and p-value from linear model. Treatments are: native, non-contaminated topsoil (A); stockpiled contaminated, untreated subsoil (SP); stockpiled, contaminated subsoil and topsoil mixed 1:1 by volume (SPA); thermally desorbed contaminated subsoil (TDU); thermally desorbed subsoil and topsoil mixed 1:1 by volume (TDA).

blending subsoil or TDU with topsoil, may conserve resources while enhancing reclamation success compared to subsoil or TDU alone.

Saprophytic Fungi

Saprophytic fungi biomarker abundance did not vary among any of the treatments or models (Table 6). The fungal abundance in our study was more similar to reclaimed oil road (30-yr old) soil than undisturbed prairie soil, which is expected due past disturbances and annual crop production (Viall et al., 2014). Saprophytic fungi predominantly decompose SOM and plant litter and provide significant amounts of C for plant growth (Talbot et al., 2013). Changes to plant community, land use, and other abiotic factors can have profound effects on fungal community properties (Ngosong et al., 2012; Köhl et al., 2014; Bauer et al., 2017; Shi et al., 2019). In our study saprophytic fungi was moderately associated with organic C (r = 0.32) and NO_3-N percentage (r = 0.34), but not with P (r = -0.06), suggesting P was not a significant

limiting factor and fungi may be more limited by C/N ratios (Baer et al., 2003). Remediation activities certainly severely impacted native fungi across all soil treatments, but fungal biomarker abundance in all treatments were similar to the replaced A topsoil control after four years of no-till crop production. No-till agricultural practices lead to consistent accumulation of plant litter (i.e. crop residue) and provide energy sources for fungi (Karlen et al., 1994; Leichty et al., 2020). Therefore, it can be concluded that fungal communities have recovered to stable numbers, however, there is likely differences yet between what was observed in our study and nearby undisturbed topsoil. Follow-up investigations into the fungal recovery in remediated soils could include diversity assessments to observe redundancy or species richness.

Soil Bacteria

Total biomarker abundance of non-filamentous bacteria significantly varied among soil treatments only (Table 6). Abundance was similar between treatments A, SPA, and TDA. Meanwhile, SP and TDU subsoils had the least bacteria abundance. Over 70% of microbial biomass abundance was made up of bacterial biomarkers (data not shown), therefore further analyses of bacteria were done with Gram- and Gram+ biomarker abundances. Mean ratios of Gram+ to Gram- bacteria (G+:G- ratios) varied among soil treatments (p<0.001), but not between composted manure treatments or the SxC interaction term. Past studies have utilized G+:G- ratios as indicators for SOC inputs entering the soil system (Kourtev et al., 2002; Zhang et al., 2013), and may help to assess how stressed our soil treatments are, as increased Gram- abundances can indicate soil system stress.

Overall, G+:G- ratios were most stable (i.e., closest to 1.0) where SOC was greater, as in the A topsoil. Our results agree with Viall et al., (2014), who observed a G+:G- ratio of 1.094 from undisturbed topsoil in western North Dakota. It is worth noting that because SPA soils were not subjected to TD, SOC levels were not further reduced, and may explain why SPA was statistically greater than TDU while TDA was statistically similar to TDU (Figure 3). Hence, focusing reclamation strategies on preserving or restoring SOC levels may quicken time to reclamation success by restoring these microbial groups to levels similar to the A.

Disturbed soils often result in the disruption and mineralization of more complex SOC protected in aggregates, reducing Gram+ populations, and lowering G+:G- ratios assuming more labile C inputs are

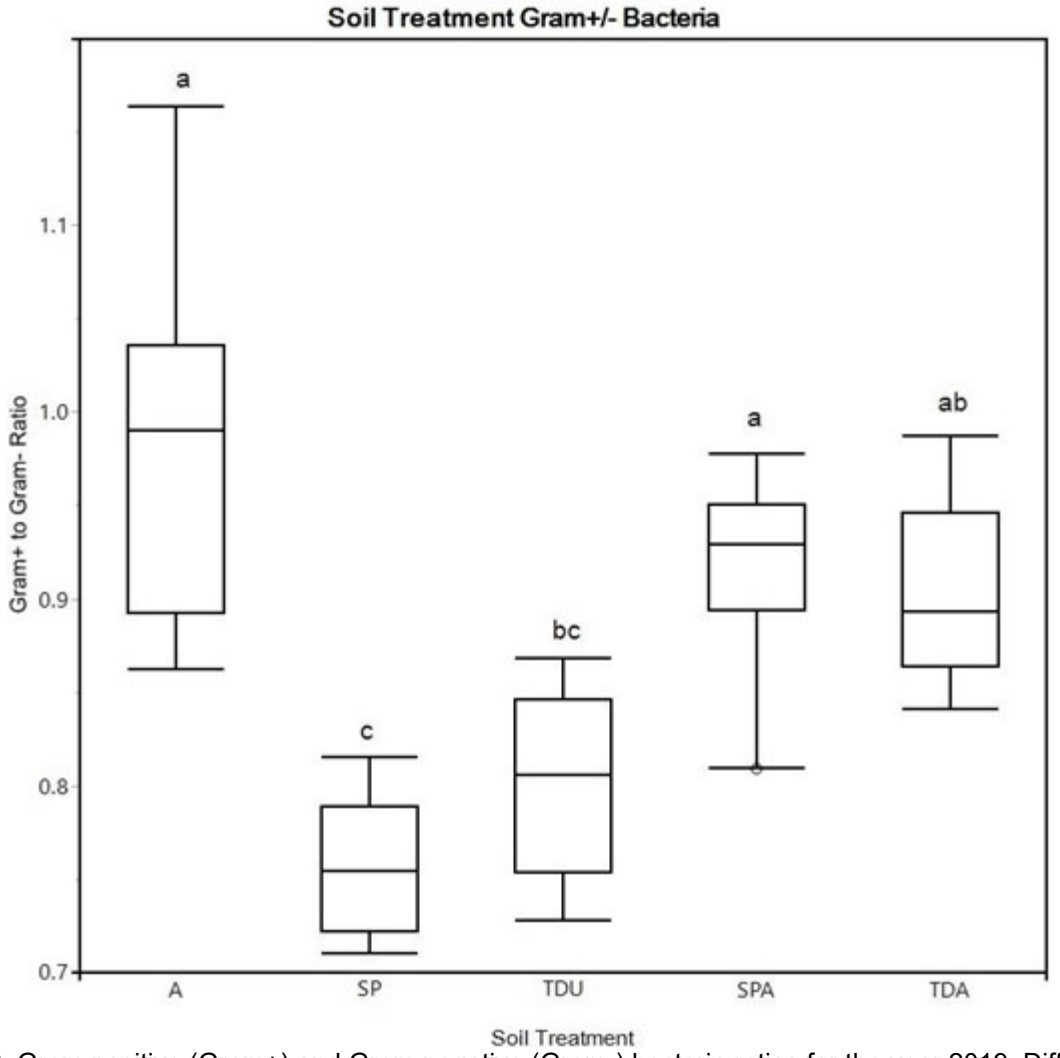

Figure 3. Gram positive (Gram+) and Gram negative (Gram-) bacteria ratios for the year 2019. Different letters within each pane indicate differences between soil types identified by Tukey's HSD test at α=0.05. Treatments are: native, non-contaminated topsoil (A); stockpiled contaminated, untreated subsoil (SP); stockpiled, contaminated subsoil and topsoil mixed 1:1 by volume (SPA); thermally desorbed contaminated subsoil (TDU); thermally desorbed subsoil and topsoil mixed 1:1 by volume (TDA).

not lost (Zhang et al., 2013). Smaller G+:G- ratios in the contaminated subsoils (SP and TDU) are likely due to reduced complex SOC and greater simple C sources such as annual crop residue. While Gram+ bacteria have been reported to be more dependent on labile SOC complex, recalcitrant forms of SOC, Gram- bacteria have been found to depend more on sources, and have decreased by over 10% during a plant litter removal chronosequence study (Fanin et al., 2019). Conversely, A topsoil is relatively unaltered soil with adequate labile and recalcitrant C concentrations to support both Gram- and Gram+ bacteria, respectively. Fortunately, mixing non-contaminated subsoil with non-contaminated topsoil for

soil remediation purposes shows a potential method to restore bacterial community balances in remediated subsoil.

Actinomycetes

Mean actinomycetes abundance varied by soil and composted manure application, and the SxC interaction term- where abundances were similar in A, SP+m, and TDA+m. The addition of A in SPA and TDA improved biomarker abundance compared to the subsoil. Hydrocarbon contamination <1000 mg kg^{-1} was determined to be nontoxic to actinomyces (Li et al., 2007), therefore the reduced abundance in actinomycetes could be reflecting changes in soil properties following the topsoil additions (Bolton et al., 1993). Nitrogen was strongly associated with actinomyces abundance (r =0.91), and is appropriate due to these microbes playing roles in degrading high C:N content materials such as cellulose and chitin, and thus being N-limited (Bhatti et al., 2017).

Conclusions

The goal of this study was to investigate the potential use of various biological metrics to quantify the status of recovery in soils remediated via TD relative to other remediation practices. Comprehensively, our results demonstrate the importance of using aboveground and belowground metrics in determining soil biological and ecological recovery, as aboveground indicators could incorrectly conclude TDU subsoils are overall more recovered than SP. In reality, soil biological characteristics in TDU were similar to SP in all but two metrics. Historically, SP and TDU were both recovering the slowest of all the soil treatments. While this was observed in our study, the increase in sorghum biomass suggests altered soil properties from TD may benefit potential plant production compared to SP when there is adequate fertilization and environmental conditions. Soil microbial communities were influenced more by topsoil and SOC than either remediation method, highlighting that the SP and TDU treatments were functioning similar ecologically, and are still recovering slowly four years after remediation. Recovery towards the A was more visible in treatments SPA and TDA, where the results were often statistically similar to treatment A, or intermediate to A and SP+TDU. Thus, showing soil blending is an appropriate method to mitigate impacts to soil biological properties from crude oil contamination and remediation disturbances. However, non-contaminated topsoil should always be the first choice during reclamation when available. Lastly, although compost or manure can improve crop yields and microbial biomass, the

application was likely too long ago or not enough initially applied to observe relic interactions in our study beyond SOC, sorghum biomass, and actinomycetes. It can be inferred from the strong correlation and association between microbial groups and SOC that biological metrics can be improved with the continued addition of composted manure or other organic amendments to improve soil biological recovery in remediated soils.

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CHAPTER 3. MICROBIAL ACTIVITY AND HARD RED SPRING WHEAT GROWTH IMPROVEMENT FOLLOWING BIOSTIMULANT APPLICATION

Abstract

Reclamation of oil and gas-disturbed soil is challenging due to diminished function (i.e. soil physical, chemical, and biological properties) from the loss of soil organic carbon (SOC) and potential mixing of topsoil and subsoil. Biostimulants are agro-products applied to soil to improve SOC formation, microbial nutrient cycling, and crop yields, suggesting their potential use in reclaiming oil and gas disturbed soils. However, studies on the ability of biostimulants to enhance reclamation in disturbed soils are limited. Therefore, research was conducted to determine if biological metrics were affected by biostimulant products in soil collected from an active pipeline installation project. The study was conducted in a greenhouse using pots consisting of the following soil treatments: TS100 (100% topsoil), TS50 (1:1 by-weight mixture of subsoil and topsoil), TS25 (3:1 by-weight mixture of subsoil and topsoil), TS12.5 (7:1 mixture of subsoil and topsoil), and TS0 (100% subsoil). Blended soil either received a liquid inoculant or biotic mulch biostimulant, and were planted with hard red spring wheat later on. Soil biological properties were generally influenced by topsoil concentration where TS50 consistently produced similar results to TS100, however, N and P were also influenced by biostimulant treatment. Additionally, wheat aboveground biomass was significantly greater in the liquid treatment while the biotic mulch stimulated greater microbial abundance and activity. Overall, these results indicate soil treatments were effective in restoring some biological properties relative to the control, but using biostimulants in mixed soils, regardless of topsoil concentration, can also improve soil biological property recovery.

Introduction

Intensive agricultural production dependent on chemical fertilizers to achieve optimal yields, and disturbance of soils for oil and gas production due to an ever-growing global population, are unique challenges facing some energy-extraction regions (Siirola, 2014; Lal, 2015; Fernando and Stika, 2021). Unfortunately, practices required to grow foodstuffs may result in the degradation of arable soil resources via accelerated erosion, depletion of soil organic carbon (SOC), and increasing salinization, all of which can lead to lowered soil microbial activity and plant growth (Tuttobene et al., 2009; Lal, 2015; Singh and Gupta, 2018; Kruger et al., 2020). Additionally, crude oil production activities have the potential to further

diminish plant growth and soil biological activity via soil mixing, pipeline installation, well pad construction and retirement, or accidental releases of crude oil or produced waters (Chapter 2; O'Brien et al., 2016; Dornbusch et al., 2020). In the Great Plains of the United States crude oil activities occur in or around the same land used for food crop production (Croat et al., 2020; O'Brien et al., 2016). For example, in addition to over 11,200 km of transmission pipeline, North Dakota has lost 9,300 ha of farmland due to development of 3,500+ well pads and access roads from 2005-2015, translating to about 2.61 ha per well pad (Fernando and Stika, 2021). Eventually, the soil will have to be reclaimed in order to restore the land back to farm productivity.

Due to declining soil productivities in many foodstuff-producing regions, there has been growing interest in crop production practices that conserve topsoil and mineral fertilizer by using relatively novel products called biostimulants (Nunes et al., 2018; Sigdal et al., 2021). Biostimulants are broadly described as agro-products containing microbes and/or naturally occurring substances employed to enhance plant growth and crop yield, reduce abiotic stress, and improve soil functioning, but do not directly provide fertilization to plants (US Environmental Protection Agency, 2020). Ingredients of biostimulants often include seaweed extracts, humic and fulvic acids, manure, industrial wastes, and living microbial cultures (Brown and Sea, 2015). Literature of specific ingredients in agricultural settings is vast and diverse, and overall these studies have reported improvement of plant growth and microbial activities, and sometimes accumulation of SOC (Kauffman et al., 2007; Calvo et al., 2014; Canellas et al., 2015; du Jardin, 2015; Van Oosten et al., 2017; Szparaga et al., 2019).

Biostimulants generally rely on bacteria, saprotrophic fungi, and other microbial groups to decompose and convert any organic materials into plant-available nutrient forms, and results in microbial CO_2 respiration (Pinto et al., 2004; Deacon et al., 2006; Hayat et al., 2010). Meanwhile, other biostimulants inoculate rhizospheres with symbiotic microorganisms, potentially improving plant root growth and quantifiable root CO_2 respiration (Thierron and Laudelout, 1996; du Jardin et al., 2015). Similarly, arbuscular mycorrhizal fungi (AMF) can aid in phosphorus (P) acquisition for plants, while also improving soil structure and SOC protection (Ingram et al., 2005; Thavamani et al., 2017). Nitrogen (N) fixing bacteria improve plant biomass production while restoring SOC into the soil system (Hayat et al., 2010). Characteristically, crop growth and soil reclamation in arid and semi-arid environments are

hindered by low SOC, nutrient availability, and sometimes lower microbial activity; suggesting biostimulants could not only help improve crop yield in degraded soils, but also improve reclamation success.

Understanding how (or if) biostimulants can enhance reclamation success and microbial recovery in soils disturbed from energy extraction (i.e. oil and gas) activities is needed. Accordingly, a two-factor greenhouse study was designed using five treatments of subsoil and topsoil in a gradient of 100% topsoil and 0% subsoil, to 0% topsoil and 100% subsoil. The soil blends were then treated with either a biotic fiber/mulch biostimulant, a liquid inoculant with no organic matter (OM) component, or nothing (control). The objectives of this study were to 1) evaluate biostimulant and soil blend effects on hard red spring wheat growth (*Triticum aestivum*), soil properties, and soil CO_2 efflux, 2) determine if AMF colonization and/or AMF abundance is influenced during P-deficit conditions, and 3) explore how the biostimulants may be influencing soil microbial abundance and percent community compositions of different microbial groups. Results from this study may serve as a baseline feasibility study for further investigations on biostimulant effectiveness in enhancing the physical, chemical, and biological properties of reclaimed or highly degraded soils.

Material and Methods

Soil Site Description and Soil Physical Properties

Native and historically undisturbed topsoil and subsoil samples were collected for use in this study from an active pipeline installation project in northwestern North Dakota (102.7639185°W, 48.2264081°N). The samples were transported overnight to North Dakota State University at Fargo, ND where they were stored in a climate-controlled greenhouse for less than 24 hr. Soil was then passed through a 10 mm sieve and air-dried at 25 °C before being stored in plastic totes in the same climate-controlled greenhouse until the start of the study. The sample location was within the Wabek-Appam sandy loams, 6 to 26 percent slopes map unit (Wabek: Sandy-skeletal, mixed, frigid Entic Haplustolls; Appam: Sandy, mixed, frigid Typic Haplustolls) (Soil Survey Staff, 2020). Soil characteristic data can be found in Table 7. Particle size analysis was conducted using an adapted hydrometer method (Gavlak et al., 2005). The topsoil was determined to be a loam and subsoil was determined to be a sandy loam. Soil samples for additional analysis were air-dried and passed through a 2-mm sieve. Field capacity (33 kPa)

values for watering purposes were determined using pressure plates (Soilmoisture Equipment Corp., Goleta, CA). Gravimetric water at field capacity was 0.18 g H_2O g^{-1} in the topsoil and 0.15 g H_2O g^{-1} in the subsoil.

Table 7. Characteristics of the soil blends used in this study. Soil organic carbon (OC), total carbon (TC), Nitrate-N (NO_3-N), phosphorus (Olsen-P), potassium (K), electrical conductivity (EC), and gravimetric water content at field capacity (FCΘg).

Soil Treatment†	SOC	TC	NO_3-N	Olsen-P	K	EC	FCΘg
	-----%----		---------mg kg^{-1}--------			dS m^{-1}	g H_2O g^{-1} oven-dried soil
TS100	2.03	2.48	34.0	10.0	309	0.50	0.18
TS50	1.62	3.11	38.0	7.50	252	0.53	0.17
TS25	1.22	3.24	40.0	6.25	223	0.53	0.16
TS12.5	1.04	3.15	41.0	5.63	208	0.54	0.15
TS0	1.04	3.99	42.0	5.00	194	0.53	0.15

† Treatments are: topsoil control, TS100; subsoil and topsoil mixed 1:1 by volume, TS50; subsoil and topsoil mixed 3:1 by volume, TS25; subsoil and topsoil mixed 7:1 by volume, TS12.5; and 100% subsoil, TS0.

Experimental Design

A randomized complete block design was used for this study, and was conducted in a greenhouse The treatments used in the two-factor experiment were a series of soil mixtures comprising of the two different soil materials, (1) topsoil: native topsoil taken from ≤15 cm depth, and (2) subsoil: native subsoil taken from the same location from ≥15 cm depth, and two biostimulants (1) ProGanics™ biotic mulch product (Profile®, Buffalo Grove, IL), and (2) SSB® liquid inoculant (Liventia™, San Antonio, TX). The soil mixtures were composed, by weight, of soils as 100% topsoil (TS100), 50%% topsoil (TS50), 25% topsoil (TS25), 12.5% (TS12.5), and 0% topsoil (TS0). Table 8 serves as an overview of the composition of ingredients in each biostimulant product, including microbial species and product base.

Soil treatments were mixed for each pot using a 15.1 L two-shell dry blender (Patterson-Kelley Company, Buffalo, NY) and mixing for 5 min. Soil was mixed by quantity of subsoil in that mixtures, where treatments with greater quantities of subsoil were mixed first. Shells were cleaned in between mixings. There were 4 soil blending replications per biostimulant block (5 soil treatments x 4 replications = 20 experimental units per biostimulant block). The SSB® was administered via watering into the blended soil, in the pots, after blending. Meanwhile, the ProGanics™ was administered by mixing it with the soil in the

two-shell dry blender. The control block received no biostimulant product. Importantly, the ProGanics™ soils were mixed after the SSB® and control soils had been mixed to reduce the risk of cross contamination. Each pot (60 total) held 2.5 kg of soil, or a volume of approximately 1,515 cm^3 and were lined with plastic bags prior to the addition of soil and water. After blending, pots were watered to field capacity with a molasses soil primer at a rate of 0.78 ml molasses L^{-1} water (rate and product provided by individuals associated with Liventia™). Soil priming is the practice of applying substrates rich in labile carbon to soils to stimulate microbial biomass accumulation and accelerate the decomposition of native SOC (Liu et al., 2017; Liu et al., 2020). The priming was recommended by SSB® manufacturers to apply it, however, it was applied to all treatments to reduce sources of error during data analysis. The SSB® product was applied at a rate of 1.1 µl SSB® pot^{-1}. ProGanics™ was mixed with the soil in the two-shell mixer at a rate of 0.5 g mulch g^{-1} soil. The water content of the pots was watered 80% of the relative field capacity of the soil during the study and was adjusted every two days.

Table 8. Biostimulant product ingredient breakdown composition.

Biostimulant	Liventia™ SSB®	Profile ProGanics™
Product Base	Water	Bark and Wood Fibers
Microbial Inoculants	*Acinetobacter calcoaceticus*	*Bacillus firmus*
	Bacillus subtilis	*Rhizophagus intraradices*
	Bacillus licheniformis	
	Bacillus thuringiensis	
	Enterobacter cloacae	
	Glomus aggregatum	
	Glomas etunicatum	
	Glomus intraradices	
	Glomus mosseae	
	Pseudomonas aeruginosa	
	Pseudomonas flourescens	
	Pseudomonas putida	
	Saccharomyces cerevisiae	
Inactive Ingredients	na	Wetting Agents

Cumulative CO_2 Efflux

Total CO_2 efflux rate (g CO_2 m^{-2} h^{-1}) was quantified by measuring CO_2 using an environmental gas monitor (EGM-4, PPSystems, Amesbury, MA) along with a soil respiration chamber (SRC-1) inserted

into a PVC ring installed in each pot (O'Brien et al., 2017). The first CO_2 reading took place prior to the first watering and all treatments remained unseeded for 14 d to allow for microbial recolonization of the soil. According to O'Brien et al. (2017), the exposure of subsoil with low biological activity to soil with greater microbial activity likely caused rapid recolonization of the pot and microbial depletion of plant nutrients (i.e. C, N, P), which may directly compete with and reduce the plant growth. Additionally, the incubation period could allow any effects of biostimulants on microbial recolonization compared to the control to be observable through the soil CO_2 efflux rate, without root CO_2 respiration inclusion.

During the recolonization period, readings occurred every three days for one week as an attempt to measure the potential "spike" of CO_2 efflux (O'Brien et al., 2017; Breker et al., 2018), after which the readings occurred every seven days until the end of the study. Headspace in the pots and efflux monitor was adjusted by:

$$A_a = A_i \times \left(\frac{HS + V}{V}\right)$$

where A_a is the final adjusted reading value (g CO_2 m^{-2} h^{-1}), A_i is the initial EGM-4 reading (g CO_2 m^{-2} h^{-1}), HS is the volume headspace (99.8 cm^3), and V is the SCR-1 volume (cm^3). Cumulative CO_2 efflux was calculated by:

$$Cumulative\ Efflux = \sum\left(\frac{A_a \times AW_c \times 24 \times n}{Aw_{co2}}\right)$$

where AW_c is the atomic weight of C (12.0 g mol^{-1}), 24 is hours in one day, n is the number of days before each reading, and AW_{CO2} is the atomic weight of CO_2 (44.0 g mol^{-1}).

Wheat Production

After a two-week incubation period 10 hard red spring wheat seeds were sowed in the pots after soaking overnight in a damp paper towel. N levels were normalized by applying 15 mg NH_4-NO_3 L^{-1} to each wheat plant. Emergence was observed DOY 73, at which the seedlings were trimmed to 5 plants per pot (Al-Karaki and Al-Omoush, 2006). On DOY 88, 1.0 mg of an Iron (Fe) chelate solution (Soygreen, CHS, Inver Grove Heights, MN) was applied to pots prior to watering due to visible deficiency symptoms occurring on older plant leaves' tips and margins. Wheat plants were planned to be sampled at the 10-12

leaf stage but the study was terminated early due to P-deficiency symptoms and complications brought forth by the COVID-19 pandemic and thus no grain yield was recorded.

Biomass and Root Sampling

Final sampling was done at the 5-7 leaf stage on DOY 95, after adequate AMF root colonization was observed. First, the plastic bags were removed from the pots and were carefully placed on clean paper so as to not disturb intact roots. Loose soil was collected and placed in sealed bags while the wheat roots were gently cleaned of soil on a flat surface. Soil was stored in a large plastic bag overnight except for a 10mg subsample for PLFA analysis, which was frozen within 1 hr of sampling. Cleaned roots were washed with water over a 2 mm screen until all visible residual soil was gone, and laid to allow excess water to drip. Once excess water was removed, a metal forceps was used to cut the aboveground biomass from the roots at the crown of the roots. A scale was then used to record the final recoverable root biomass. Finally, the cleaned and weighed roots were cut and preserved in autoclave tubes with formaldehyde-acetic-acid (FAA; by volume: 2-parts formaldehyde, 1-part glacial acetic acid, 10-parts 95% ethanol, and 7-parts deionized water) until ready to stain and examine under a microscope (Phillips and Hayman, 1970). Above-soil leaf samples were dried in a forced air oven at 60 °C for 72 hours and biomass quantified.

Soil Sampling

After being separated from the recoverable root biomass, soil was stored in closed plastic bags overnight. For C, soil was air-dried and passed through a 0.25 mm sieve. Soil organic carbon (SOC) was determined via mass loss on ignition by quantifying soil OM and converting to SOC using the conversion of 58% as noted in Pribyl (2010). Inorganic C was determined following the methods based on the gravimetric loss of soil carbonates as CO_2 in the presence of excess HCl (US Salinity Laboratory Staff, 1954). Total C was calculated as the sum of SOC and IC. Soil nutrients were analyzed by NDSU Soil Testing Lab (Fargo, ND). Soil N was reported as NO_3-N by soil water extraction using $K_2S_2O_8$. Soil P was quantified by the Olsen method (Frank et al., 1998). Electrical conductivity (EC) was quantified using a 1:1 soil-water extraction (Watson and Brown, 1998).

Microbial Biomarker Analysis

Microbial biomarker numbers were determined at the lipid extraction procedures following Buyer and Sasser (2012), followed by analysis using gas chromatography and mass spectrometry by Microbial Identification Labs, Inc. (MIDI; Newark, DL). Phospholipid fatty acid samples were prepared by freeze drying 10 g of composite soil samples from each pot (n=60), before being shipped to MIDI Labs. The results were reported as estimates of basic soil microbial groups according to fatty acid chains (actinomycetes, AMF, bacteria, fungi, and protists/eukaryotes). The neutral lipid fatty acid (NFLA) analysis was included to provide a measure of AMF in the field soils to complement the PLFA biomarker associated with AMF (16:1w5c) (Sharma and Buyer, 2015). The NFLA extraction and quantification follow the PLFA methodology with the addition of an internal standard of 19:0 trinonadecanoin glyceride and collection of the neutral lipid fraction in chloroform elution (Sharma and Buyer, 2015). Peaks were identified using Sherlock Chromatographic Analysis System software and the PLFAD2 peak naming table (MIDI, 2021). The community composition of each microbial group is reported in terms relative abundance (nmol fatty acid g^{-1} soil) as opposed to absolute terms.

Quantification of Mycorrhizal Colonization

Wheat root colonization percentages by AMF were estimated following a modified staining procedure adopted from Koske and Gemma (1989) and Phillips and Hayman (1970). As stated, roots were cut at the crown of the plant and were preserved in FAA immediately after separation from soil. After preservation, the roots were rinsed in deionized (DI) water to remove FAA, and roots $\leq$ 1 mm were carefully cut from larger fibrous roots with metal forceps and cut into 1-cm segments. Commonly, the primary, older roots are larger and contain more pigmentation, potentially complicating the clearing process than smaller, younger roots (Phillip and Hayman, 1970). Roots greater than 1 mm in diameter are too large to place on microscope slides under the slide covers and were therefore not considered for examination.

Segments were then placed in 40 mL test tubes with 10% potassium hydroxide (KOH; 10% weight/volume) aqueous solution and submerged in 90 °C water bath for 15 min for clearing. In this process, the pigmentation is cleared/reduced from roots to allow for increased contrast between the plant root and fungal biomass when stained (Phillips and Hayman, 1970). Next, segments were rinsed

thoroughly with DI water and stained in lacto-glycerol trypan blue (by volume: 1-part lactic acid, 1-part glycerol, 1-part water, 0.00066-part trypan blue) for 15 min. Finally, the stained root segments were rinsed and placed in a watch glass beaker cover with DI water. Stained root segments were randomly selected by stirring the roots, and allowing them to settle. Once settled, a 1-cm root segment was extracted with tweezers. This was repeated 10 times. The 10 selected segments were mounted on 22x40 mm glass slides with glycerol for viewing.

The procedure for estimating percent root colonization was modified from Allen and Allen (1980). Under 40x magnification, five passes were taken across all ten root segments for a total of 50 observation points per slide and thus experimental unit. In accordance to the procedure, each observation point was marked for the presence (+) or absence (-) of AMF structures including AMF hyphae, vesicles, or arbuscules. Finally, percent root colonization was determined as the total observation points with a positive score over the number of total observed views. No AMF taxonomic information was determined.

Statistical Analysis

Both the soil mixtures and biostimulant applications were each considered factors in data analyses. Accordingly, all response variable results were calculated with two-factor analysis of variance (ANOVA), with soil mixture and biostimulants as the factors at $\alpha = 0.05$, and the combined model being *soil x biostimulant*). Tukey's Honestly Significant Difference post-hoc test was done on all response variable's differences in means. All ANOVA analyses was done in SAS (SAS version 9.4, SAS Institute, Cary, NC) using Proc GLM. Linear regression was used for observing the relationships between various biological and chemical data collected, reported as correlation coefficients " *r* ". SAS Proc REG (SAS version 9.4, SAS Institute, Cary, NC) was run for all regressions.

Results and Discussion

Soil Properties

Soil properties varied significantly among soil treatments except for EC (Table 9). However, only NO_3-N, P, and EC varied significantly in the biostimulant effect, and there were no statistical differences in the *soil x biostimulant* model for any variables tested. Expectedly, the concentration of SOC, NO_3-N, P, and K generally decreased as subsoil content increased, while pH increased with subsoil content. Similar mean values among some mixtures with low amounts of topsoil, such as TS25 and TS12.5 for SOC, or

TS25-TS0 for P, may have been a result of natural soil heterogeneity, or because the mixing ratio is too low to affect levels at the scale of this study despite initial levels being different. In the soil treatments, increases in SOC was likely evidence of accumulation of microbial biomass and unrecoverable plant root biomass (Table 10), and not formation of recalcitrant soil C as this study's timeframe may be too short to accurately observe significant stable SOC accumulation (Grandy and Robertson, 2007; Zanatta et al., 2007; Tejada et al., 2011b; Kallenbach et al., 2016). Soil organic carbon was also positively related to total microbial biomass and unrecoverable root biomass ($p<0.05$; Table 11). This suggests that soil blending may influence SOC greater than the examined biostimulants in the short-term, and may require longer-term studies to understand how these products influence SOC pools overtime.

In the biostimulant treatments, NO_3-N was greatest in the Control and SSB® and least in ProGanics™ (Table 9). Interestingly, treatment with ProGanics™ consistently resulted in reduced NO_3-N across all soil blends (Figure 4A), except in TS100 where NO_3-N was similar among biostimulants. This observation of reduced NO_3-N levels among soil treatments may suggest reduced nitrifying microbial activity and on-going recolonization of native nitrifying bacteria in ProGanics™ (Prosser, 2005). Alternatively, it may also be direct evidence of greater microbial N-immobilization in the blended soils treated with ProGanics™ (Table 10). Among biostimulant treatments within each soil blend, P was significantly greater in the SSB® than ProGanics™ or the control (Figure B), which may have been a result of enhanced P-solubilization via AMF as SSB® contained three AMF inoculants (*Glomus (G.) aggregatum, G. etunicatum, and G. mosseae*). Ultimately, with the exception being N and P, chemical parameters were influenced by soil treatment and not biostimulant amendment, suggesting biostimulants can improve nutrient availability by enhancing microbial processes compared to the control, but they are not as capable at improving nutrient availability across the blended soil treatments alone.

Table 9. ANOVA of selected soil properties (with standard deviation) for each treatment.

Treatment	Levels	SOC	TC	N	P	K	EC	pH
		----------% by weight---------		----------mg kg^{-1}----------			dS m^{-1}	
Soil (S)	TS100 [a]	2.17 (0.14) a[b]	2.60 (0.17) b	236 (32.5) a	5.75 (0.13) a	333 (19.2) a	0.48 (0.04)	7.88 (0.04) a
	TS50	1.58 (0.11) b	2.87 (0.27) ab	195 (25.0) b	5.08 (1.31) a	265 (14.7) b	0.49 (0.05)	8.08 (0.06) b
	TS25	1.35 (0.12) c	3.18 (0.14) a	173 (26.6) c	3.83 (0.72) b	236 (13.0) c	0.48 (0.04)	8.20 (0.06) c
	TS12.5	1.24 (0.80) cd	3.23 (0.47) a	159 (30.0) c	3.58 (0.79) b	216 (4.31) d	0.49 (0.03)	8.26 (0.07) c
	TS0	1.12 (0.13) d	3.29 (0.63) a	135 (37.0) d	3.33 (0.65) b	192 (11.3) e	0.49 (0.04)	8.34 (0.05) d
	P-value	***	*	***	***	***	ns	***
Biostimulant (B)	Control	1.46 (0.46)	2.90 (0.58)	197 (35.2) a	4.15 (1.50) b	245 (47.9)	0.51 (0.03) a	8.15 (0.18)
	SSB®	1.52 (0.43)	3.12 (0.31)	196 (38.0) a	4.75 (0.85) a	250 (52.1)	0.51 (0.03) a	8.17 (0.16)
	ProGanics™	1.50 (0.36)	3.08 (0.42)	146 (43.2) b	4.05 (1.23) b	251 (55.3)	0.45 (0.02) b	8.15 (0.17)
	P-value	ns[c]	ns	***	*	ns	***	ns
S x B		ns	ns	ns	ns	ns	ns	ns

*p<0.05, ***p<0.0001

[a] Treatments are: topsoil control, TS100; topsoil and subsoil mixed 1:1 by volume, TS50; topsoil and subsoil mixed 3:1 by volume, TS25; topsoil and subsoil mixed 7:1 by volume, TS12.5; and 100% subsoil, TS0.

[b] Values not followed by a common letter within columns and effect are significantly different at α= 0.05 in a post-hoc Tukey's Honestly Different test.

[c] ns, not significant

Table 10. ANOVA of biological properties (with standard deviation) for each treatment.

Effects	Levels	Aboveground Dry Biomass	Recoverable Root Biomass	Cumulative CO_2 Efflux	AMF Root colonization
		g	g	g CO_2-C m^{-2}	%
Soil (S)	TS100 [a]	0.66 (0.10) a[b]	2.72 (0.82) a	119 (27.8) a	70.0 (26.1) a
	TS50	0.58 (0.09) a	2.27 (0.51) ab	111 (37.1) a	65.8 (27.2) a
	TS25	0.45 (0.07) b	1.96 (0.35) b	99.8 (34.1) ab	55.2 (28.8) b
	TS12.5	0.47 (0.06) b	1.96 (0.63) b	100 (34.8) ab	60.5(30.4) ab
	TS0	0.40 (0.07) b	2.24 (0.56) ab	87.4 (34.5) b	64.2 (26.4) ab
	P-value	***	*	*	*
Biostimulant	Control	0.50 (0.11) b	2.29 (0.61)	81.3 (22.4) b	61.2 (28.5)
(B)	SSB®	0.55 (0.14) a	2.37 (0.66)	86.1 (15.9) b	61.9 (27.4)
	ProGanics™	0.49 (0.10) b	2.02 (0.62)	143 (21.0) a	66.3 (29.5)
	P-value	*	ns	ns	ns
S x B		ns[c]	ns	*	*

*p<0.05, ***p<0.0001

[a] Treatments are: topsoil control, TS100; topsoil and subsoil mixed 1:1 by volume, TS50; topsoil and subsoil mixed 3:1 by volume, TS25; topsoil and subsoil mixed 7:1 by volume, TS12.5; and 100% subsoil, TS0.

[b] Values not followed by a common letter within columns and effect are significantly different at α= 0.05 in a post-hoc Tukey's Honestly Different test.

[c] ns, not significant

Table 11. Correlation coefficients " *r* " for Nitrogen (N), Phosphorus (P), Potassium (K), Soil Organic Carbon (SOC), Dry Biomass, Recoverable Root Biomass, Total Microbial Abundance, ***absolute*** abundances of AMF, Percent Colonization (via AMF), ***and absolute*** abundances of Actinomycetes, Gram+ Bacteria (Gram+), Gram- Bacteria (Gram-), Eukaryotes, Saprotrophic Fungi, and CO_2 efflux (Cumulative).

Variable		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	N	-														
2	P	0.63 **	-													
3	K	0.72 **	0.71 **	-												
4	SOC	0.75 **	0.74 **	0.93 **	-											
5	Dry Biomass	0.60 **	0.59 **	0.71 **	0.75 **	-										
6	Recoverable Root Biomass	0.32 *	0.29 *	0.27 *	0.29 *	0.40 *	-									
7	Total Microbial Abundance	0.48 **	0.61 **	0.87 **	0.83 **	0.61 **	0.27 *	-								
8	AMF Abundance	0.09	0.02	0.07	0.11	0.00	0.05	0.03	-							
9	Percent Colonization	0.11	0.22	0.21	0.27	0.08	0.05	0.29	0.01	-						
10	Actinomycetes Abundance	-0.06	-0.15	-0.07	-0.13	-0.09	-0.10	-0.10	0.74 **	-0.18	-					
11	Gram+ Abundance	-0.08	-0.18	-0.11	-0.16	-0.11	-0.13	-0.13	0.71 **	-0.22	0.99 **	-				
12	Gram- Abundance	-0.07	-0.17	-0.11	-0.13	-0.11	-0.10	-0.13	0.83 **	-0.18	0.97 **	0.97 **	-			
13	Eukaryotic Abundance	-0.21	-0.17	-0.19	-0.24	-0.13	-0.10	-0.17	0.23	-0.28 *	0.53 **	0.62 **	0.55 **	-		
14	Saprotrophic Fungi Abundance	-0.18	-0.27 *	-0.23	-0.20	-0.17	-0.13	-0.20	0.58 **	-0.18	0.65 **	0.73 **	0.77 **	0.64 **	-	
15	CO_2 Efflux	-0.20	0.07	0.34 **	0.30 *	0.09	-0.16	0.52 **	-0.11	0.12	-0.06	-0.05	-0.08	0.01	-0.06	-

*p<0.05, **p<0.01

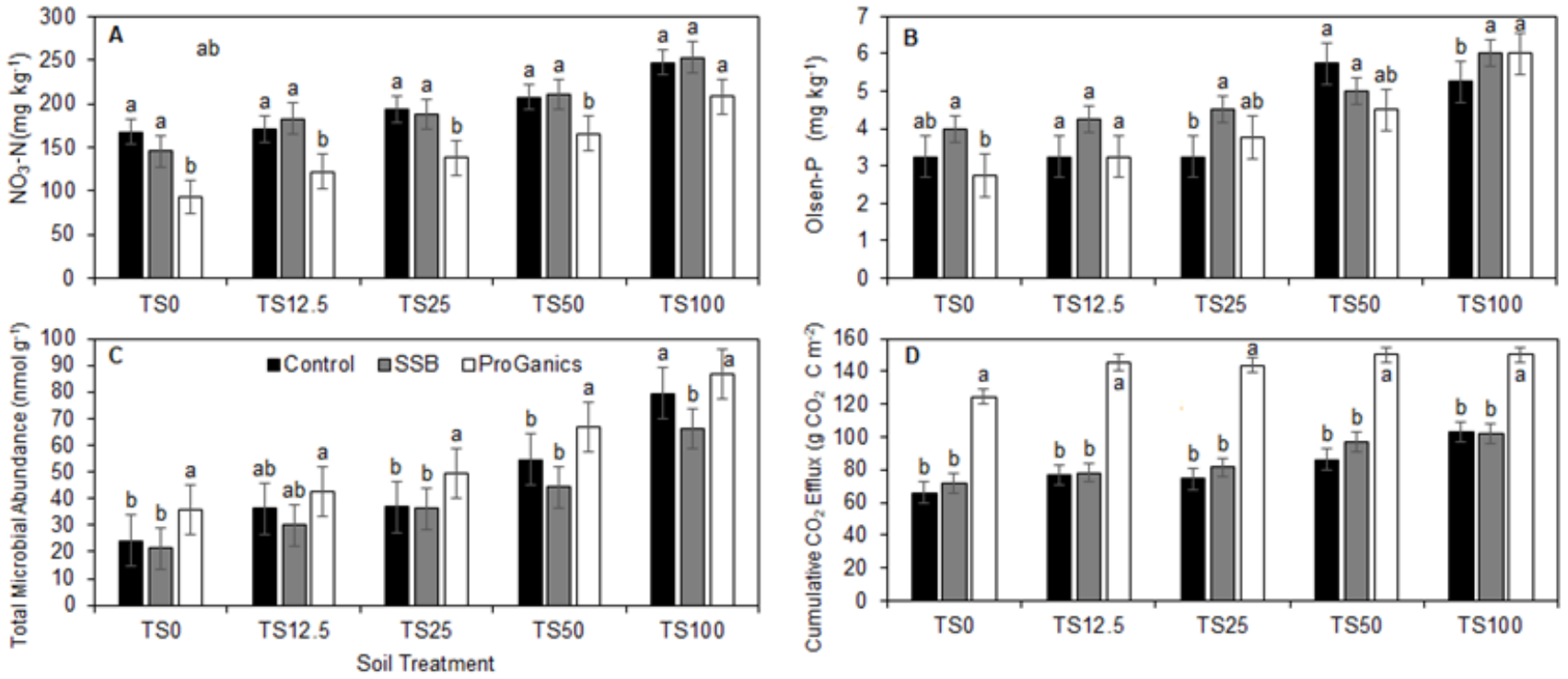

Figure 4. Nitrate-N ($NO3_3$-N; panel A), Phosphorus (Olsen-P; panel B), Total Microbial Abundance (panel C), and Cumulative CO_2 efflux (panel D) across all biostimulant treatments and clustered according to soil blend ratio with error bars. Different letters indicate significant differences between values between biostimulant treatment Control, SSB®, or ProGanics™ in each soil blend. Soil Treatments are: topsoil control, TS100; topsoil and subsoil mixed 1:1 by volume, TS50; topsoil and subsoil mixed 3:1 by volume, TS25; topsoil and subsoil mixed 7:1 by volume, TS12.5; and 100% subsoil, TS0. Biostimulant treatments are: No biostimulant Control, Liventia SSB®, and Profile ProGanics™.

Biological Properties

Soil treatment, biostimulant amendment, and the *soil x biostimulant* model had significant effects on aboveground dry biomass production (p<0.0001; Table 10). Dry biomass was greatest in TS100 and TS50 compared to treatments TS25, TS12.5, and TS0. The 1:1 blended subsoil to topsoil produced more vegetative growth than TS0, and similar to TS100 agrees with the findings of Croat et al., (2020), and results previously reported in Chapter 2 of this thesis. On average, TS25, TS12.5, and TS0 achieved aboveground growth only 71% of TS100, which is slightly lower, but similar to, the findings of Power et al. (1981) where spring wheat grown in a 3:1 subsoil to topsoil mixture of reclaimed North Dakota soil post-coal extraction produced approximately 80% of the relative maximum yields. In our study, dry biomass production was positively influenced by to N, P, K, recoverable root biomass, total microbial abundance, and most strongly by SOC (p<0.01; Table 11). Meanwhile, treatment SSB® produced significantly greater dry biomass than the control (0.50 g) and ProGanics™ (0.49 g). Additionally, SSB® soils were observed to contain significantly greater P-availability among treatment blocks, and could explain how SSB®-treated pots increased wheat biomass, and suggests SSB® was superior in enhancing plant growth due to increased P mobilization from its AMF inoculants.

Regardless, the addition of topsoil to subsoil only improved wheat growth when mixed at a 1:1 by weight ratio (Table 10). This mixing threshold was also reported in O'Brien et al. (2017), where wheat grown in 1:1 mixture of subsoil and topsoil produced significantly greater biomass than subsoil only, and is likely due to greater concentrations of SOM, and associated benefits OM has on nutrient cycling and availability (Mummey et al., 2005; Anderson et al., 2008; GIll et al., 2012; Table 10). O'Brien et al. (2017) also reported mixing ≤25% topsoil with subsoil reduced biomass production up to 40% due to nutrient competition in the rhizosphere, brought forth by microbial nutrient demand reducing plant growth. This trend was not observed in our study and demonstrates that the two-week incubation period before planting allowed sufficient time for microbial recolonization to avoid severe plant-nutrient stress (Breker et al., 2018). A possible explanation for the similar biomass observations from TS0, TS12.5, and TS25 was that they simply did not receive a large enough proportion of topsoil to noticeably improve crop growth. This finding could be useful for field-scale remediation projects where soil mixing occurs in order to allow sufficient microbial recolonization and avoid mixed subsoil to topsoil at ratios greater than 1:1. Bartsch et

al., (2022) reported even after crude oil contamination of subsoil, and subsequent remediation via thermal desorption and natural degradation, a 1:1 blend ratio produced crop yields similar to the topsoil control. However, root biomass analysis was not performed in that study.

Total recoverable root biomass significantly varied among the soil treatments, but was not different among the biostimulants or in the *soil x biostimulant* model (Table 10). Treatments TS25 and TS12.5 contained significantly less recoverable root biomass than TS100, which contained the greatest quantity of recoverable root biomass. Interestingly, both TS50 and TS0 were intermediate to the control, TS25, and TS12.5. Treatment TS50 may have had similar root biomass to TS100 because there were adequate levels of SOC in the 1:1 mixture to promote biological activity (O'Brien et al., 2017). Meanwhile, statistically similar root biomass levels in TS0 relative to TS100 could be attributed to nutrient deficiency from no topsoil. Nutrient deficiency has been reported to stimulate root growth and increase root-to-shoot ratios because of potential stress responses that lead to the accumulation of sugars in plant roots, stimulating root growth and soil exploration, but not shoot growth (Cakmak et al., 1994; Hermans et al., 2006). This could explain why TS0 root growth was similar to TS100 while biomass was not. Finally, TS12.5 and TS25 may either have contained just enough topsoil, plant nutrients, and biotic activity that stress-related root growth was inhibited, while still not having enough plant nutrients for aboveground biomass production. Nevertheless, despite TS12.5 and TS25 containing 12.5% and 25% topsoil, respectively, aboveground biomass production was not improved. Within the boundaries of this study the TS50 would be the most beneficial soil blending ratio to promote root growth and reduce stress-related root growth over TS25, TS12.5, and TS0 when topsoil is limited or not available.

Cumulative Soil CO_2 Efflux

During the 40-day laboratory study, cumulative soil CO_2 efflux significantly varied among soil blends and biostimulant treatments, but not in the *soil x biostimulant model* (Table 10). The greatest cumulative soil CO_2 efflux was observed in TS100 and TS50 with 119 and 111 g CO_2-C m^{-2}, respectively, and was significantly greater than TS0. Measuring CO_2 efflux is indicative of the rate of soil organic matter decomposition by soil microbes, and is influenced by land management and use, and contains a positive relationship with temperature, moisture, and aeration, until a certain point (Frank et al., 2006). Soil disturbances such as tillage or excavation can also stimulate CO_2 respiration due to increased aeration

and may suggest a net loss of C to the atmosphere (Reicosky et al., 1997). While topsoil addition benefitted CO_2 efflux in all blending ratios (Table 10), additional sources of soil CO_2 respiration may come from plant roots, or soil fauna, and alter interpretation (Kuzyakov, 2006). In our study, there was no soil macrofauna or aboveground plant biomass inside the respiration chamber during readings so contributions to cumulative efflux are assumed to be limited to wheat roots and soil microbes.

Additionally, increased soil respiration rates may suggest increased SOC accumulation and greater microbial abundance in undisturbed soil (Binet et al., 1998; Frank et al., 2006). In a North Dakota field study of wheat growing in silt loam soil, CO_2 efflux was 1.9 g CO_2-C m^{-2} day^{-1} and 2.8 g CO_2-C m^{-2} day^{-1} under a wheat-fallow and grassland land use, respectively (Frank et al., 2006). Our study achieved similar daily rates, averaging 3.0 g CO_2-C m^{-2} day^{-1} in TS100 and 2.0 g CO_2-C m^{-2} day^{-1} in the control. Since Frank et al. (2006) conducted efflux measurements continuously in summer and winter (where climatic conditions are not as conducive to soil microbial respiration, such as low soil temperature, soil water content, or certain land uses), the reported daily CO_2 efflux rate may have been slightly lower than our results (Wagai et al., 1998; Frank et al., 2006). Ultimately, the subsoil blending at any of the ratios in our study provide evidence that blended soils can facilitate microbial activity similar to topsoil at significantly lower total microbial abundances. Therefore, analyzing how CO_2 efflux responds to biostimulant treatments with various ingredients may be necessary to determine if biostimulants can further encourage organic matter degradation and SOC accumulation.

Among biostimulant treatments, ProGanics™ respired significantly greater cumulative g CO_2-C m^{-2} than SSB or the control, regardless of soil blend (Figure 4D). The control and SSB® treatments did not vary significantly (Table 10). ProGanics™ produced a daily rate of 3.6 g CO_2-C m^{-2} day^{-1}, which is greater than the TS100 alone. Kruger et al. (2020), reported respiration rates of 2.4 to 2.7 g CO_2-C m^{-2} day^{-1} in soil respiration chambers soil CO_2 efflux applied with ProGanics™ and is near, but slightly less than our results. Greater daily efflux rates in this study may be due to the presence of plant roots and the microbial primer applied that contains labile C, which were absent in Kruger et al. (2020). No other studies with ProGanics™ were identified. Overall, the inoculants *Bacillus firmus* and *Rhizophagus intraradices* when applied with seaweed extracts and polysaccharides (in ProGanics™), was the best method for encouraging CO_2 efflux. Conversely, SSB® may not have experienced increased CO_2 efflux due to the

lack of other ingredients which can provide soil organic matter for microbial decomposition. Lastly, the SSB® may not have improved the cumulative CO_2 efflux if the introduction the microbial species created a soil ecosystem where microbial activity was limited by resource competition. For example, four *Glomus sp*. (AMF), three Gram negative *Pseudomonas sp.* and three Gram positive *Bacillus sp.*, which are all saprotrophic bacteria (Table 8).

Soil CO_2 efflux was positively related to K, SOC, and total microbial abundance ($p<0.05$), and may explain why soil respiration was greater in the ProGanics™ soil than the control or SSB®. Approximately 94% of ProGanics™ (by-weight) is OM including tree fibers, polysaccharides, seaweed extract, and humic acids, and have all been positively reported with microbial activity and plant growth (Battacharya et al., 2015; Canellas et al., 2015; Costa et al., 2018). Additionally, the product contained inorganic fertilizer and labile C materials that have been reported to increase soil CO_2 efflux by over 24% compared to non-fertilized soil (Rui et al., 2016; Yang et al., 2017). Ultimately, Proganics™ demonstrated potential for increasing microbial abundance, activity, and mineralization of C substrates in disturbed soils, however, the activity did not result in improved plant growth suggesting additional reclamation methods should be considered during soil reclamation if greater crop growth and yield is desired.

AMF Colonization

Root colonization via AMF significantly varied among soil treatments and in the *soil x biostimulant* model, but not among biostimulant treatments (Table 10). Our study tested whether root colonization would be positively associated with subsoil content due to P scarcity (Liu et al., 2016; Deng et al., 2014; Chapter 2), however, P availability did not display any strong association with percent colonization (Table 11). Colonization was greatest in soil treatments TS100 and TS50, and least in TS25. Our results suggest similar colonization percentages in TS100 and TS50 were likely due to containing increased OM from the topsoil compared to the other blended treatments (Yang et al., 2011). Colonization percentages in TS12.5 and TS0 may have been a result of a P-deficiency response from the wheat that TS25 did no encounter from containing enough topsoil to avoid a stress response (Kahiluoto et al., 2001). Additionally, root colonization percentages were negatively related to eukaryotic abundance ($p<0.05$; Table 11). This may be from AMF reducing the amount of carbohydrates in root exudates which also reduces food sources for rhizosphere bacteria and their protist predators (Henkes et al., 2018). Ultimately, soil treatment and AMF

abundance did not directly reduce the ability of AMF-plant symbiotic relationships to form, and was consistent with Bartsch et al. (2022). Nutrient availability, and therefore topsoil concentration, may influence the overall success of the association in terms of plant biomass production and P uptake in greenhouse settings.

Biostimulant treatments did not significantly increase root colonization percentages compared to the control or SSB®. ProGanics™ contained inorganic fertilizer in its product, which has been observed to decrease root colonization percent in various crops due to decreased AMF activity and increased P availability (Douds et al., 1993; Bakhshandeh, 2017; Shahabivand et al., 2018). Information about SSB® is not known, but generally AMF inoculation is reported to have inconsistent results on root colonization, as the symbiosis is driven by P dynamics and also dependent on host plant species and genotypes (Venegas et al., 2021). Thus, subsoil properties beyond P may be affecting the frequency and effectiveness AMF-plant mutualistic relationships that ProGanics™ nor SSB® can ameliorate in this short-term study. Interestingly, root colonization in the *soil x biostimulant* model in TS25 Control and ProGanics™ treatments were more statistically similar to TS0 despite containing 25% topsoil. Ultimately, AMF microbial species can continue to form symbiotic relationships with host plants in subsoil, in blended subsoil with topsoil, and were not negatively affected by AMF abundance.

AMF Community Composition

Mean AMF community composition percent (marker 16:1w5c) from the PLFA analysis varied among biostimulant treatments, but not among soil treatments or the *soil x biostimulant* model (Table 12). In the biostimulant treatments, AMF composition was significantly greatest in the control (4.99%), and least in the ProGanics™ and SSB® treatments. Following a linear regression analysis, percent community composition of AMF, but not absolute AMF abundance ($p>0.05$; Table 11), contained a positive relationship with P-availability ($R^2= 0.23$, $p<0.0001$), which disagrees with results in Chapter 2 of this thesis where absolute AMF abundance contained a positive relationship with P (Liu et al., 2016; Huang et al., 2020). Also, absolute AMF abundance was positively associated to other microbial groups (Table 11).

Comparing studies involving AMF inoculation is inherently complex due to differing inoculation rates and species of biostimulants, but similar studies have reported that AMF inoculation of soils increases soil AMF biomass. However, those effects were not observed in this study or other studies

(Alkan et al., 2006; Köhl et al., 2015). Additionally, Janoušková et al. (2013) observed *Glomus intraradices, Gl. claroideum,* and *Gl. mosseae* inoculum had inconsistent results on AMF abundance depending on what species were introduced and what species were already present in the soils. One explanation offered by the authors was competition between native AMF species and the inoculum resulted in a depletion of nutrients and a decline in AMF community composition percent. This suggests AMF abundances may have been lower in SSB® and ProGanics™ compared to the control as a result of both soils receiving AMF inoculum, whereas, the control did not receive any additional AMF inoculants. Greater AMF composition in the control treatment could also be a result of residual mycelium biomass being assimilated into other microbes in the soil (Ngosong et al., 2012). Overall, AMF community composition percentage was not an accurate proxy for predicting crop yield potential in remediated soils, evident by variation in wheat biomass production in soil treatments despite similar AMF community composition values across the same treatments (Table 11). However, absolute AMF abundance had a positive relationship to aboveground biomass yield (Table 10), and may be used as a proxy for crop

Table 12. Mean microbial group percent composition (and standard deviation) of arbuscular mycorrhizal fungi (AMF), saprophytic fungi, actinomycetes, gram-positive bacteria, gram-negative bacteria, and eukaryotes.

Effects	Levels	AMF	Total Microbial Abundance	Gram+ Bacteria	Gram- Bacteria	Eukaryotes	Saprophytic Fungi	Actinomycetes
		%	nmol g^{-1} soil			%		
Soil (S)	TS100 [a]	3.96 (1.49)	77.4 (10.4) a	39.2 (2.02)	32.2 (1.56) a	1.02 (0.54) a	3.06 (1.44)	20.6 (1.68) c
	TS50	3.38 (2.00)	55.2 (10.9) b	40.3 (3.71)	30.9 (2.71) ab	0.41 (0.53) b	2.98 (2.00)	22.0 (2.39) bc
	TS25	3.27 (1.80)	40.8 (7.86) c	39.8 (3.10)	30.8 (2.33) ab	0.52 (0.79) b	3.09 (1.70)	22.5 (2.30) abc
	TS12.5	3.16 (2.02)	36.3 (7.13) c	40.0 (3.52)	30.2 (1.69) ab	0.60 (0.91) ab	3.01 (1.77)	23.1 (2.53) ab
	TS0	2.84 (1.88)	27.1 (7.60) d	40.1 (2.95)	28.7 (2.81) b	0.82 (1.25) ab	3.39 (2.08)	24.1 (3.34) a
	P-value	ns[c]	***	ns	*	***	ns	*
Bios-	Control	4.99 (0.42) a[b]	46.2 (19.8) b	37.8 (0.62) b	31.6 (1.40) a	0.20 (0.46) b	2.98 (0.67) b	22.4 (1.62) b
timulant	SSB®	2.47 (2.28) b	39.6 (16.8) c	42.0 (4.15) a	29.1 (3.23) b	0.26 (0.57) b	1.43 (1.36) c	24.7 (2.97) a
(B)	ProGanics™	2.51 (0.76) b	56.3 (19.4) a	39.8 (1.41) b	30.9 (1.80) a	1.56 (0.67) a	4.91 (0.95) a	20.3 (0.92) c
	P-value	***	***	*	*	***	***	***
S x B		ns	ns	ns	ns	*	ns	ns

*P<0.05, ***p<0.0001

[a] Treatments are: topsoil control, TS100; subsoil and topsoil mixed 1:1 by volume, TS50; subsoil and topsoil mixed 3:1 by volume, TS25; subsoil and topsoil mixed 7:1 by volume, TS12.5; and 100% subsoil, TS0.

[b] Values not followed by a common letter within columns and effect are significantly different at α= 0.05 in a post-hoc Tukey's Honestly Different test.

[c] ns, not significant

Total Microbial Abundance

In the biostimulants effect, total microbial abundance was greatest in the ProGanics™, and least in SSB®, while no differences in SOC were observed. ProGanics™ consistently increased total microbial abundance within soil blends compared to SSB® and sometimes the control (Figure 4C), demonstrating the effectiveness of ProGanics™ to encourage rapid soil microbial recolonization. Additionally, total microbial abundance was likely greatest in the ProGanics™ treatments after 40 d because of labile C sources in the amendment such as wood material, polysaccharides, seaweed extract, and humic acids. Labile C sources can quickly be assimilated into microbial biomass, and have all been positively reported with microbial biomass (de Graaff et al., 2012; Brackin et al., 2014; Francioli et al., 2016; Ramirez et al., 2020). Although some constituents of ProGanics™ may have negative effects on certain microbial groups, such as mineral fertilizer on fungi (Gryndler et al., 2006), saprotrophic fungi in our study were only negatively related to P (p<0.05; Table 11). Therefore, the combined effects of the ProGanics™ ingredients likely resulted in the increased total microbial abundance. In Brackin et al. (2014), sucrose addition to soil microcosms increased total microbial biomass 1.6 to 2.0-fold after 30 d, but declined after labile inputs were mineralized. Similarly, de Graaff et al. (2010) reported 1 to >4-fold increases of bacterial and fungal biomass after additions of labile C and plant residue, and overall agrees with our finding of a 1.2 to 1.4-fold increase of total microbial abundance in ProGanics™ over the other two biostimulant treatments. While positively associated to aboveground biomass production (Table 11), greater total microbial abundance did not reflect similar effects on aboveground wheat growth, notably in SSB®.

Conversely, SSB® had the lowest total microbial abundance but produced similar soil CO_2 efflux as the control (Table 10). The SSB® inoculant product had five species of fungi (four arbuscular mycorrhizal fungi; one yeast), and eight species of bacteria including Gram- and Gram+, aerobic and anaerobic bacterium. Lower total microbial abundance is likely a response to soil ecosystem changes following soil inoculation of microbial species in different groups with different metabolic pathways, microbes in the same group with similar metabolic pathways, and intermixing soil nutrients thereby increasing resource competition between microbial species in scarce space (Ghoul and Mitri, 2016). Inter-species and inter-group competition also likely altered soil microbial group abundance, community

structure, and species diversity, however, community composition percentage may stabilize overtime (Ghoul and Mitri. 2016; Eldridge et al., 2017). Also, this study did not aim to analyze species diversity or taxonomy.

Unlike total abundance, community composition percentages (Table 12) demonstrate SSB® increased the content of soil actinomycetes and Gram-/+ bacteria compared to ProGanics™ and the control. This was likely a result of the high proportion of bacterial species in SSB®. Gu et al. (2020) reported microbial inoculation of soil increased bacterial composition compared to mineral fertilizer and the control. Additionally, Gu et al. (2020) reported that the increased bacterial composition allowed for greater aboveground biomass in inoculated soils, while also increasing root growth in two of the three inoculation treatments. In our study, inoculation with SSB® was observed to increase aboveground dry biomass production (Table 11), suggesting inoculation can aid plant growth in disturbed soils from enhanced plant nutrient accumulation and perhaps increased exudation of beneficial enzymes or compounds (Hayat et al., 2010).

Soil Bacteria (Non-Filamentous)

Community composition percentage of Gram+ bacteria significantly varied among biostimulants but not among soil blends or in the *soil x biostimulant* model (Table 12). Treatment with SSB® contained the greatest percent community composition of Gram+ bacteria compared to ProGanics™ and the control. Additionally, absolute abundance of Gram+ bacteria was positively related to AMF, Gram- bacteria, saprotrophic fungi, and actinomycetes ($p<0.01$; Table 11). Gram- bacteria percent community composition significantly varied in the soil and biostimulant treatments, but not in the *soil x biostimulant* model. In the soil treatments, Gram- percent composition was greatest in TS100, least in TS0, and intermediate among the other soil blends, suggesting bacterial total biomass is not greatly disturbed from topsoil and subsoil blending. What's more, absolute Gram- bacteria abundance was positively related with AMF, Gram+ bacteria, saprotrophic fungi, and actinomycetes ($p<0.01$; Table 11), while Gram- bacterial community composition was least in SSB® compared to ProGanics™ and the control. Overall, Gram+ bacteria were greater in community composition percentage over Gram-, while both microbial groups were influenced more by biostimulant treatment than soils blend.

Notably, biostimulant treatment SSB® inoculated soils with three Gram+ bacterial species (*Bacillus (B.) subtilis, B. licheniformis, and B. thuringiensis*), and five Gram- bacterial species (*Acicetobacter calcoaceticus, Enterobacter cloacae, Pseudomonas (P.) aeruginosa, P. flourescens, and P. putida*). ProGanics™ only contained one Gram+ bacterial inoculant (*B. firmus*). Bacterial inoculation is usually done to promote plant growth, but directly or indirectly, can result in shifts in bacterial group composition. For example, rhizospheres inoculated with Gram+ (*B. subtilis*) and Gram- bacteria (*P. flourescens*) have been observed to significantly alter the overall bacterial community composition, diversity, and significantly increase specific species abundances over others (Gadhave et al., 2018; Jiménez et al., 2020). Although no taxonomic analyses were conducted, the addition of these species may have altered native bacterial populations. Because ProGanics™ and the control were similar, it could be inferred the addition of *B. firmus* did not result in any Gram+ bacterial community shifts. Similarly, the lack of Gram- bacterial inoculants in the ProGanics™ biostimulant likely resulted in the community composition percentage to be similar to the control despite other ingredients. However, the addition of both Gram+ and Gram- bacteria in SSB® influenced community composition by altering C pools and substrate fluctuations (Zhang et al., 2013).

Eukaryotes

Eukaryotic community composition percentage, most commonly soil protists, significantly varied in the soil, biostimulant, and *soil x biostimulant* model (Table 12). Protist community composition was greatest in the TS100, and was least in TS50 and TS25. Among the biostimulants, protists were most abundance in the ProGanics™ treatment with 1.65% composition, and least in the control and SSB® with 0.20 and 0.26% composition, respectively. It was also observed eukaryotes were positively related to all other soil microbial group abundances, likely reflecting topsoil's affinity for microbial activity and abundance ($p<0.01$; Table 11). Water availability and soil moisture have been shown to regulate soil eukaryotic diversity, abundance, and density (Kennedy, 1993; Geisen et al., 2014; Geisen et al., 2018), which suggests ProGanics™ may have increased the water holding capacity and/or soil moisture of the soil treatments. However, gravimetric water content at field capacity (-33 kPa) nor wilting point (-1500 kPa) varied significantly among any soil treatments (data not shown) despite the already present SOC's ability to increase plant available water (O'Brien et al., 2016), or ProGanics™ being reported to increase

soil water-holding capacity ≥900% (Kruger et al., 2020). Ultimately, further investigations into gravimetric pressures among soil treatments to determine plant available water could help further determine the effects of biostimulants containing OM on protists populations.

Saprotrophic Fungi

Saprophytic fungi percent composition varied in the biostimulant only (Table 12). Percent composition was greatest in ProGanics™ and least in SSB®. Absolute saprotrophic fungi biomarker abundance was positively related to all other microbial groups, and negatively related to P (p<0.05; Table 11). Reduced fungal composition in SSB® may have been a result of intergroup competition following inoculation; where increased bacteria and actinomycetes abundance effectively lowered the composition of saprotrophic fungi; or by consumption of introduced yeasts and other fungi by soil bacteria and protists (Botha, 2011; Ballhausen and de Boer, 2017). ProGanics™ may have had the greatest saprotrophic fungi due to the availability of suitable food sources compared to SSB®, as wood fibers and other OM has been observed to enhance fungal species composition (Kubartivá et al. (2008), which would support increased colonization of the soil matrix and rhizosphere (Boddy and Hiscox, 2016). Biostimulant ingredients can therefore influence soil microbial composition, including microbial inoculants and/or non-living components.

Actinomycetes

Actinomycetes composition varied by soil and biostimulant treatment, but not the *soil x biostimulant* model (Table 12) where composition was significantly greater in TS0 and least in TS100. In the biostimulant treatments, actinomycetes composition was greatest SSB® and least in ProGanics™. The actinomycetes biomarker abundance was positively related to all other microbial groups (p<0.05; Table 11). Subsoil may have contained greater composition of actinomycetes as result of decreased percentages of other microbial groups, such as Gram- bacteria. Greater actinomycetes composition in SSB®, may be due native actinomycetes filling in a soil niche where native and inoculated species with similar metabolic and environmental niches were competing for resources. Additionally, variability between actinomycetes and composition in the soil and biostimulant may have a been a result of a competitive edge in metabolizing soil C substrates, leading to actinomycetes out competing saprotrophic fungi (Lewandowski, et al., 2015).

Conclusions

The goal of this study was to determine the effects of two different biostimulant products on soil biological properties, wheat growth, and soil microbial communities in various blended topsoil and subsoil ratios in a greenhouse study. Topsoil concentration influenced soil biological properties and wheat responses more than biostimulants. Soils blended below a 1:1 ratio demonstrated low potential for reclamation success and soil productivity due to declines in microbial activity and ecosystem functioning. Fortunately, this study also demonstrates reclamation strategies may benefit from 1:1 soil blending when facing topsoil scarcity. While the studied biostimulants were not as effective as topsoil concentration in influencing soil biological properties, their use still influenced microbial-driven nutrient systems when compared to the control treatment. SSB® was effective at increasing P-availability and resulted in increased wheat dry biomass growth. Meanwhile, ProGanics™ did not affect wheat growth but resulted in increased microbial N-immobilization, microbial biomass, and CO_2 efflux. This phenomenon may explain why plant growth was not influenced in ProGanics™ and should be considered where crops are being grown on treated soils. In regards to the soil ecosystem, biostimulants showed potential to benefit microbial recolonization of blended soils compared to the control, but individual microbial group responses were dependent on biostimulant components such as woody fibers high in recalcitrant C, or specific microbial inoculants which can shift microbial community composition. Overall, our greenhouse study reveals potential uses for biostimulants in improving microbial recolonization and processes in disturbed soils in the short-term, but choosing products that improve plant function, microbial activity, and soil properties, such as microbial diverse biostimulants, may be the best choice. Further field evaluations of biostimulant effects on soil properties and plant growth are needed to fully evaluate their potential use in reclamation.

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OVERALL CONCLUSIONS

The objective of this research was to 1) investigate how thermal remediation vs natural degradation influenced soil biological recovery following crude oil-contamination and 2) observe the effects of biostimulant products on soil biological properties in disturbed soils. The findings suggest topsoil and/or SOC concentrations in reclaimed soil drives soil biological recovery greater than either remediation method. Additionally, blending topsoil and subsoil (1:1) was successful in significantly improving soil biological function recovery, which is important because soil biological processes are necessary to restore to rebuild nutrient pools, SOC, and sustainable land uses. However, although thermally treated and naturally degraded soils may be similar from a soil ecological perspective, the alteration of subsoil properties following TD allowed for greater sorghum growth compared to the contaminated subsoil when environmental conditions were met, and may not be observed if different crops are produced. Further, although biostimulants likely do not influence soil biological properties as significantly as topsoil concentration, their use still significantly influenced nutrient pools, plant growth, and soil microbial abundance compared to the control. Biostimulants may play a vital role in improving reclamation success by encouraging microbial function and plant growth, but further field evaluations of biostimulants in reclamation settings are needed to fully validate their potential use (or economical feasibility) in reclamation, and to determine how different ingredient mixtures or rates can be utilized for a desired outcome. From this research, a best-case scenario for reclamation may include the application of biostimulants to blended topsoil and subsoil to enable greater short-term biological property recovery, and not only does this present an opportunity to conserve fragile topsoil resources by reducing the quantity of topsoil entering landfills and the cost being acquiring new topsoil by allowing degraded or less-ideal subsoils to be utilized, but it may allow greater long-term soil function.

APPENDIX

Table A1. List of fatty acid chain biomarkers used for Gram positive (Gram+) and Gram negative (Gram-) bacteria abundances and the Gram+ : Gram- ratio.

Gram+		Gram-		
11:0 iso	18:0 10-methyl	10:0 2OH	16:0 2OH	19:1 w7c
11:0 anteiso	19:1 w7c 10-methyl	10:0 3OH	18:1 w8c	19:1 w6c
12:0 iso	20:0 10-methyl	12:1 w8c	16:1 w9c	19:0 cyclo w7c
12:0 anteiso	17:1 iso w9c	12:1 w5c	16:1 w7c	19:0 cyclo w6c
13:0 iso	17:0 iso	13:1 w5c	14:0 3OH	20:1 w9c
13:0 anteiso	17:0 anteiso	13:1 w4c	16:1 w6c	20:1 w8c
14:1 iso w7c	18:0 iso	13:1 w3c	16:1 w4c	20:1 w6c
14:0 iso	17:1 iso w10c	12:0 2OH	16:1 w3c	20:1 w4c
14:0 anteiso	17:1 anteiso w9c	14:1 w9c	17:1 w9c	20:0 cyclo w6c
15:1 iso w9c	17:1 anteiso w7c	14:1 w8c	17:1 w8c	21:1 w9c
15:1 iso w6c	19:0 cyclo w9c	14:1 w7c	17:1 w7c	21:1 w8c
15:1 anteiso w9c	19:0 iso	14:1 w5c	17:1 w6c	21:1 w6c
15:0 iso 15:0 anteiso	19:0 anteiso	15:1 w9c	17:0 cyclo w7c	21:1 w5c
17:0 10-methyl	20:0 iso	15:1 w8c	17:1 w5c	21:1 w4c
22:0 10-methyl	22:0 iso	15:1 w7c	18:1 w7c	21:1 w3c
18:1 w7c 10-methyl		15:1 w6c	18:1 w6c	22:1 w9c
17:0 10-methyl		15:1 w5c	18:1 w5c	22:1 w8c
22:0 10-methyl		14:0 2OH	18:1 w3c	22:1 w6c
18:1 w7c 10-methyl		17:1 w4c	19:1 w9c	22:1 w5c
		17:1 w3c	19:1 w8c	