IMPROVEMENTS IN THE SAMPLING METHOD OF THE WHEAT RHIZOSPHERE AND SOIL MICROBIOME

By

URMILA MANANDHAR

Master of Science in Microbiology

Tribhuvan University

Kathmandu, Nepal

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Thesis Approved:

Dr. Michael Anderson

(Thesis Adviser)

Dr. Shiping Deng

Dr. Andrea Jilling

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Author,
Urmila Manandhar

Name: URMILA MANANDHAR

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Abstract: The Rhizosphere microbiome is extremely important for improving soil and plant health. Though various methods have been proposed for the sampling rhizosphere microorganisms, these still lacks standardization. Molecular analysis based on 16S rRNA sequences revealed that the choice of sampling methods influences the end results of the data analysis. The first step in this process is the efficient extraction of good quality DNA with high yield and purity from the sample. Similarly, the choice of DNA extraction method plays a major role in determining the outcome of the analysis. Here we compared the conventional “pull and shake” method with our current “core” method for sampling of the rhizosphere soils. Phylogenetic analysis based on 16S rRNA showed significant differences in terms of microbial community structure in terms of beta diversity, taxonomic distribution of microbial community and ANCOM analysis while alpha diversity did not show any statistically significant differences. We also compared four different DNA extraction methods across eight contrasting soils including a rhizosphere soil sample. The result indicated that the DNA extraction method did not have a measurable effect on alpha diversity and taxonomic distribution of microbial community. However, there was a significant difference in terms of beta diversity and ANCOM analysis across DNA extraction methods. Therefore, improvements in both rhizosphere sampling and DNA extraction methods have the potential to better represent the actual rhizosphere microbiome present in the soil.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

My thesis work is divided into two projects. The first project compares two distinct methods for sampling rhizosphere soil, and the second project compares four different DNA extraction methodologies. Both projects utilize 16S rRNA sequencing data and advanced bioinformatics and statistical tools to analyze the results. The Rhizosphere is central to almost everything related to plants and soil interactions, so improving the soil and DNA extraction and sampling methodologies will provide a more accurate and reliable representation of the microbial community.

1.1 Wheat Rhizosphere Sampling Methods

1.1.1 Importance of Rhizosphere

The rhizosphere is one of the most biologically active regions in the soil where important interaction between microbes and plants occurs (Nihorimbere, Ongena, Smargiassi, & Thonart, 2011; Singh, Millard, Whiteley, & Murrell, 2004). The region is inhabited by tens of thousands of microbial species (Mendes et al., 2011) forming an interacting web of biological activity. The rhizosphere microbiome is known to benefit the plant by enhancing plant growth, development and health in many undefined ways, including: the improvement of soil nutrient extraction efficiency (el Zahar Haichar,

Santaella, Heulin, & Achouak, 2014; Jones, Nguyen, & Finlay, 2009), protection against soil-borne diseases (Lazcano et al., 2021) and provision of growth promoting substances (de Santi Ferrara, Oliveira, Gonzales, Floh, & Barbosa, 2012) as well as defense against abiotic stresses (J. Yang, Kloepper, & Ryu, 2009). Therefore, the rhizosphere microbiome is crucial for understanding crop soil interactions and in developing methods for increasing agricultural yield and productivity (Hinsinger, Bengough, Vetterlein, & Young, 2009).

1.1.2 Current Methods for Extracting and Defining the Rhizosphere

Research into the rhizosphere microbiome is critical for understanding plant microbe environment interactions and in developing and improving ways to grow more productive crops. The rhizosphere is defined as the area around the root surface that influences the plant (Hartmann, Rothballer, & Schmid, 2008), a vague definition at best. Rhizosphere research is highly dependent on the methodology used to differentiate the boundaries between rhizosphere and bulk soils. Since, the zone around the roots where root exudates are secreted varies significantly along the root axis (C.-H. Yang & Crowley, 2000), involving root hairs and fine root structures embedded in a complex and heterogeneous soil matrix, there is no accurate and consistent method for defining the rhizosphere soils. As Giddens and Todd noted “precise method(s) of determination of rhizosphere microbial numbers in the root zone do not exist” (Giddens & Todd, 1984). It is because we do not know the optimum zone to which secretion of root exudates can be experienced (Angle, Gagliardi, & Mclntosh, 1996) that research into this critical plant soil interface is based on a foundation that problematical at best.

The most common technique for the extraction of soil around the roots involves the manual and careful removal of the roots from the soils with the shaking off of the soil with a defined and consistent shake (Han et al., 2009; Smalla et al., 2001). Soil that remains attached to the roots after the plant is uprooted and shaken is commonly referred to as rhizosphere soils. Usually, most of the research in the rhizosphere depend upon this conventional pull and shake method for sampling rhizosphere soil. Although, the shake method is used most frequently, there is no definite agreement concerning the exact procedure. Even a slight variation in the procedure can likely have a great impact on the result. More importantly, any manual removal of soil is prone to detach much of the fine root structure including fine branched roots and even finer root hairs. These fine root structures are likely home to an undermining number of microorganisms that remain unaccounted for when removed using the conventional shake method. This lack of definition in terms of the rhizosphere introduces considerable uncertainty concerning results derived from such a method. Therefore, studies that use the shake method are likely to be biased toward old roots that resist the shaking, compared to young fine roots. What is needed is an alternative method that takes into account the fragility of the rooting system when sampling, what we refer to as the rhizosphere (Macrae, Lucon, Rimmer, & O'donnell, 2001). In this research we develop a novel approach termed the core technique to characterizing the rhizosphere which takes into account both coarse and fine root structure of the wheat plants. Therefore, we hypothesize that core method will represent an improved more representative method of the true overall rhizosphere than the current pull and shake method.

1.1.3 Proposed Method for Improving Rhizosphere Representation

Soil grown in a confined environment with an optimized number of plants based on plant growth produces a growth saturated environment. Under growth saturating conditions all the soil could be considered as rhizosphere soil. Therefore, extracting a soil core from such a system would constitute a rhizosphere sample. Taking a similar core from a similar growing system but without plants (termed the blank) would reveal the bulk soil or soil that is not influenced by plant life. Extracting and sequencing DNA from both the Core soils under saturating conditions provides a way to characterize the rhizosphere microbial community in total. Here we propose a method that utilizes soil coring that enables us to collect both fine roots named the core method. Comparing core with the shake method we hypothesize will be statistically different in terms of community structure and diversity. Therefore, the objective of our study is to demonstrate what we consider to be an improved and more representative microbiome sequence base sampling of the rhizosphere based on the Core technique under growth saturating conditions in comparison to the shake method.

1.2 DNA Extraction Methods

Extracting DNA from soil at the necessary levels of yield and purity is one of the most crucial steps for gene sequencing of the soil microbiome (Young, Rawlence, Weyrich, & Cooper, 2014) Yield and purity vary greatly with the choice of DNA extraction method (Abdel-Latif & Osman, 2017; Anderson, 2018). Laboratory-based DNA extraction methods and commercial kits are viable options for DNA recovery, with each methods following three basic steps: cell lysis, precipitation, and purification (R.

Rojas-Herrera, J. Narváez-Zapata, M. Zamudio-Maya, & M. Mena-Martínez, 2008). On the whole, silica-based purification methods forms the basis of most purification techniques (Anderson, 2018). In the silica gel method, DNA binds to silica surface due to the interaction between DNA phosphate and surface silanol groups and hydrophobic bonding between DNA base and silica hydrophobic region (Shi, Shin, Hassanali, & Singer, 2015). Another way to purify soil DNA involves the use of gel filtration chromatography which separates molecules according to their molecular size, usually in spin column format. This technique has two phases, the stationary phase consists of gel matrix of hydrated beads of certain narrow range of pore sizes and the mobile aqueous phase. When the aqueous solution passes through the gel matrix, larger molecules are eluted first whereas smaller molecules get trapped inside the beads and travel a longer distance forcing them to elute out later (Robinson, 2014).

1.2.1 Improvement in DNA Extraction Methods

Improvements in DNA extraction technology is an ongoing goal for many researchers and commercial entities. Anderson (2018) reported a procedure that uses gel filtration chromatography to purify DNA from soil to a level routinely sufficient for sequencing. This method was found to be superior in terms of yield to a popular silica gel based commercial kit. However, the gel filtration technique was only examined on a limited range of soils and was not demonstrated using rhizosphere soils. Further experimentation revealed that rhizosphere soils were difficult to extract to a consistent level of purity relevant for microbiome sequencing using both Anderson and commercial procedures. Similarly, another research conducted by Rojas, et al (2012) claimed that silica method was the good choice for DNA extraction from most soils. According to the

author, the method as outlined is simple, cheap and yields good quality DNA with high molecular weight (R. Rojas-Herrera, J. Narváez-Zapata, M. Zamudio-Maya, & M. J. M. B. Mena-Martínez, 2008). But this method also failed to purify the DNA at an acceptable level of purity. Therefore, there is the need of standardized protocol that can extract DNA from wide range of contrasting soil samples effectively. We hypothesize that a combination of both gel filtration chromatography and silica gel may be the best approach overall. In this method, silica-based adsorbents bind DNA allowing contaminants to pass thorough and be discarded, further purifying the DNA beyond the Anderson technique.

1.2.2 DNA Sequencing: The Current Gateway to the Microbiome

Recent practices that identify microorganisms use modern molecular methods like DNA hybridization and PCR based gene sequencing (Colella, Shen, Baggerly, Issa, & Krahe, 2003; V. Torsvik & L. Øvreås, 2002) etc. are critical to the characterization of the microbial community and their associations with the plant growth and development. Historically, microorganisms were identified after culturing on artificial media based on their morphological characteristics. Unfortunately, less than two percent of microorganisms can be routinely cultured in laboratory leaving the vast majority uncharacterized. Gene sequencing with 16S rDNA or 16S rRNA are two of the most powerful methods used to taxonomically identify which microbes are present and to semi quantitatively determine in what amounts. Microbiome sequencing using the 16S gene can provide a high-resolution measurement of microbial diversity (V. Torsvik & L. J. C. o. i. m. Øvreås, 2002), as well as characterize the microbial community structure to the genus-species level (Singh et al., 2004). Significant understanding of microbial

communities associated with plants has been gained through these sequencing methodology (V. Torsvik & L. J. C. o. i. m. Øvreås, 2002). Thus, the overall objective of this research is to 1) compare the DNA yield, purity, and microbial community structure of both the silica based, gel filtration based and gel filtration plus silica-based DNA extraction techniques compared the most popular commercial kit across a wide range of soils.

CHAPTER II

LITERATURE REVIEW

2.1 Wheat Rhizosphere Sampling Methods

2.1.1 Rhizosphere and its Importance

The Rhizosphere as first coined by Hiltner is the area within the soil under the influence of the plants which may extend couple of millimeters away from the root surface (Girish & Ajit, 2011). It is a home to a diverse microbiota such as bacteria, fungi, nematodes, protozoa, parasites, viruses, and algae (Meena et al., 2017). It can contain up to 10^{11} bacteria per gram of soil (Egamberdieva et al., 2008) and more than 30,000 prokaryotic species (Mendes et al., 2011). The microbial community in the rhizosphere is called rhizosphere microbiome (Ali, Naveed, Mustafa, & Abbas, 2017) and can be categorized depending on the physical location around or within the plant root. Microorganisms residing on the surface of the roots are called rhizoplane microorganisms. Those rhizoplane organisms reside on a thin biofilm layer on the root surface while the rhizosphere microorganisms reside in the immediate adjacent soil volume. Microorganisms residing within the roots are termed endophytic microorganisms (Bhromsiri & Bhromsiri, 2010; Gaskins, Albrecht, & Hubbell, 1985). Bulk

soil is the area outside the rhizosphere and thus not influenced by the roots and root exudates. There is a significant difference in the microbial abundance, microbial biomass, and diversity in the rhizoplane, rhizosphere, and bulk soils (Barillot, Sarde, Bert, Tarnaud, & Cochet, 2013). Usually, rhizosphere harbors greater microbial populations than bulk soil (Fan, Weisenhorn, Gilbert, & Chu, 2018), probably due to the fact that plants secrete up to 40% of their photosynthates into the rhizosphere (Bais, Weir, Perry, Gilroy, & Vivanco, 2006) thereby providing plenty nutrients for the growing population. This phenomenon is called as rhizosphere effect. However, the rhizosphere has a less diverse microbial communities than the bulk soil (Fan et al., 2018) most likely due to a plant selection mechanism associated with the secretion of exudates (Buee, De Boer, Martin, Van Overbeek, & Jurkevitch, 2009).

2.1.2 Root Exudates

Root exudates are chemicals secreted by the roots of living plants into the rhizosphere soils (Hayat, Ali, Amara, Khalid, & Ahmed, 2010). They contain ions, water, enzyme, free oxygen, mucilage, and carbon rich primary and secondary metabolites (Nardi et al., 2000). These exudates can be divided into two classes. Low molecular weight exudates typically composed of amino acids, organic acids, sugar (Rougier, 1981) whereas high molecular weight exudates include mucilage (polysaccharide containing pentose and hexose sugar and uronic acids) and proteins (Abbott & Murphy, 2003; Walker, Bais, Grotewold, & Vivanco, 2003). Root exudates serve as the food for microbial community to power their activities and in return plants benefit by the many types of microbial activities (el Zahar Haichar et al., 2008). Root exudates help plants in a number of ways: encouraging the formation of soil aggregates, (Kato-Noguchi, 2004),

preventing the soil from dehydrating, mobilizing nutrient minerals for plant use (Narula, Kothe, & Behl, 2012), attracting beneficial microbes, and repelling pathogenic microbes (Kumar et al., 2007) and structuring the overall rhizosphere microbial community (Walker et al., 2003) in ways that help promote plant growth and health (Abbott & Murphy, 2003). The exudation process is not just a one-way street. Plants also communicate with the microbes present in the rhizosphere by chemical signaling through their secretion of root exudates (Bais et al., 2006). Plants select their microbial community of interest by secreting specific root exudates (Vives-Peris, de Ollas, Gómez-Cadenas, & Pérez-Clemente, 2020).

There are many factors that affect exudate production and the quality of exudates including the plant species present. Plants differ in types and quantities of exudates they produce, and this difference is known as plant species effect (Singh & Mukerji, 2006). Other parameters that affect exudate production, include pH, soil type, oxygen status, light intensity, soil temperature, nutrient availability, and the microbial community structure (Nihorimbere, Ongena, Smargiassi, & Thonart, 2011). These parameters may influence exudate production more than the plant species effect (Singh & Mukerji, 2006). It was also found that exudate production was greater during maturing stage than earlier stage of development (Wacquant, Ouknider, Jacquard, & Soil, 1989).

2.1.3 Soil Microbial Diversity

Microbial diversity can be defined as the range of features or degree of difference between organisms in a particular environment (Bing-Ru, Guo-Mei, Jian, & Gang, 2006). Soil microbial diversity is composed of species diversity and genetic diversity, as well as

ecosystem biodiversity (Solbrig, 1991). We can view the diversity from three different aspects like diversity based on cell morphology, biochemical or metabolic process, and DNA sequence. There are enormous number of diversities of soil microorganisms that exist in the world, and it is still not possible to estimate how vast is the extent of microbial diversity (Patel, 2021). It may be because we lack sufficient taxonomic knowledge and appropriate methodology to understand the complex microbial diversity in nature (Bing-Ru et al., 2006).

2.1.4 Microbial Diversity Metrics

Microbial diversity measure is important for revealing community structure and dynamics (Lozupone & Knight, 2008). There are several ecological diversity indices available such as Alpha (α), Beta (β) diversity to measure microbial diversity and to study the diversity and richness of organisms in different ecological environments (Liao, Huang, & Huang, 2007). An Operational Taxonomic Unit (OTU) is an operational definition of classifying groups of closely related individuals. OTU's are microbial genomic sequences clustered by sequence similarity and are used to classify bacteria based on sequence similarity (Mandal et al., 2015). Alpha diversity is determined based on OTU number and distance between different taxa. Alpha diversity metric measures diversity within the sample gives us some quantitative metrics to compare those samples and incorporates richness and evenness (Willis, 2019). It tells us how similar or different are the organisms in the community from each other. Total number of species in an environment is known as species richness. Similarly, evenness counts for abundance of species and phylogeny is weighed by evolutionary history or phylogenetic tree. Faith PD (Phylogenetic Diversity) is one of the metrics of alpha diversity. It brings richness and

phylogeny that shares evolutionary history. Similarly, Shannon diversity index is another nonphylogenetic alpha diversity metric which incorporates measures of richness and evenness between different environments. Pielou's Evenness looks at the distribution of features within the samples rather than looking at how many are there. It focuses on the number of taxa in a community where more taxa indicate more diversity. It tells how evenly distributed the number of features per taxa in a sample are (Bolyen et al., 2019).

Beta diversity measures the unique differences between the samples. The composition of one sample is compared using some metric of diversity like unweighted or weighted unifrac (Bolyen et al., 2019). Bray-Curtis dissimilarity is the quantitative measure of community dissimilarity and, is based on abundance between two samples at species level. Beta diversity ranges in value from 0 to 1. 0. A 0 rating means both samples share the same species at exact the same abundances, whereas 1 means both samples have completely different species abundances. Jaccard distance is a qualitative measure of community dissimilarity and does not include abundance information. It is based on presence or absence of species. It measures differences in microbial composition between two samples. 0 means both samples share exact the same species. 1 means both samples have no species in common. Similarly, unifrac measurement is based on the fraction of branch length that is shared between two samples or unique to one or another sample. Unweighted unifrac is based on sequence distance where it does not include abundance information whereas weighted unifrac includes both sequence and abundance information and is weighted by relative abundance (https://twitter.com/panphlan).

ANCOM (Analysis of Composition of Microbiome)

One of the basic goals of microbiome research is to find out which microbes have influential effect on experimental units. For this process, it is necessary to determine which microbial taxa differ in abundance between the groups (differentially abundant taxa) (Lin & Peddada, 2020). There are numerous statistical analyses proposed and used in the past studies to determine differentially abundant taxa between two or more groups, but only few of them are useful. Most of the recent technologies consider OTUs as fundamental bases to study microbiota at a community wide level. However, comparing microbial composition based on OTUs only can introduce false result in the study (Mandal et al., 2015). So, to mitigate this problem, a novel statistical approach was introduced known as ANCOM (Analysis of composition of microbiome). Sequencing data is compositional data and is based upon percentage of total and this kind of data is difficult to analyze or is not effectively analyzed using any other kinds of analyzes including ANOVA (Bolyen et al., 2019). Only ANCOM was developed to analyze these kinds of compositional data. It is the new emerging statistical tool that identifies individual taxa whose relative abundances are significantly different across groups. It compares microbiome composition based on abundance of taxa between two or more groups (Mandal et al., 2015). Because of its solid statistical foundation ANCOM analysis was included in the Qiime2 package for microbiome analysis.

2.1.5 Planting Density

Planting density is one of the important agronomic factors influencing biomass production and grain yield. It affects biomass production, stem width, germination and emersion of seed, number of leaves per plant and many other factors. High planting density causes the reduction in yield since there is greater inter-plant competition for

things like essential nutrient minerals, light, water. (Nitisha & Girjesh, 2013). On the other hand, low plant density causes a decrease in productivity due to an increase in number of weeds, weed competition and not using all available soil nutrients and soil volume adequately (Allard, 1999). In current study, research is conducted to observe the effect of different planting density on overall biomass of plant.

2.1.6 Difficulties in Defining Rhizosphere Soil

Rhizosphere influence is thought to occur predominantly within a few millimeters from root surface; however, it is difficult to predict boundary between rhizosphere and soil not influenced by the plant roots (Angle, Gagliardi, & Mclntosh, 1996). Additionally, it depends on the individual plant root system and even the single root. Normal definition of rhizosphere is easy to describe, however, mechanistic definition of rhizosphere is obscure. Therefore, it is difficult to compare rhizosphere population among different studies. Since roots influence the soil around them by depositing dead cells, secreting root exudates, absorbing water and nutrients, the definition of the rhizosphere is exceedingly important in any plant microbe interacting study. Research has shown that roots play influential role in determining rhizosphere microbiome (Helmisaari & Brunner, 2006).

2.1.7 Sampling of Rhizosphere Soils

Fine roots are typically primary roots having the diameter less than 2 mm and helps in absorbing water and nutrients (Du & Wei, 2018). They are heavily branched and fragile and get detached easily from older roots when they are taken out of soil. Several procedures for sampling rhizosphere soil have been proposed. However, in the past much

research depend upon conventional pull and shake method for rhizosphere soil sampling. Angle (1996) obtained the rhizosphere soil by careful shaking the root followed by placing in solution. Similarly, Smalla (2001) obtained the rhizosphere soils by vigorous shaking the non-rhizosphere soil and then placing them in the distilled water. Though there are some variations in the sampling procedure proposed by many authors, they all follow the same shake method for rhizosphere soil sampling. Though shake method is used most frequently in the research, it does have some limitations. Shake method is variable in terms of the amount of energy used to dislodge non rhizosphere soil in addition to other factors that may affect the amount of soil adhering to the root system, like: soil texture, soil moisture (Luster, Göttlein, Nowack, & Sarret, 2009), type of root system (Hinsinger, Gobran, Gregory, & Wenzel, 2005) and how energetically you shake the plant. So, the shake method tends to focus on older portion of roots because these are typically larger and contain stronger and more fibrous root structures. We tend to lose most of the fine roots when we pull the plant out from the soil. Fine roots likely harbor a distinct set of microorganisms more associated with an actively growing root tissue than the older roots. Therefore, the research that uses the shake method is likely to be biased towards the older roots.

Another method is necessary for retaining the fine root structure when sampling the rhizosphere. Here we develop and compare a technique referred to as the CORE technique, that retains more of the fine root structure compared to the standard shake method.

2.2 DNA Extraction Methods

2.2.1 Limitation of Cultural Technique

Soil as a complex and heterogenous habitat harbors numerous diverse microbial communities (Curtis, Sloan, & Scannell, 2002). However, less than 1% of total soil micro-organisms are culturable (Amann, Ludwig, & Schleifer, 1995; Brock, 1987; Hawksworth, 1991; Luo, Qi, Xue, & Zhang, 2003; Yuexin, Homstrm, Webb, & Kjelleberg, 2003) greatly limiting our ability to characterize soil communities. So, the use of cultural technique cannot represent the total micro-organisms present in the soil environment (Amann et al., 1995).

2.2.2 Importance of DNA Sequencing

Molecular technique based on nucleic acid like sequencing helps to identify many uncharacterized microbial communities (Liesack & Stackebrandt, 1992; Ward, Weller, & Bateson, 1990). DNA sequencing is a useful tool to explore microbial diversity and community present in the environment and it is widely used in modern microbiology and biotechnology (Yi & Chun, 2015). The introduction of this type of non-culturable technique unravels not only the identities within the microbial community but also opens the door to evaluate species diversity, richness, and evenness (D. N. Miller, 2001), critical aspects of community characterization. Furthermore, the use of 16S rRNA in DNA sequencing helps to identify numerous phylogeny related genes within the bacterial genome. The hypervariable regions of 16S rRNA gene sequences provide species-specific signature sequences useful for bacteria identification. This sequencing technique helps to identify the pathogenic bacteria and also discovers new species that was never

been successfully cultured in laboratory (Woese & Fox, 1977). Usually, a cut off value of 3% divergence is used as a level of sequence divergence that separates species form higher taxonomic classifications. (Stackebrandt et al., 2002; Tindall, Rosselló-Móra, Busse, Ludwig, & Kämpfer, 2010; Wayne et al., 1987). In other words, prokaryotic species have more than 97% 16S rRNA gene sequence homology. An operational taxonomic unit (OTU) or phylotype is used to classify the groups of closely related individuals based on DNA sequences similarity (Yi & Chun, 2015). Several databases of 16S rRNA gene sequences are publicly accessible and are the main source for species circumscription (Yi & Chun, 2015).

2.2.3 Problems Encountered during DNA Extraction Method

The effective use of sequencing techniques requires the evaluation of methods prior to sequencing such as DNA extraction and purification of nucleic acids from soils and sediments (Leff, Dana, McArthur, & Shimkets, 1995). Pure and high-quality DNA is required for successful DNA sequencing (Abdel-Latif & Osman, 2017). However, efficient extraction and purification of nucleic acid from soils is challenging, arduous and time consuming (D. N. Miller, 2001). Numerous extraction methods have been proposed and evaluated (Anderson, 2018; Jia, Han, Zhao, & Zhou, 2006; Rojas-Herrera, Narváez-Zapata, Zamudio-Maya, & Mena-Martínez, 2008; Zhou, Bruns, & Tiedje, 1996), however all these methods may not be equally efficient at capturing the soil community sequence signatures when applied to the wide range of contrasting soils found in nature. Several difficulties like incomplete rupturing of cell, DNA sorption to soil surface, inhibitory substances like humic and fulvic acids, damage to DNA are encountered

during DNA extraction process, all creating bias in the DNA study (D. Miller, Bryant, Madsen, & Ghiorse, 1999).

Several types of extraction methods have been developed and classified as either direct or indirect extraction methods. Direct extraction method involves direct lysis of cells within the soil sample (Ogram, Sayler, & Barkay, 1987; Van Elsas, Mäntynen, & Wolters, 1997) whereas indirect extraction method involves the removal of the cells from soil the sample and then subsequent lysis (Berry, Chiocchini, Selby, Sosio, & Wellington, 2003; Jacobsen & Rasmussen, 1992) and sequencing. Either direct or indirect methods are used to extract DNA from soils and sediments in a number of published studies (Holben, Jansson, Chelm, & Tiedje, 1988; Steffan, Goksøyr, Bej, & Atlas, 1988). Both methods have pros and cons, but usually direct method is used most frequently since it yields more DNA and is more effective at extracting DNA from recalcitrant microbial species than the indirect method. However, in the direct method, PCR inhibitory substances are also extracted along with DNA (Holben et al., 1988). DNA is extracted either by physical disruption, chemical lysis or enzymatic lysis. Primarily, humic acids are the major contaminants coextracted in the DNA extraction process (Anderson, 2018; Saeki, Ihyo, Sakai, & Kunito, 2011; Wilson, 1997), however sometime metals and polysaccharides can pose a problem in extracting high quality DNA (Straub, Pepper, & Gerba, 1995; Tsai & Olson, 1992). Usually, these inhibitory substances are removed using spin column packed with various resins including silica gel (D. N. Miller, 2001). In fact, most of the commercial kits use silica-based purification for removing impurities. However, these processes are time consuming involving multiple steps which increase the price of the DNA extraction process (D. Miller et al., 1999).

2.2.4 Some common DNA Extraction Methods

DNA can be isolated from any source of biological materials using chromatography-based DNA extraction method (M Carpi, Di Pietro, Vincenzetti, Mignini, & Napolioni, 2011). Size exclusion chromatography, ion exchange chromatography, affinity chromatography and mini spin column are some of the examples of chromatography-based DNA extraction methods (Adeli & Ogbonna, 1990; Lindblom & Holmlund, 1988). Yield and purity vary greatly with the choice of chromatography method used for DNA recovery. Since the traditional DNA extraction method involves the use of hazardous chemicals like phenol and chloroform, and more labor, chromatography-based DNA extraction methods have become more popular in these recent days for DNA purification (M Carpi et al., 2011) due to their inherent safety and their reduction in labor.

2.2.4.a Gel Filtration Chromatography

Gel filtration chromatography (GFC) or gel permeation chromatography also known as size exclusion chromatography separates the nucleic acids or proteins based on their hydrodynamic volume (M Carpi et al., 2011) passing throuh a porous matrix with pores of specified size. Generally, the selection of matrix used in GFC depends upon the size of the DNA molecule which can be anywhere from 15 kDa for oligonucleotides to several million kDa for large DNA fragments. Typically, the chromatography matrix is packed into a small centrifugal column and the DNA loaded on top of the matrix. The DNA is forced via low speed centrifugation through the matrix. Choice of matrix type with respect to pore size and matrix materials are available for DNA purification from

commercial sources. Usually, three types of matrices are popularly used for DNA purification: dextrans (Sephadex), agarose (Sepharose) and allyl dextran bisacrylamide (Sephacryl) (Ó'Fágáin, Cummins, & O'Connor, 2011). Sephacryl S 300 HR column were used in the previous studies (Adamski, Husen, Marks, & Jungblut, 1992; Anderson, 2018; Mu et al., 1995) and have several advantages due their greater matric rigidity than the other matrix types. In GFC, we have two phases namely a mobile phase and a stationary phase consisting of porous matrix of defined pore sizes. When the sample is loaded on top of the matrix column, the molecules larger than the pore size elute faster having a less tortuous path, while molecules with smaller size elute later having a more tortuous path around and through the gel filtration beads. Hence, elution occurs in the decreasing order of molecular size (Ó'Fágáin et al., 2011). Gel filtration matricies are not as common as silica based matricies, but have been used in soil DNA purification previously (Anderson, 2018; Dijkmans, Jagers, Kreps, Collard, & Mergeay, 1993; Jackson, Harper, Willoughby, Roden, & Churchill, 1997; LaMontagne, Michel Jr, Holden, & Reddy, 2002; Miller, 2001; Rochelle, Fry, John Parkes, & Weightman, 1992; Roose-Amsaleg, Garnier-Sillam, & Harry, 2001).

2.2.4.b Silica-gel Chromatography

Since silica binds DNA molecules specifically (Höss & Pääbo, 1993), DNA adsorbs and becomes immobilized on the silica surface in the presence of high concentrations of chaotropic salts such as guanidine hydrochloride or sodium iodide at a defined pH (Tan & Yiap, 2009). Once bound the DNA molecule can be washed with ethanol solution and then eluted by washing with low salt solution containing buffer or pure water. This method is available either in a spin column or microchip format, takes

less time, may not involve any hazardous chemicals, and is cost effective as compared to most DNA extraction methods. Multiple wash steps also help in removing contaminants from the extract. Low salt elution buffer is typically used to elute the DNA during the final step of DNA purification by centrifugation. Most of the commercial kits use silica-based technologies (Dhaliwal, 2013). Many studies have successfully used silica-based DNA extraction method for their DNA extraction methods ((Devi et al., 2015; Juniper, Cambon, Lesongeur, & Barbier, 2001; D. Miller et al., 1999; Rojas-Herrera et al., 2008). Silica based chromatography systems are among the easiest, most consistent, and least laborious of all the systems available in commercial formats. However, this does not mean that they are the best (Anderson 2018).

2.2.4.c Anion Exchange Chromatography

Anion exchange chromatography is also commonly used in plasmid isolation kits (Dhaliwal, 2013) and somewhat in commercial DNA extraction kits. In this technique, purification occurs due to the specific interaction between negatively charged phosphate groups of nucleic acid and positively charged surface molecules on the chromatography matrix. When we add the sample to the cationic matrix, then anionic DNA gets attracted to and binds to the silica gel in the presence of low salt buffer. DNA can then be washed of impurities and eluted by increasing the concentration of a positive counter anion. (Budelier & Schorr, 1998). This approach can also use a centrifuge with centrifugal columns containing the matrix to simplify and commercialize the DNA purification system as with the silica-based system, with binding occurring in low salt, washing with low or medium salt solution and elution with high salt conditions.

2.2.4.d Organic (Phenol-Chloroform) Extraction

This is one of the conventional methods used to extract and purify DNA (Dhaliwal, 2013). In this approach DNA is partitioned between the organic chloroform phenol phase and the aqueous phase. First cells are lysed by adding SDS or proteinase K. A protease is added to denature protein followed by phenol or mixture of both phenol and chloroform for further purification, and then the mixture is shaken and centrifuged to remove protein precipitates in the organic phase. Finally, ethanol or isopropanol is added to recover and precipitate purified DNA from the aqueous phase (McKiernan & Danielson, 2017). Though this method helps to recover high molecular weight DNA, it is time consuming, uses hazardous chemicals and involves multiple steps which increases sample contamination and errors during extraction (Köchl, Niederstätter, & Parson, 2005).

2.2.5 Limited Soil Samples Creates Bias in the DNA Extraction Method

Though there are many DNA extraction methods proposed for DNA recovery from soil, soil sample diversity in most studies is often very narrow (Anderson 2018). Since the soil samples used in the previous studies were limited, studies with an expanded array of soil types are necessary. Studies focusing on microbial community characterization take place in a wide range of soil types and environments. Thus, it becomes necessary to examine many soils of diverse types whenever evaluating soil extraction methods. One of the most recalcitrant of all the soils includes the rhizosphere samples. The Rhizosphere is in fact one of the most important of all the soil samples due to its importance in crop production and plant growth and development. Unfortunately,

there are few research projects which includes rhizosphere soils in their studies of DNA extraction methodology (Jia et al., 2006; Qi, Wang, Xing, Zhao, & Chen, 2012). So, there is the urgent need to compare the effectiveness of different DNA extraction method using diverse range of soils including the rhizosphere to effectively evaluate soil extraction methods. In the present study, four DNA extraction methods; silica based, gel filtration based and gel filtration plus silica-based DNA extraction and commercial DNeasy PowerSoil Pro kit were used to extract DNA directly from diverse range of soil including the rhizosphere. We hypothesize that gel filtration plus silica-based DNA extraction method will recover highest quality DNA from soils. We assume adding a silica-based step after the gel filtration step will further purify DNA from more recalcitrant soil types. Thus, the overall objective of this research is to compare the reliability of both the silica based, gel filtration based and gel filtration plus silica-based DNA extraction techniques compared to the current most popular commercial kit across a wide range of soils in terms of DNA yield, purity, and microbial community structure.

CHAPTER III

METHODS

3.1 Wheat Rhizosphere Sampling Methods

3.1.1 Developing Growth Saturated Environment for Creating Rhizosphere Sample

When the soil is saturated with a large number of plants growth saturation is obtained. When plants are growth saturated, we assume that root growth has reached a point where the enclosed root system has explored the entire soil volume. Under such a condition all soil will be termed rhizosphere soil. Therefore, extracting a soil core from such a system would constitute a rhizosphere sample. We hypothesize that a growth saturated environment will exist within the planting box when a further increase in planting density does not result in an increase in shoot biomass. An experiment was performed to determine the planting density where growth saturation occurs based on our assumption.

3.1.2 Soil Sampling and Arranging the Boxes inside the Green House

An Easpur soil obtained from the Stillwater Farm was used to determine the growth saturating planting density for our experiment. A total of 26 Kg of homogenized Easpur soil was added to each of 35 Tray-10 boxes (Stuewe and Sons, OR) inside the

Western Road Departmental greenhouse #315. Homogenization was performed using a large cement mixer to completely homogenize the soil system. Soil was added to the mixer and mixed for at least 10 minutes. The soil was then dumped into a large wheelbarrow and mixed again using a shovel. Then an equal volume of soil was distributed across all experimental units. The process was repeated about 15 times until all boxes were filled (26 Kg of soil on average). Thus, all boxes contained a near identical soil mixture. Spring wheat seeds (var. Brick) were planted 1 inch deep in the soil contained in the Tray 10 box to yield a planting density of 5, 10, 15, 20, 25, 30, 35 seeds/box. Each planting density was replicated five times. A set of 5 boxes were set up with homogenized Easpur soil but without any plants as a blank control. The plants were watered, and no nitrogen was added to the boxes. Boxes were moved every alternate day at the time of watering so that each box experienced the variety of spatial environments throughout plant growth. The plants were harvested after 47 days corresponding to Feekes 9 growth stage. Plant shoots were cut and shoots fresh weights for each plant grown inside the box determined. At harvest, aggregate plant shoot weight for each box was determined and plotted against number of plants. The plant density where additional aggregate biomass did not result in increased total biomass was determined (Figure 1). This planting density was assumed to be the optimum density where growth saturation of soil took place. Subsequent experiments used this density.

Twenty Tray 10 boxes ($33*33*25\ cm^3$) containing experimental material were set up and filled with homogenized Easpur soil as above. Wheat seed was planted to a 1 “depth. Plants were watered and unfertilized as indicated above. At Feekes 9 stage plant shoots were harvested and weighed for fresh weight biomass. From each box, we created a grid of 20 positions from which to sample using the core method. Selection of

sampling position was done using the randomization feature in the Excel spreadsheet. Galvanic based soil moisture readings were taken to determine if there were differences among boxes in terms of soil moisture. Within each Tray 10 box a total of 3 subsamples were taken. A soil core was taken to 15 cm depth using a coring tool (PRO PLUGGER, USA). The soil core was removed and weighed and placed in a labeled plastic bag. Prior to grinding, visible roots, and pebbles were removed. The soil core was homogenized at high speed using a blender (NINJA, USA) in 700 mL of water with 5 mL detergent (Dawn Ultra Detergent) for 1 minute. Sample (Liquid) was collected immediately using a pipette into one 15 mL tube and three 2 mL tubes. The samples were labelled carefully and immediately stored at -21°C and then transported to the laboratory where they were stored at -70°C. These constituted the CORE samples.

To obtain the shake samples all the procedures were same as the core method except instead of grinding the whole core sample, we manually removed root tissue from the soil. The roots were then shaken vigorously three times to remove non-rhizosphere soil. Those selected roots were kept inside the 50 ml tubes and weighed. The same detergent was added to the tube. The tubes were closed and shaken vigorously. The soil detergent mixture was then passed through the 1 mm sieve filter to remove any additional root particles. Finally, the sample was collected in one 15 ml tubes and three 2 ml tubes as with the core samples, labelled carefully, and stored at -21°C and then at -70°C for DNA extraction these were labeled shake samples.

3.1.3 DNA Extraction Process

Three samples from each box were taken making a total of 60 samples. Of the 60 samples 9 were randomly selected for use as CORE samples, 9 as shake samples. An

additional 9 samples were selected from cores from a blank Tray 10 box which did not contain any wheat plants. From these cores we followed the Power Soil DNA extraction method outlined in this thesis. All extracted DNA were of high yield and purity (260/280 nm ration > 1.80). The DNA were stored at -70C and then shipped to a commercial sequencing facility under frozen conditions.

3.1.4 Sequencing and Molecular Analysis

We sent our extracted DNA to Novogene (Novogene, CA) for DNA sequencing. Sequencing was performed on the V5-V7 as amplified region of the 16S rRNA gene, using 799 F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC) as forward and reverse sequence primers, respectively. The forward and reverse primers were selected due to their resistance to amplify plant-based plastid or mitochondrial DNA. Raw sequences were downloaded from the Novogene server and uploaded to a laptop computer (The processor of my computer is Intel(R) Core (TM) i5-4200U CPU @ 1.60GHz 2.3GHz with 4.00 GB of installed RAM and the operating system is Windows 10 Enterprise) computer in Fastq manifest format. A manifest file was created indicating forward and reverse sequence identities for each sample using a text editor (Nano) for use by Qiime2 instructions. Novogene demultiplexed the sequences and these sequences were uploaded as a Qiime2 artifact file for computation. After uploading, a summary file was created in Qiime2 which indicated the quality of the sequences at each nucleotide position as well as overall sequence numbers for each sample. The sequences were denoised using Qiime2 to eliminate PCR errors including chimeric sequences from the amplicon reads. Denoising using DADA2, also corrected errors in marginal sequences, removed singletons, joined denoised paired-

end-reads and then dereplicated those sequences. In denoising step, we truncated the size of our sequences according to the Qiime2 denoising analysis so that sequences retained a quality factor greater than 30. After denoising we constructed a sample metadata file using a text editor which provides our treatment structure for the denoised data. Qiime2 then created a feature table which indicated the number of each sequence obtained in each sample. We conducted phylogenetic taxonomic analysis using Qiime2 using the NCBI (National Centre for Biotechnology Information) database. We then analyzed for alpha diversity using Faith's Phylogenetic Diversity (a qualitative measure of community richness that incorporates phylogenetic relationships between the features) were measured. Alpha diversity was used to measure the diversity within the sample or community. In contrast, Beta diversity was used to measure the differences in microbial communities from different samples. We analyzed differences in beta diversity using the Bray-Curtis distance metric. We aimed to observe any significant community dissimilarity in different samples using beta diversity analysis. Additionally, principal coordinate analysis was performed to determine if there was a difference between core, shake and blank samples in terms of overall microbial community structure. We assigned the taxonomy to the sequences by using classifier Greengenes 13_8 99% OTU to determine what taxonomic levels (e.g., species, genus, family, …). We then summed up all of the OTUs expressed as a percentage of total and plot the data in a stacked bar plot for each sample. The final analysis on this compositional data performed using differential abundance testing or ANCOM (Analysis of composition of microbiome). This test is the most recent test which statistically analyzed the microbiome compositional data in two or more treatments. We used this statistical method to identify

features that were differentially abundant across sample groups (Bolyen et al., 2019). The flow diagram of DNA sequencing and molecular analysis is given in figure 2.

3.1.5. Statistical Analysis

Bacterial taxonomies were assigned using the pretrained 16S rRNA V3–V4 classifier based on the Greengenes reference database. Comparisons of alpha diversity were conducted using Kruskal–Wallis test. Kruskal-Wallis is a non-parametric version of one-way ANOVA. Permutational multivariate analysis of variance (PERMANOVA) (Tang, Chen, & Alekseyenko, 2016) was performed with 999 permutation and used to test the associations between microbial beta diversity and the treatments. Principal coordinates analysis (PCoA) was used to visualize sample dissimilarities (Caporaso et al., 2010; Vázquez-Baeza, Pirrung, Gonzalez, & Knight, 2013) based on the unweighted UniFrac distance metrics. We used a two-way analysis of variance (ANOVA) and Tukey HSD post hoc for pairwise comparison.

3.2 DNA Extraction Methods

3.2.1 Soil Samples Collection and Processing

Eight soil samples were collected for the experiment from a diversity of soils based upon analysis obtained from the Oklahoma Soil Survey (URL) (Figure 7). Soil series name and taxonomic name were provided for all soils except for potting-mix soil based on Web Soil Survey (USDA) (Table 3). Among these, six soil samples which we termed: Clay, Easpur, Garden, Kirtland, Natural, Teller samples were collected from top 15 cm of the soil from fields in the Stillwater area on August 2019. Care was taken to select locations with reported variations with respect to texture and organic matter

content. These soils were air dried for one day, screened for unwanted pebbles, roots dried, leaves removed, and soil clods broken down and passed through a 2 mm sieve and mixed well. Samples were kept in sterile plastic bags and stored at -4^{O}C for later extraction in a 50 ml centrifuge tube. In addition to these soils, we evaluated our soil extraction using a commercial potting soil (Miracle Gro). The potting soil was air dried, homogenized and ground to a powder in a coffee grinder. Rhizosphere soils were also analyzed. This soil was collected from the roots of a green bean plant grown in Stillwater, OK in the backyard of Dr. Anderson. The plant was uprooted, shaken vigorously three times to remove non rhizosphere soil. The roots along with soils attached to roots were washed in a clean bowl with water which is approximately equal to the volume of soil taken. About 2 mL of detergent was added to dissolve soil aggregates and then the solution was stored in a clean plastic bottle at 4^{O}C. Since this rhizosphere soil has water, and the soil settles down at the bottom of the 50 ml tube, we shook the tube vigorously prior to sampling each time. Then approximately 800 µL of mixture was drawn from the tube quickly into four 2 mL tubes. All soils were mixed and sampled into 2 ml tubes in like manner. Tubes were centrifuged at 14,000 g for two minutes to pellet the soil particles. Remaining supernatant was poured from the tubes and the tube plus soil weighed to determine the soil weight to volume ratio.

3.2.2 DNA Extraction Methods and Principle

Each of the DNA extraction methods used the direct method for extracting DNA, where the DNA is extracted using bead-beating extraction. In the direct method, cell lysis occurs by both mechanical and chemical disruption with DNA being released into a buffered solution. The benefit of direct methods is that it tends to have high DNA yields

but less quality because of the soil organic content released containing more contaminants like humic acids, proteins, metals, etc. (Kozdrój & van Elsas, 2000; Liesack, Janssen, Rainey, Ward-Rainey, & Stackebrandt, 1997) which disrupt the soil purification processes. In contrast, indirect methods of DNA extraction utilize enzymatic methods to dissolve away cell walls and release cell content without agitation. These methods tend to have higher quality DNA compared to direct method, but often suffers from insufficient extraction of the bacterial fraction from soil (Van Elsas, Mäntynen, & Wolters, 1997). Commercial kits mostly use the direct method to lyse the cell (Anderson, 2018). Here we compare the popular PowerSoil kit with our laboratory made DNA extraction method along with the laboratory silica-based kit. All procedures in this study use the direct extraction approach. The laboratory DNA extraction method or Gel Filtration chromatography incorporates a Sephacryl S-300 HR gel filtration matrix in spin column format as a chromatography method for the purification process.

3.2.2.a Gel Filtration Chromatography Method

We followed all the steps according to the protocol developed by Anderson et. al 2018. This method consists of four main steps: - beadbeating extraction, ammonium acetate precipitation, isopropyl alcohol precipitation, and Sephacryl 300 S HR spin column chromatography.

Beadbeating Extraction

Each soil weighing 250 mg was added to four 2 ml bead beating tubes with one scoop (150 µL) each of 0.1, 0.5, and 2.5 mm glass beads (Bio Spec Products). A total of 1000 µL extraction buffer (50mM TRIS pH 8.0, 50mM disodium EDTA, 500mM NaCl and 4% (w/v) of SDS) was then added, and tubes were subjected to bead beating for two

minutes at 1000 rpm. The tubes were then centrifuged at 14,000 g for five minutes to pellet the soil and beads leaving the DNA in the supernatant. The supernatants were carefully pipetted into a new 2 mL tube and the volume determined. A half volume of ammonium acetate (7.5 M) was added and the tube vortexed for ten seconds. The tube was placed on ice for five minutes to allow the contaminants to precipitate and then the tube was centrifuged again at 14,000 g for five minutes to pellet the contaminants.

Isopropyl Alcohol Precipitation

Approximately 900 µL of the supernatant was transferred to new 2 mL tubes, an equal volume of ice-cold isopropyl alcohol was added, and the tubes were mixed and placed on ice at least for 10 minutes to precipitate the DNA. The samples were centrifuged at 14,000 g for five minutes to get the DNA pellet. The supernatants were discarded, and the tubes were inverted and drained for another five minutes. The pellets were resuspended using 50 µL of nano pure water. At the point of suspension, the four multiple extractions were combined together by serially suspending pellets into the 50 µL of Nanopure water before gel filtration chromatography. The four multiple extractions were combined into one sample representing 1 gm of soil as one experimental unit. All the samples were placed on ice until the next step began.

Sephacryl 300 S HR Spin Column Chromatography

i. Preparation of Spin Column Chromatography

The DNA in the extracted sample was further purified using spin gel filtration chromatography. For this technique, we prepared the Sephacryl column following the exact protocol from the paper by Anderson et al (2018). To make spin column, we took a 600 µL and a 2 mL tubes and cut off their caps. Then we made a hole on the bottom of

the 600 µL tube with a dissecting needle, placed about a 1 cm^2 piece of Kimwipe wadded up on its bottom and used a 200 µL pipette tip with cut end to compress the Kim wipe down to its bottom of the tube. The paper wad served as a paper frit to prevent the loss of gel matrix upon centrifugation. We placed the 600 µL tube on the top of 2 mL catch tube. Then we added the suspended Sephacryl 300S HR gel matrix (Sigma Aldrich, Sweden) to the 600 µL tube and centrifuge at 1000 g for 2 minutes to pack the columns, and to force the eluent to flow out into the catch tubes. We repeated the addition of resuspension mix until the column was approximately 500 µL filled with Sephacryl 300S HR gel matrix.

ii. Sample Loading to Freshly pPepared Sephacryl 300 S HR Spin Column Chromatography

About 50 µL of sample was loaded onto Sephacryl 300 S HR spin column on top of a new 2 mL collection tube and centrifuged at 1000 g for 2 minutes. About 50 µL of gel filtration buffer (50 mM TRIS, 100 mM NaCl, pH 8.0) was added to the top of the column and centrifuged at 1000 g for 2 minutes. The column was placed on top of a new catch tube and 100 µL of gel filtration buffer was added to the column to elute the DNA. The sample was centrifuged at 1000 g for 2 minutes to elute the sample. The sample was placed on ice prior to performing Nanodrop spectroscopic analysis (Anderson, 2018).

3.2.2.b Silica gel Method

As with the gel filtration chromatography method, this method has four steps, beadbeading, ammonium acetate precipitation, isopropyl alcohol precipitation, and silica gel purification. The first three steps are the same for the gel filtration chromatography and silica gel methods, with only the fourth step differing.

Silica gel Preparation

About 0.26 g of silica dioxide (SIGMA-ALDRICH, USA) was added to 2 mL tube and approximately 2 mL of Nanopure water was added to the tube. The tube content was resuspended by vortexing for few seconds till the homogeneous mixture was obtained and the tube centrifuged at 1000 g for 2 minutes and the supernatant discarded leaving the silica gel pellet. This rinsing was repeated two more times (Rojas-Herrera et al., 2008a).

Silica gel Purification

About 100 µL of 5M K acetate was added to the silica gel pellet in the 2 mL tube, incubated for 5 minutes at 65°C and on ice for 5 minutes. Then the 100 sample from isopropyl alcohol step after precipitation and pellet drying was added to the silica solution in a 2 mL tube, mixed and then centrifuged at 14,000 g for two minutes and the liquid collected in 2 mL tube was discarded. Then 500 µL of 70% ethanol was added to 2 mL tube, the tube vortexed and centrifuged at 14000 g for two minutes and the supernatant discarded. This washing process was repeated two more times. 50 µL of nanopure water was added to column and the contents resuspended by vortexing. The sample was incubated at 50°C for five minutes, centrifuged at 14000 g for two minutes. Approximately, 60 µL of sample was removed carefully and collected in 600 µL tube. Samples were kept on ice for later Nanodrop analysis.

3.2.2.c Gel Filtration Chromatography Method Followed by Silica gel Method

The method is similar to the previous two, with the exception that silica gel purification was performed after the extracts had been subjected to gel filtration chromatography. Here the 100 ul coming off the gel filtration column was added to the

silica matrix and the procedure was continued as indicated above for the silica gel chromatography.

3.2.2.d PowerSoil Kit Method

Extractions using the commercial kit (DNeasy PowerSoil Pro Kit, Qiagen, Germany) was performed according to the manufacturers' procedures. These procedures involve the use of their proprietary extraction buffer, precipitation of contaminants and then binding, washing and eluting of the DNA from the silica membrane column.

3.2.3 DNA Quantitation

The DNA purity is an important parameter to measure. The purified DNA was quantitated at 260 nm using a Nanodrop spectrophotometer (Thermo-Scientific, Waltham, MA, USA) and crude purity was determined based on the ratio of 260/280 nm and 260/230 nm. Typically, a spectrophotometric 260/280 ratio of 2.00 is considered optimum, with greater than 1.80 acceptable for most PCR-based procedures (Jia, Han, Zhao, & Zhou, 2006). Ratio of 260/280 nm is used to measure the purity of nucleic acids and to detect protein, phenol, or other contamination in the sample. On the other hand, the 260/230 ratio is used as secondary measure of nucleic acid purity primarily detecting humic and fulvic acid contaminations (Wilfinger, Mackey, & Chomczynski, 1997). DNA has maximum absorbance at 260 nm while polyphenol derivatives like humic acids, fulvic acids and other polyphenol types have maximum absorbance at 230 nm. The ratio represents contaminants from polyphenol derivatives. Generally, a ratio of 2.0 is an indication that DNA has very low contaminants. Generally, a higher ratio is better indicating a lower level of DNA contaminants (Abdel-Latif & Osman, 2017).

3.2.4 Sequencing and Molecular Analysis

We sent our extracted DNA to Novogene (Novogene, CA) for DNA sequencing. Sequencing was performed on the V5-V7 amplified region of the 16S rRNA gene, using 799 F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC) as forward and reverse sequence primers, respectively. Raw sequences were downloaded from the Novogene server and uploaded to my computer (The processor of my computer is Intel(R) Core (TM) i5-4200U CPU @ 1.60GHz 2.3GHz with 4.00 GB of installed RAM and the operating system is Windows 10 Enterprise) in Fastq manifest format. A manifest file was created indicating forward and reverse sequence identities for each sample using a text editor for use by Qiime2 program. Novogene demultiplexed the sequences after sequencing and these sequences were uploaded as a Qiime2 artifact file for computation. De multiplexing identifies specific treatment with each sample. After uploading, a summary file was created in Qiime2 which indicated the quality of the sequences at each nucleotide position as well as overall sequence numbers. The sequences were denoised using Qiime2 to eliminate PCR errors including chimeric sequences from the amplicon reads. It also corrects errors in marginal sequences (in case of DADA2), removes singletons, joins denoised paired-end-reads and then dereplicates those sequences. In denoising step, we truncated the size of our sequences according to the denoising analysis. We constructed a sample metadata file using a text editor to provide our treatment structure for the denoised data. Qiime 2 then created a feature table which indicated the number of each sequence obtained in each sample. Qiime2 then created the phylogenetic tree for taxonomic analyses. Alpha diversity using Faith's Phylogenetic Diversity (a qualitative measure of community richness that incorporates phylogenetic relationships between the features) was measured. Alpha diversity is used to

measure the diversity within the sample or community. In contrast, Beta diversity is used to measure the differences in microbial communities from different samples. We analyzed differences in beta diversity using the Bray-Curtis distance metric. We aimed to observe any significant community dissimilarity in different samples using beta diversity analysis. Additionally, principal coordinate analysis was performed to determine if there is a difference in terms of overall microbial community structure due to DNA extraction methods and soil types. We assigned taxonomy to the sequences using the classifier based on Greengenes database 13_8 99% OTU to determine at what taxonomic levels (e.g., species, genus, family, …) difference was observed. We then summed up all of the OTUs expressed as a percentage of total and plotted the data in a stacked bar plot for each sample. The final analysis on this compositional data was performed using differential abundance testing or ANCOM (Analysis of composition of microbiome). This test is used to compare the composition of microbiomes in two or more populations. We use this statistical method to identify features that are differently abundant across sample groups (Bolyen et al., 2019). The flow diagram of DNA sequencing and molecular analysis is given in figure 2. Statistical analysis for this project was exactly the same as described in subsection 3.1.5 under “Statistical Analysis”.

CHAPTER IV

RESULTS

4.1 Wheat Rhizosphere Sampling Methods

The current method for sampling rhizosphere soil includes conventional pull and shake method. Basically, shake method is the manual removal of plant from the soil and then vigorously shaking of the loose soil leaving the attached soil on the roots called as the rhizosphere soils. The soil that is separated easily from root after shaking is called the bulk soil (Riley & Barber, 1971; Tsutomu, 1989).

4.1.1 Summary of Sequencing Results

We submitted 27 DNA samples from three respective treatments, coreshake and blank to Novogene for DNA sequencing. We obtained 4,122,318 raw DNA sequences count from Novogene which were then reduced to 1,021,492 after the denoising step. The number of clean raw DNA sequences counts, and DNA sequences count after denoising step for each treatment was given in the table 1. For microbial community composition, we performed alpha diversity, and beta diversity analysis. We classified the obtained sequences to the level of phylum. Additionally, we performed ANCOM to find the differentially abundant features in the study.

4.1.2 Developing Growth Saturated Environment for Creating Rhizosphere Sample

Wheat plants were grown in homogenized Easpur soil without the fertilizer to Feekes stage 9. Upon harvest, plant shoots were cut and shoots fresh weights for each plant grown inside the box determined. Aggregate plant shoot weight for each box was determined and plotted against number of plants. Each planting density was replicated five times. The result from figure1 revealed that the 20 wheat plants per box had the highest shoot biomass of 793.54 g followed by 30 wheat plants per box having shoot biomass of 759.54 g. The result demonstrated that after specific point, plants total biomass did not increase with increasing plant number. So, we assumed that was the point where growth saturation occurred. Thus, the optimum planting density was 20 plants per box. Therefore, we planted 20 wheat plants per box for our upcoming experiment from which we would harvest the rhizosphere soil for microbiome analysis purposes. Our further experiment focused on the sampling method of rhizosphere soils with two different approached: They were shake method and core methods.

4.1.3 Alpha Diversity Analysis of Bacterial Communities

In this study, we observed a high number of good quality reads ranging between 33955 – 45931. The average sequence number was 37822 reads per sample. From the boxplot (Figure 3), it is seen that there was no significant difference between the treatments in terms of the microbial diversity as determined by the Faith Phylogenetic Diversity (PD) index ($p = 0.0003$). However, Kruskal-Wallis pairwise comparison table showed that the blank differed significantly from core ($q < 0.0010$) and shake methods (q

< 0.0014) while core did not differ significantly from shake method (q > 0.7573) (Figure 3).

4.1.4 Beta Diversity Analysis of Bacterial Communities

Beta diversity analysis across the treatment was carried out through which principal coordinate analysis (PCoA) plots were generated and visualized by Emperor component of Qiime 2 using the unweighted UniFrac metrics. PCoA (Principal Coordinates Analysis) plots were used to visualize differences in community composition across sample categories. The first principal component (PC) accounted for 8.008%% of the variation in the data, and the second PC accounted for 5.857% of the variation in the data. While going through pairwise Permanova test analysis indicated that there was a significant difference between the blank and core ($p < 0.0015$), blank and shake ($p < 0.0015$) and core and shake ($p < 0.0180$). Obvious clusters were formed between blank and core and blank and shake. The Permanova result further confirmed that the sampling method had a significant influence on the microbial community (Figure 4).

4.1.5 Taxonomy Community Structure

Phylogenetic analysis of bacterial communities in each sampling method at the phylum level is summarized in Figure 5. Thirty-five phyla were identified, among them two phyla were uncharacterized. The most abundant phyla across all treatments were Actinobacteria, Proteobacteria, Firmicutes and Acidobacteria. These top four phyla jointly accounted for more than 93.73% in blank, 91.23% in core and 90.44% in shake treatments of the total microbial sequences obtained. However, the mean relative abundance of top four phyla in case of individual treatment i.e., in case of blank treatment

was Actinobacteria (56.78%), followed by Proteobacteria (25.52%), Firmicutes (9.81%), Acidobacteria (1.62%), in core treatment was Actinobacter (54.45%), followed by Proteobacteria (25.67%), Firmicutes (8.28%), Acidobacteria (2.84%), whereas in shake treatment was Actinobacteria (49.62%), followed by Proteobacteria (27.11%), Firmicutes (8.84%), Acidobacteria (4.87%)of total sequences in all samples. The remaining other reads in the microbial population structure were associated with Armatimonadetes, WS3, TM6, Chlamydiae, AD3, Chlorobi, TM7, Elusimicrobia, FBP, Fibrobacteres, Cyanobacteria, OD1, Thermi, Spirochaetes, FCPU426, NKB19, BRC1, OP3, Tenericutes, GNO2, WS4, GNO4, MVP-21, WPS-2, WS2 with different relative abundance to the microbial population. The major difference noticed was with the shake treatment having relatively less percentage of Actinobacter and higher percentage of Acidobacter than two other treatments (Figure 5). The summary table for each phylum with their respective total reads, and mean was shown in table 4.

4.1.6 ANCOM Analysis

ANCOM statistical analysis was performed at genus level to unravel the individual taxa whose relative abundances are significantly different across groups with the result was shown as a volcano plot. The shake treatment displayed one genus (Fibrobacteria, W = 555) with significantly higher relative abundance than core and blank treatment (Figure 6).

4.2 DNA Extraction Methods

4.2.1 DNA Purity (260/280 ratio)

Out of four extraction methods, PS method had the highest DNA purity 1.9 followed by GFC 1.84, whereas purity for the GFC-SG and SG methods were 1.67 and 1.47 respectively. These methods (GFC-SG and SG) yielded most impure samples and failed to purify DNA at an acceptable level of 1.8 sufficient for DNA sequencing. On the other hand, PS and GFC methods met 1.80 threshold for DNA sequencing for most of the soils. These methods were effective in removing the contaminants from most soils. Least variability among soil types was from PS then GFC then GFC-SG then SG. Each technique was statistically significantly different from each other (p- value < 0.0001) (Figure 8).

DNA purity was evaluated based on soil types. Eight soils were evaluated across 4 extraction techniques. Purity varied with soil and procedure (p-value < 0.0001). GFC failed to reach the 1.8 ratio with rhizosphere and potting mix while all others were sufficiently pure for sequencing. PS showed sufficient purity across all soils. Only the potting mix soil differed significantly across PS and GFC where PS was numerically superior to GFC. PS kit provided purity greater than 1.80 for all soils while GFC method did not in the case of two soils (Potting-mix and Rhizosphere). Only one soil showed higher purity in GFC method compared to PS (Kirtland) (Figure 8) but these differences were not significant.

4.2.2 DNA Purity (260/230 ratio)

Out of four extraction methods, GFC method (1.77) was found to be best in removing the polyphenol derivatives while PS was the least (0.97). GFC, SG and SG methods had the ratio of 1.57 and 1.45 respectively. Least variability among soil types

was from GFC and highest was from PS method. Each technique was statistically significantly different from each other with a p - value =0.0001) (Figure 9).

8 soils were evaluated across 4 extraction techniques. Purity varied with soil and extraction methods (p-value < 0.0001). Only the potting mix soil did not differ significantly across PS and GFC where GFC was numerically superior to PS (Figure 9).

4.2.3 DNA Yield

Since the DNA purity for GFC, SG and SG methods had lowest purity and the DNA obtained through these two methods did not meet the minimum purity threshold enough to be sent for DNA sequencing. Yield data are only applicable if the purity is above 1.8 and both GFC-SG and SG methods had the purity lower than 1.8 ratio. So, further analyses were not done for these methods and only two methods (GFC and PS) were included for further comparison and analyses. Out of two methods, PS method had the highest DNA yield of 30.55 µg/g while GFC method yielded 4.67 µg/g on average per gram of soil. However, high standard error was observed with the PS method which showed a high variation in the data than in GFC method (Figure 10). A significant difference in the yield across extraction methods and soil type ($p < 0.0001$) was found. For PS method, all the soil type had significantly higher yield than GFC method except for the potting-mix soil. In the PS method, the highest yield was obtained from rhizosphere soil (74.76 µg/g) and least was from potting mix (3.40 µg/g) whereas in case of GFC, the highest yield was from natural soil (7.29 µg/g) and least from Easpur soil (3.30 µg/g) per gram of soil (Figure 10).

4.2.4 Alpha Diversity Analysis of Bacterial Communities across DNA Extraction Methods and Soil Types

In this study, we observed a high number of good quality reads (clean reads) ranging between 77978 – 169020 per sample. The number of DNA sequences after denoising step for each treatment are given in the table 3. The average sequence number (clean reads) was 141746 reads per sample. Illumina 16S Metagenomic Sequencing Protocol considered 15000 – 100,000 reads per sample to be sufficient for microbiome diversity analysis (Zielińska et al., 2017). The boxplot confirmed that there was no significant difference in the microbial diversity due to DNA extraction methods as determined by the Faith Phylogenetic Diversity (PD) index ($p = 0.17$) (Figure 11). The range of Faith-PD values for GFC method was (69.03 – 147.23) and for PS method was (52.22 – 152.97). The average faith-PD for GFC and PS methods were 88.26 and 87.22 respectively (Figure 11).

There was a significant difference in the microbial diversity across soil types between two extraction methods ($p < 0.0001$). Out of eight soil types, only Kirtland and Easpur soils differed significantly across methods. In case of PS method, we observed the highest faith-PD with Teller soil and least with clay soil and potting-mix soil had the highest variation observed. With GFC method, garden soil had the highest faith-PD and the least with natural soil, and the natural soil was found to have highest standard error compared to other soil types (Figure 11). On the other hand, with Kruskal Wallis pairwise test further suggested that there was a significant difference between clay and Easpur, clay and potting-mix, clay and rhizosphere, clay and teller, Kirtland and potting-mix,

Kirtland and rhizosphere, Kirtland and teller, natural and potting-mix, natural and rhizosphere, natural and teller soils (Data not shown).

4.2.5 Beta Diversity Analysis of Bacterial Communities

Beta diversity analysis across DNA extraction method was carried out through which principal coordinate analysis (PCoA) plots were generated and visualized by Emperor visualization using the unweighted UniFrac metric. PCoA (Principal Coordinates Analysis) plots were used to visualize differences in community composition across sample categories. The analysis revealed that there was a significant effect ($p < 0.015$) of DNA extraction method on beta diversity. It was observed that there was a correlation between the bacterial community and DNA extraction methods which further suggested that microbial composition obtained through GFC methods differed significantly from PS in terms of beta diversity (Figure 12).

Similarly, beta diversity analysis performed on the unweighted UniFrac distance matrix represented through PCoA revealed significant clustering ($p < 0.004$) of different soil types based on PERMANOVA statistical analyses confirming that microbial community structure differed across soil types. It further indicated that there was a correlation between bacterial community and soil types (Figure 12).

4.2.6 Taxonomy Community Structure across DNA Extraction Methods

Phylogenetic analysis of bacterial communities in each DNA extraction method at the phylum level is summarized in Fig 13. The overall community structure was remarkably similar between the two treatments. In both treatments, thirty-eight phyla were identified, among them two phyla were uncharacterized. The relative abundance of

top nine phyla in case of gel filtration chromatography were Actinobacteria (45.61%), followed by Proteobacteria (36.15%), Firmicutes (5.84%), Acidobacteria (4.98%), Gemmatimonadetes (2.51%), Chloroflexi (1.94%), Verrucomicrobia (0.63%), Nitrospira (0.67%) and others (1.66%), whereas in case of PowerSoil kit were Actinobacteria (47.18%), followed by Proteobacteria (32.99%), Firmicutes (7.93%), Acidobacteria (4.80%), Gemmatimonadetes (2.04%), Chloroflexi (1.57%), Verrucomicrobia (0.86%), Nitrospira (0.73%) and others (1.90%) of total sequences in all samples. The remaining other reads in the microbial population structure were associated with Bacteroidetes, Planctomycetes, Armatimonadetes, WS3, TM6, Chlamydiae, AD3, Chlorobi, TM7, Elusimicrobia, FBP, Fibrobacteres, Cyanobacteria, OD1, Thermi, Spirochaetes, FCPU426, NKB19, BRC1, OP3, Tenericutes, GNO2, WS4, GNO4, MVP-21, WPS-2, WS2 with different relative abundance to the microbial population. In case of GFC method and PS method, these top three phyla jointly accounted for 87.60% and 88.10% respectively of the total microbial sequences obtained (Figure 13). The summary table for each phylum with their respective total reads, and mean was shown in table 5.

4.2.7 ANCOM Analysis

ANCOM statistical analysis was performed at genus level to unravel the individual taxa whose relative abundances are significantly different across groups and the result were shown as a volcano plot. The PS kit method displayed three genera with significantly higher relative abundance than GFC method while GFC method showed only one genus with significantly higher relative abundance then PS kit method. The three genera that were found differentially abundant in PS kit method were Rhodococcus (W = 30898) (orange dot), Patulibacter (W = 29907) (blue dot), Arthrobacter (W =

29726) (yellow dot) and Cryptosporangium (W = 28815) (cyan dot) was found to be differentially abundant in GFC method (Figure 14).

CHAPTER V

DISCUSSION

5.1 Wheat Rhizosphere Sampling Methods

For the rhizosphere soil sampling, several sampling methods have been proposed which include both conventional pull and shake method (Timonin, 1947), drying the entire root system (Turpault, 2006) and washing off the root materials adhering to roots (Naim, 1965). Angle et. al (1996) reviewed for the first time all the steps involved in sampling rhizosphere soil and suggested that some critical techniques need to be followed (Angle, Gagliardi, & Mclntosh, 1996). Like for collecting root, he suggested to take the entire root system as far as possible. Timonin (1947) was the first who came with the idea of shaking the root to separate the bulk soil from rhizosphere soil (Timonin, 1947). Naim et. al (1965) proposed the method for sampling rhizosphere soils where entire root system is carefully pulled out from the soil and adhering soil particles are carefully removed, then the root system was shaken continuously for 5 minutes in water (Naim, 1965). Turpault et.al (2006) collected the rhizosphere soil samples by drying the root system. The soil that fell off after drying is considered as bulk soil and rest of the soil still attached to dried root after subsequent shaking is considered as rhizosphere soils

(Turpault, 2006). Macrae et. al (1995), Gahoonia et.al (1991) introduced the slicing technique where soil sampling is carried out at 0.5 mm interval from a root map (Gahoonia & Nielsen, 1991; Macrae, Lucon, Rimmer, & O'donnell, 2001). Though many sampling methods are proposed, there is still lack of standardized sampling method for rhizosphere soil samples. It is in our opinion one of the primary obstacles in rhizosphere research.

Though the conventional pull and shake method is commonly used, the method itself cannot be considered as the standardized sampling method for rhizosphere soil samples. Macrae et. al (1995), reported the poor resolution of the pull and shake method for sampling DNA when they applied the pull and shake method for sampling rhizosphere and bulk soil (Macrae et al., 2001). The method is variable and depends upon many factors like soil texture (clay soil vs sandy soil), soil moisture (Luster, Göttlein, Nowack, & Sarret, 2009), types of root system, energetics of the shake (Angle et al., 1996) and tends to focus on older portion of the roots. For clay soil, even after shaking the root, big chunk of soils still adheres to root particles while for clay soil, most of the soils detached after shaking. Shaking also depends upon the operator's preferences to determine how forcefully the root is shaken. Luster et.al suggested to be cautious while analyzing the results since the amount of soil adhering to soil largely depends upon the soil texture and soil moisture. The sampling method should not be biased, and a standardized approach adopted. Variability in method would affect the final results representing the actual microbiome present in the soil. Even a slight variation in the sampling method introduces bias in the soil-plant- microbial investigation of rhizosphere. Therefore, we came up with a new approach for rhizosphere sampling called the core

method. The core method involves growing plants at a density where their growth is saturated in a particular confined container and then taking cores from the soil which should contain soil that is under the influence of the plant. When applying this method, we found the significant differences in the microbial composition compared to the standard shake and blank soils.

In this study, we also present how the different sampling methods in the end influences the final analysis of the microbial community structure. We used Faith PD metric to measure the alpha diversity across methods. Though the shake method had higher diversity than the core method, the difference was not significant, it suggested that the sampling method did not influence on the alpha diversity. the Faith pd was significantly higher in shake and core method compared to blank suggesting that the rhizosphere soils regardless of sampling method was more diverse than the blank. Microbial communities in the unvegetated soil are comparatively lower in diversity than vegetated soil (Banks, Mallede, & Rathbone, 2003). There were no plants grown in the blank samples, so plant-microbe interaction was absent which might have cause lower diversity in the blank samples.

Principal component analysis was performed on all three treatment samples. The first principal component (PC) accounted for 8.008%% of the variation in the data, and the second PC accounted for 5.857% of the variation in the data. Though microbial communities in all treatments were significantly different from each other, microbial communities in shake treatment were comparatively different from the blank and core samples. This difference could be due to the nature of treatment used. Blank treatment did not have any plants. Core treatment contained the entire root system where fine roots

were retained while shake treatment was left with the roots after vigorous shaking which mainly consisted of older roots. So, the microbial community in each of these treatments differed due to the differences in the treatment used.

In terms of community structure analysis, all the three treatments exhibit the similar relative abundance except for phylum, Actinobacteria and Acidobacteria. Shake treatment was enriched with relatively higher percentage of phylum, Acidobacteria while the relative abundance of Actinobacteria was lower with shake method. As described earlier, shake method tends to focus on older portion of roots because most of the fine and young roots detached after vigorous shaking. It is reported that older roots have increased number of Actinobacteria (Thirup, Johnsen, & Winding, 2001) (Watt, Magee, & McCully, 2008). However, our result contrasts with this finding. The increased number of Acidobacteria in shake method indicated that older roots prefer Acidobacteria, it might be because Acidobacteria can degrade secondary cell walls with lignin and other complex molecules (Kirby, 2005). The result showed some similarities in relative abundance between the core and blank samples. While taking the rhizosphere soil sample using the core method with the coring tool having the diameter of 6 cm, the entire root system was taken as a sample. So, the core samples might have some soil which were less affected by the plant roots. Therefore, core and blank samples shared some similarities. On the other hand, for shake method, only the soil adhered to root after vigorous shaking were considered as rhizosphere samples even though the majority of adhered roots were older roots.

ANCOM statistical analysis was performed to unravel the main genera responsible for the differences at genus level between three treatments. ANCOM analysis

is the appropriate method for analyzing compositional data sets such as those presented here. Interestingly, one different genus Fibrobacter, was found to differently abundant with shake method. It is difficult to answer the reasons for these differences. It is predicted that Fibrobacter will likely reside on the older roots. Cellulose is one of the components of roots along with water, lipids, carbohydrate, and proteins (Tao, Zhang, Wang, & Christie, 2008). Fibrobacter is known to degrade cellulose, and perhaps older roots are more likely to harbor Fibrobacter. However, there could be other differentially abundant genera associated with one of these methods, but they might be below detection limit, so they did not appear in the result.

5.2 DNA Extraction Methods

There are numerous studies which have evaluated different DNA extraction methods using soils with contrasting characters (Bürgmann, Pesaro, Widmer, & Zeyer, 2001; de Lipthay, Enzinger, Johnsen, Aamand, & Sørensen, 2004; Frostegård et al., 1999; Lloyd-Jones & Hunter, 2001; Miller, Bryant, Madsen, & Ghiorse, 1999; Niemi, Heiskanen, Wallenius, & Lindström, 2001; Steffan, Goksøyr, Bej, & Atlas, 1988). Both the choice of DNA extraction method and soil type influence on the yield and purity of DNA as well as on the microbial community structure were shown previously (Zielińska et al., 2017). Efficiency of DNA extraction methods end up with variable results due to the differences in microbial cell wall, cell membrane structure and character of soils (Carrigg, Rice, Kavanagh, Collins, & O'Flaherty, 2007; Krsek & Wellington, 1999). It is reported that problematic soils when using different DNA extraction methods showed significantly higher levels of differences in yield and purity of DNA (Gabor, de Vries, &

Janssen, 2003; Islam, Sultana, Joe, Cho, & Sa, 2012; Krsek & Wellington, 1999; Robe, Nalin, Capellano, Vogel, & Simonet, 2003). It is because different soil microorganisms have different susceptibilities to various cell lysis methods (Daniel, 2005). It is suggested to use the multiple extraction methods for the same single sample to increase the chance of recovering all organisms present in the tested sample (Morgan, Darling, & Eisen, 2010). Morgan et. al further reported that final representation of microbial community structure not only depends upon the DNA extraction method, but also on sequencing technology and bioinformatics tools used, which are beyond our consideration in this thesis. However, one should know the limitations and alternative extraction method before engaging in microbial community characterization by sequencing. Therefore, it is essential to know the primary goal of the research before selecting the extraction method.

In this study, we observed that quality and quantity of DNA and microbial community profile varied across DNA extraction methods and soil types. Since the result and their interpretation can be influenced by the selection of DNA extraction method, choosing the appropriate DNA extraction method is crucial in every microbiome research program (Zielińska et al., 2017).

Looking at the DNA purity across all extraction methods, both SG method and GFC-SG method ended with high impurities. All the DNA samples obtained by these two methods were brown in color which indicated presence of humic acids in the samples (Zielińska et al., 2017). As humic acid contains similar charge and size characteristics like DNA, it exhibits absorbance at both 230 and at 260 nm and hence interferes in measuring DNA concentration and humic acid content (Fatima, Pathak, & Rastogi Verma, 2014). Additionally, both DNA and contaminants can absorb light at 260 nm

causing the overestimation of DNA yield (Heaton & Keer, 2008). We suspect silica method brought impurities and contamination in the extraction process. Therefore, we found more impurities in the GFC-SG than GFC method itself. Hence, it was confirmed that the introduction of silica step in the process was not helpful in removing the impurities from the sample. However, our result contrasts with the finding by Rojas et. al (2008) where they obtained DNA with high yield and purity. This difference might be because the same DNA extraction method might not work for diverse range of soils, and they used silica method on limited range of soils. On the other hand, PS kit method had the highest and most consistent DNA purity (260/280) for most of the soils. It performed well even for the stubborn rhizosphere soils. However, when considering humic acids, PS had the lowest purity (260/230) among four extraction methods. This lowest purity is probably due to the presence of polyphenol derivatives contaminants. High contaminants with phenolic compounds are one of the problems with the PS commercial kit method and since humic acids are known to interfere with PCR procedures including sequencing the presence of these acids may be problematical for these procedures.

GFC method yielded second highest purity (260/280) and highest 260/230 ratio among four extraction methods. This method was successful in removing most impurities from the samples, so it yielded second highest purity among four extraction methods. The use of Sephacryl S-300 HR, gel filtration matrix in GFC method helps to trap the contaminants like humic acids while allowing the DNA to flow through the column (Anderson, 2018). One of the most fascinating natures of Sephacryl S-300 HR, gel filtration matrix is that it can trap even small DNA oligomers (DNA < 180 bp) within the GFC matrix and thereby reducing chimera formation during downstream polymerase

chain reaction (PCR) (Zhou, Bruns, & Tiedje, 1996) a major problem with DNA sequencing efforts.

While analyzing the purity across soils, clay soils showed the highest purity despite having highest percentage of organic matter (4.74) among eight soil types. Organic matter is generally considered as the major source of inhibitors that is coextracted with DNA during extraction process (Yeates, Gillings, Davison, Altavilla, & Veal, 1998). However, the contrasting result was shown by our study where clay soil despite having high organic matter showed the highest purity.

Looking at the overall yield across two DNA extraction methods, PS method had comparatively highest yield when compared with the GFC method. These results differed from Anderson 2018 where GFC method yielded more DNA. As the commercial kits are upgraded frequently, the kit quality may have been improved dramatically within the last 4 years. So, the DNA yield with PS method in our study may have been much better due to these changes than the previous study conducted by Anderson et. al (2018) which used an earlier kit.

Along with DNA yield and purity, cost and time needed for the DNA extraction are sometimes important when selecting the appropriate extraction method. Though PS kit method was found to be the better choice for extracting more DNA from wide range of soils, especially for rhizosphere soil, it is expensive as compared to laboratory based GFC method. Furthermore, these commercial kits typically expire within one year of purchase, so researchers often find a need to repurchase new kits even when all components have not been used within a given time. Kit components are proprietary in nature and are subject to change without notice (Zielińska et al., 2017) as indicated

earlier. The other important drawback of commercial kits is that the company often upgraded the quality of kits, so it becomes hard to find the same kit when necessitating their use for comparative purposes over time. But with the laboratory- based method, researcher can buy reagents in bulk and prepare however much the project needs. The bulk reagents typically have a long shelf life compared to commercial kits, (Anderson, 2018) so they can be stored for many years. This makes the cost of extraction method cheaper, overall. Despite these limitations, the PS kit method should be chosen when maximizing yield and purity are of primary importance or when working with rhizosphere soils or commercial potting mix with much organic content. The PS kit method offers the further advantage of requiring only around 45 minutes to extract DNA, while laboratory- based method needs 4 hours to complete DNA extraction process. However, for the non-rhizosphere soil, GFC is the great alternative and is cheaper than most commercial kit methods.

Evaluation of DNA extraction method should not be limited to the kit with the best DNA yield and purity, but also on the characterization of the sequences themselves (Zielińska et al., 2017). In this study, we present data showing that the choice of DNA extraction method has significant influence on the analysis of microbial community structure.

We use faith PD metric to measure the alpha diversity across extraction methods. We observed that the extractions yielded similar phylogenetic diversity. Our result contrasts with other finding where they obtained differing level of phylogenetic diversity across DNA extraction methods (Zielińska et al., 2017). When comparing the alpha diversity across all soils there were statistically significant differences based upon soil

and extraction procedure with respect to the Teller soil which showed the highest diversity in the PS compared to GFC technique. In contrast the Kirtland soil exhibited higher diversity in the GFC than in the PS technique. The other soils: Easpur, clay, natural, garden, potting and rhizosphere soils did not differ in terms of microbial diversity as measured by Faith PD across extraction techniques.

The highest diversity was found in the Teller extracted with PS while the lowest diversity was found in the Natural, and clay soil extracted in both PS and GFC technique. Both the clay and natural soils come from undisturbed and unmanaged environments while the Teller came from a highly managed field with a long history of wheat production. Furthermore, the Teller was much more coarse textured than was the clay soil while the clay and natural soils had much higher levels of organic matter than the Teller. On the other hand, highest diversity was exhibited by Kirtland soil in the GFC. Kirtland soil had comparatively low pH (5.5) and low organic matter content. It is found that under low pH conditions, decomposition is faster causing the decrease in soil organic carbon (Wang et al., 2019). High diversity of bacteria in Kirtland soil might be due to that the organic matter present in the Kirtland soil was labile and diverse range of bacteria could decompose it faster. At this time, it is not exactly clear why these significant differences in microbial diversity arise among these eight soils, but it may have something to do with the organic content, although the Teller soil would be a significant outlier in that case.

Principle coordinate analysis revealed that there was a significant difference between extraction methods. However, the analysis revealed a large variation between the replicates of each extraction method. Similar finding was observed in Zielinska et.al

(2016), where they also found the large variation between the replicates in each kit method (Zielińska et al., 2017). However, microbial community was significantly different across soil types. There was clear clustering of samples of each soil which suggested that soil had a significant influence on the microbial community. The analysis further indicated that microbial composition of each soil is phylogenetically related to their own groups than others. However, from the figure a large variation between the replicates of some soil were observed especially in case of potting-mix.

While looking at the taxonomy community structure, we found the similar trends of taxonomic groups of soil bacteria in many previous studies in the literature. Twelve most dominant phyla across the globe are found to be Proteobacteria, Actinobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Verrucomicrobia, Bacteroidetes, Gemmatimonadetes, Firmicutes, Armatimonadetes, TM7 and WS2 (Delgado-Baquerizo et al., 2018). Acidobacteria is one of the most predominant phyla encountered in soils and sediments (Dunbar, Barns, Ticknor, & Kuske, 2002; Liles, Manske, Bintrim, Handelsman, & Goodman, 2003; Tringe et al., 2005). In the study conducted by Wei et. al (2020), Proteobacteria, Chloroflexi, Bacteroidetes and Actinobacteria were the top predominant bacterial phylum (Wei et al., 2020). Proteobacteria and Actinobacteria were the most predominant phyla found in different soil samples in the study conducted by Habtom et. al (2019) (Habtom et al., 2019). Similar result was reported by Kardayi et. al (2021) where Proteobacteria, Actinobacteria and Acidobacteria were the most abundant phyla (Karadayı, 2021). After the analysis of microbial composition of soil samples from diverse habitats, Jesmok et. al (2016) stated that bacteria share the same main bacterial classes up to 75% based on the relative abundance chart (Jesmok, Hopkins, & Foran,

2016). This might be one of the reasons why our study coincides with other findings. It is still unknown what factors contribute the variation in the taxonomic groups of soil bacteria across soil types. However, it is assumed that these phyla which are found to be dominant across the globe are critical drivers, or indicators of key soil processes worldwide (Delgado-Baquerizo et al., 2018). It is predicted that they share some phenotypic traits or life-history strategies that make them able to thrive under specific environmental conditions (Barberán, Caceres Velazquez, Jones, & Fierer, 2017; Fierer, Morse, Berthrong, Bernhardt, & Jackson, 2007). Additionally, it is assumed that various environmental factors like soil pH, soil moisture, temperature, plant species might be responsible for this variation (Lauber, Hamady, Knight, & Fierer, 2009; Maestre et al., 2015; Ramirez et al., 2014; Schloss, Girard, Martin, Edwards, & Thrash, 2016; Zhou et al., 2016). The main conclusion from our results is that both GF and PS yield similar results in terms of community structure at the level of Phyla.

Sequencing data comprises an example of compositional data analysis. Compositional data typically has trouble fitting to conventional normalized distribution with any sorts of accuracy. The ANCOM analysis overcomes these limitations by fitting the data to a more appropriate distribution. ANCOM statistical analysis was performed as part of the Qiime 2 package to unravel the main genera responsible for the differences at genus level between two extraction methods. Interestingly, four different genera (Rhodococcus, Patulibacter, Arthrobacter and Crptosporangium) were found to be significantly different with one of these methods. These all bacteria are gram positive, aerobic except Arthrobacter and belong to phylum Actinomycetota. These bacteria have great agricultural importance because all of them are involved in the degradation of

environmental pollutant. Like Rhodococcus widely known for its ability to degrade the recalcitrant toxic environmental pollutants (Liu et al., 2019). Similarly, Patulibacter is able to degrade ibuphren (Almeida et al., 2013). Arthrobacter can biodegrade various environmental pollutants like glyphosate, atrazine, nicotine, fluorine (Mongodin et al., 2006). Cryptosporanium is capable of degrading organic matter (EUZéBY, 1997). However, it is difficult to answer the reasons for these differences without further investigations why these bacteria were found to be differentially abundant in specific extraction method. We do not perform ANCOM for different soils. As per the ANCOM rule, if we expect more features are changing between the groups, we should not use ANCOM as it will introduce more error in the result. Therefore, we may expect that many features are changing across soil.

CHAPTER VI

CONCLUSION

6.1 Wheat Rhizosphere Sampling Methods

Our results suggests that the rhizosphere soil sampling method is one of the factors responsible for determining the end result of the microbial community analysis both at taxonomic and functional level. It can be concluded that the core and shake method are fairly comparable in terms of alpha diversity but differed in terms of beta diversity and differed slightly in terms of the differences at the level of genera according to the ANCOM analysis with only one genera difference. The differences associated with Beta diversity is likely due to the inclusion of microorganisms associated with the entire root system in terms of the Core method and with the loss of fine roots in association with the Shake methods. Furthermore, the Core method is likely to contain soil that is less influenced by the plant compared to the Shake method showing more resemblance to the Blank. Thus, use of either the Core or shake method is advisable under the proper interpretation as indicated above. Finally, the Core is likely the best method to represent the entire root system.

6.2 DNA Extraction Methods

Recovering DNA with high yield and purity is the first critical step and plays a crucial role in gene sequencing. We compared different DNA extraction methods with wide range of contrasting soils to come up with the conclusion concerning the best DNA extraction method viable for all soil types. We found that despite the drawbacks that come with the PS kit method, we recommend it for rhizosphere soils because it outperformed all other methods in terms of DNA yield and DNA purity. However, looking at non-rhizosphere soil, GFC method might be preferable because it performed as well as PS kit method and, also does not come with same drawbacks that the PS kit method has. While observing the microbial community structure, we concluded that soil type has a greater influence on determining the microbial community structure other than DNA extraction methods. We predict that PS kit method might be the better choice to present the closest assessment to the actual structure of the microbial population and using this kit, it might be helpful to find noble microorganisms that can be used to increase soil fertility, crop productivity and to remediate soil contamination.

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APPENDICES

Table 1: Raw DNA sequences count, and DNA sequences count after denoising step in each treatment.

Treatment	Blank	Core	Shake
Raw DNA Sequences Count	1308899	1393179	1420240
DNA Sequences Count After Denoising	327393	340679	353420

Table 2: Physical and chemical Characteristics of eight different soil types.

Soil Name	Clay	Easpur	Garden	Kirkland	Potting mixed	Natural	Rhizosphere	Teller
Vegatation	Forest (Cider tree behind OSU Cross Country)	All areas are prime farmland	Green beans were planted and soil enriched with high organic matter	Stillwater farm	NA	Grassy meadow	Backyard near garden soil	Perkin Expt Centre (Wheat field)
Texture class	Clay loam	Sandy loam	Sandy clay loam	Loam	NA	Silt loam	NA	Sandy loam
Sand (%)	22.5	60	47.5	27.5	NA	35	NA	67.5
Silt (%)	40	27.5	27.5	50	NA	52.5	NA	25
Clay (%)	37.5	12.5	25	22.5	NA	12.5	NA	7.5
pH	6.4	6.3	7.7	5.5	NA	6	NA	6.3
N03(lbs/A)	3	21	2	33	NA	10	NA	27
P(lbs/A)	13	58	40	279	NA	37	NA	40
K(lbs/A)	323	176	279	260	NA	211	NA	205
Top SO4(lbs/A)	7.317	NA	23.696	9.022	NA	11.335	NA	1.03
Ca(lbs/A)	6127	1896	7698	2782	NA	2551	NA	1181
Mg(lbs/A)	1450	505	787	804	NA	633	NA	331
Fe(lbs/A)	31.4	16.2	19.6	84.8	NA	95.6	NA	11.9
Zn(lbs/A)	1.6	0.6	131.6	0.9	NA	1.9	NA	0.6
B(lbs/A)	0.41	0.124	0.357	0.108	NA	0.091	NA	0.078
Cu(lbs/A)	0.7	0.7	1.9	1.1	NA	0.6	NA	0.4
%OM	4.74	0.64	4.12	1.52	NA	4.19	NA	0.78
Latitude, Longitude	36.13800°, -97.075710°	36.11848°, -97.09839°	36.07175°, -97.04412°	36.12133°, -97.09306°	NA	36.13881°, -97.08086°	36.07176°, -97.04455°	35.99120°, -97.04509°

Table 3: Soil series and taxonomic names of soils based on Web Soil Survey (USDA).

Soil Name	Soil Series Name	Taxonomic Name
Clay	Masham silty clay loam	clayey, mixed, active, thermic, shallow Udic Haplustepts
Easpur	Easpur loam	fine-loamy, mixed, superactive, thermic Fluventic Haplustolls
Garden	Grainola-lucien complex	Haplustalfs/Haplustolls
Kirtland	Kirkland silty loam	fine, mixed, superactive, thermic Udertic Paleustolls
Potting mix	NA	NA
Natural	Kirkland silty loam	fine, mixed, superactive, thermic Udertic Paleustolls
Rhizosphere	Renfrow loam	fine, mixed, superactive, thermic Udertic Paleustolls
Teller	Teller fine sandy loam	fine-loamy, mixed, active, thermic Udic Argiustolls

Table 4: Raw DNA sequences count, and DNA sequences count after denoising step in each extraction method.

DNA Extraction Method	Total Sum of Raw DNA Sequences	Total Sum of DNA Sequences after Denoising
GFC	5687718	1567576
PS Kit	5651923	1486880

Table 5: Nine most abundant phylum (Total sum, percentage and mean) identified in Blank, Core, and Shake treatment respectively.

Blank treatment

Phylum	Total Reads	Percentage	Mean
Actinobacteria	20654.56	57.0209	2294.951
Proteobacteria	9282.889	25.62721	1031.432
Firmicutes	3568	9.850156	396.4444
Acidobacteria	590.7778	1.630957	65.64198
Gemmatimonadetes	1177.778	3.251484	130.8642
Chloroflexi	245.5556	0.677904	27.28395
Nitrospirae	290.4444	0.801828	32.2716
Verrucomicrobia	94.44444	0.260732	10.49383
Bacteroidetes	114.5556	0.316253	12.7284
Planctomycetes	65.33333	0.180365	7.259259
Others	138.4444	0.382203	15.38272

Core treatment

Phylum	Total Reads	Percentage	Mean
Actinobacteria	20609.11	55.0395	2289.901
Proteobacteria	9714.556	25.94407	1079.395
Firmicutes	3134	8.369783	348.2222
Acidobacteria	1074.667	2.870047	119.4074
Gemmatimonadetes	1288.889	3.442157	143.2099
Chloroflexi	594.1111	1.586656	66.01235
Nitrospirae	321	0.857275	35.66667
Verrucomicrobia	191.5556	0.511576	21.28395
Bacteroidetes	196	0.523445	21.77778
Planctomycetes	155.2222	0.414543	17.24691
Others	165.1111	0.440952	18.34568

Shake treatment

Phylum	Total Reads	Percentage	Mean
Actinobacteria	19483.11	50.04995	2164.79
Proteobacteria	10644.44	27.3444	1182.716
Firmicutes	3472.889	8.921466	385.8765
Acidobacteria	1912.556	4.913143	212.5062
Gemmatimonadetes	1112.667	2.858317	123.6296
Chloroflexi	945.5556	2.429027	105.0617
Nitrospirae	439.5556	1.129169	48.83951
Verrucomicrobia	314.2222	0.807202	34.91358
Bacteroidetes	192.4444	0.494368	21.38272
Planctomycetes	187.2222	0.480953	20.80247
Others	222.6667	0.572006	24.74074

Table 6: Nine most abundant phylum (Total sum, percentage and mean) identified in GFC and PS kit method, respectively.

GFC

Phylum	Total Reads	Percentage	Mean
Actinobacteria	679160	45.61%	16979
Proteobacteria	538342	36.15%	13458.55
Firmicutes	87026	5.84%	2175.65
Acidobacteria	74151	4.98%	1853.78
Gemmatimonadetes	37496	2.52%	937.40
Chloroflexi	28866	1.94%	721.65
Verrucomicrobia	9427	0.63%	235.68
Nitrospirae	9943	0.67%	248.58
Others	24730	1.66%	20.61

PS Kit Method

Phylum	Total Sum	Percentage	Mean
Actinobacteria	738416	47.18%	18460.40
Proteobacteria	516366	32.99%	12909.15
Firmicutes	124122	7.93%	3103.05
Acidobacteria	75093	4.80%	1877.33
Gemmatimonadetes	31865	2.04%	796.63
Chloroflexi	24594	1.57%	614.85
Verrucomicrobia	13398	0.86%	334.95
Nitrospirae	11482	0.73%	287.05
Others	29762	1.90%	24.80

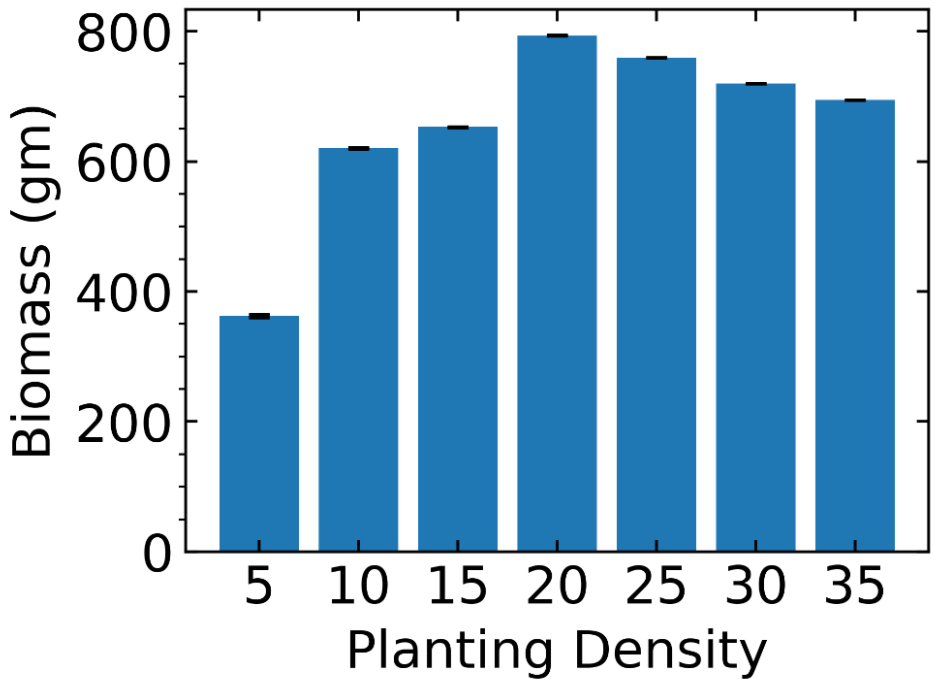


Figure 1: Bar graph showing the total average biomass of wheat plants across different planting density. Errors bars represent ± standard deviation of the mean.

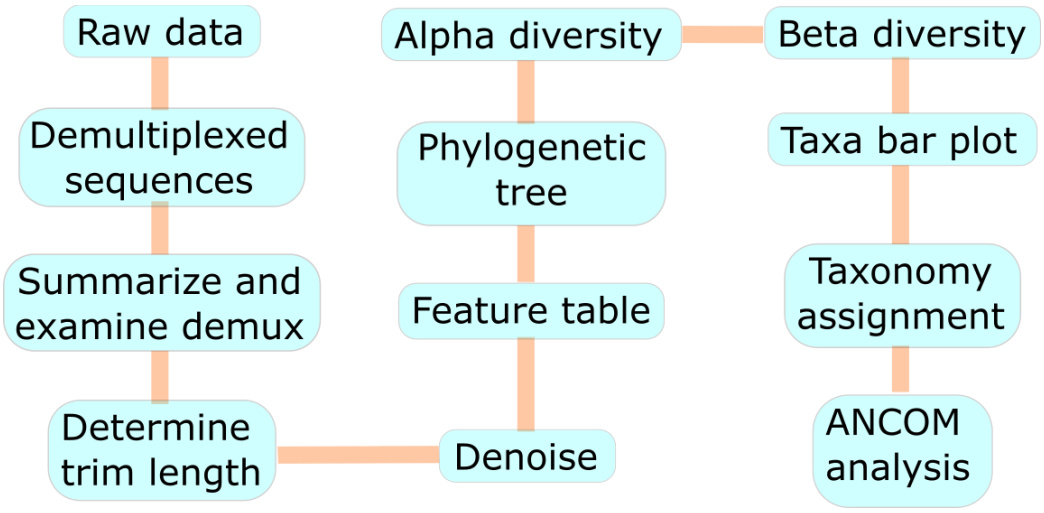
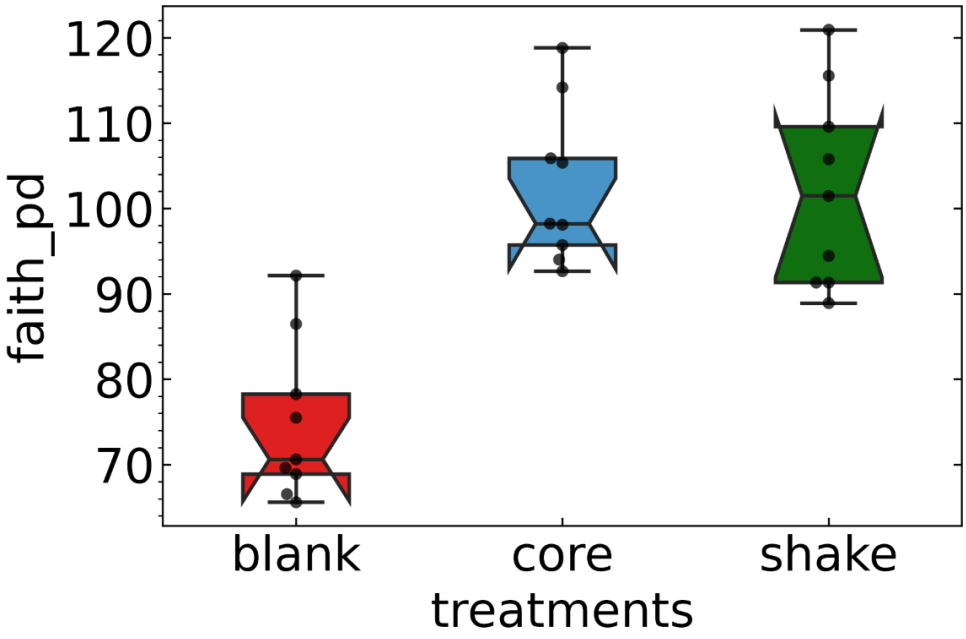


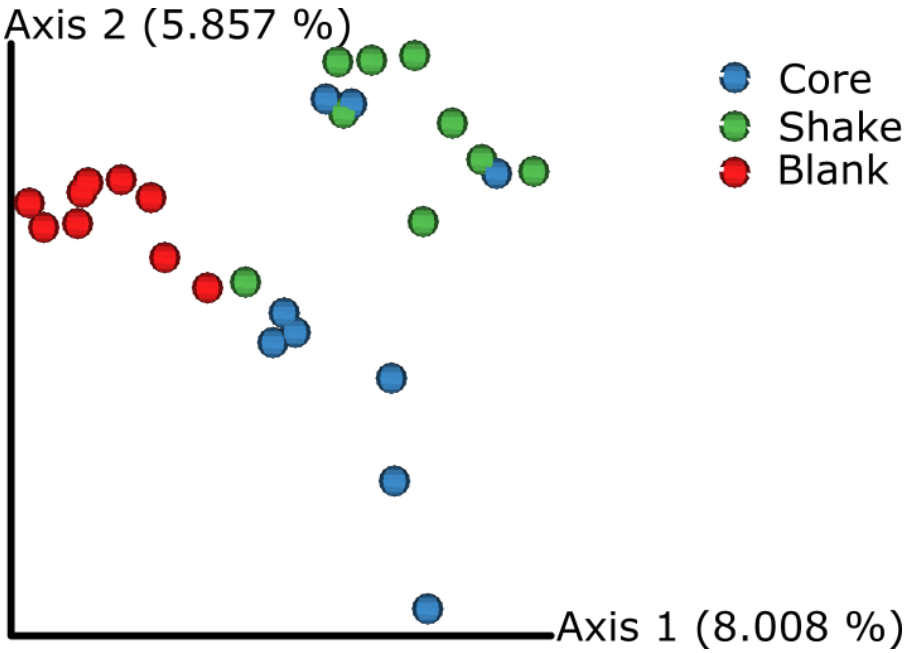
Figure 2: Flow diagram of DNA sequencing and molecular analysis.



Kruskal-Wallis Pairwise Comparison

Treatment	q-value
Blank * Shake	0.0010
Blank * Core	0.0014
Core * Shake	0.7573

Figure 3: Boxplots displaying Faith Phylogenetic Diversity (PD) index plotted across treatments (a), bar graph showing Faith -PD for each treatment (b). Different letters on top of bars represent significant differences at $p < 0.0001$ for Tukey's HSD. Errors bars represent ± standard deviation of the mean. Kruskal-Wallis pairwise comparison table is shown close to figure 3.



Pairwise PERMANOVA Comparison

Treatment	q-value
Blank * Shake	0.0015
Blank * Core	0.0015
Core * Shake	0.0180

Figure 4: A two-dimensional plot generated from principal coordinate analysis (PCoA) using the unweighted UniFrac Metric showing the bacterial community diversity different treatments. Pairwise PERMANOVA comparison table is shown close to figure 4.

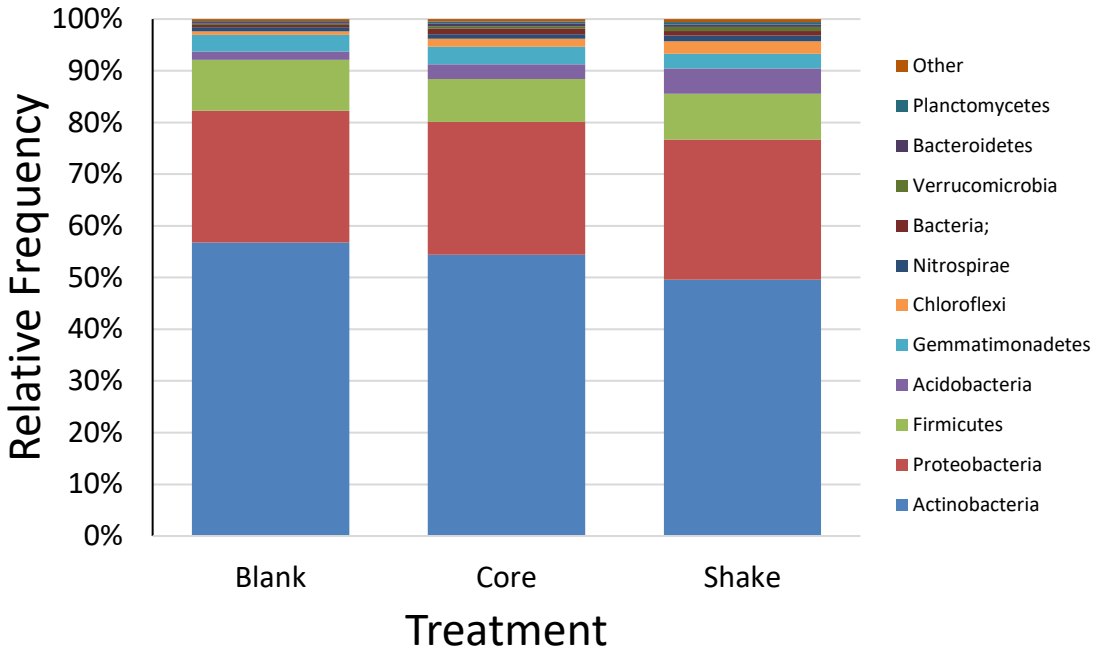
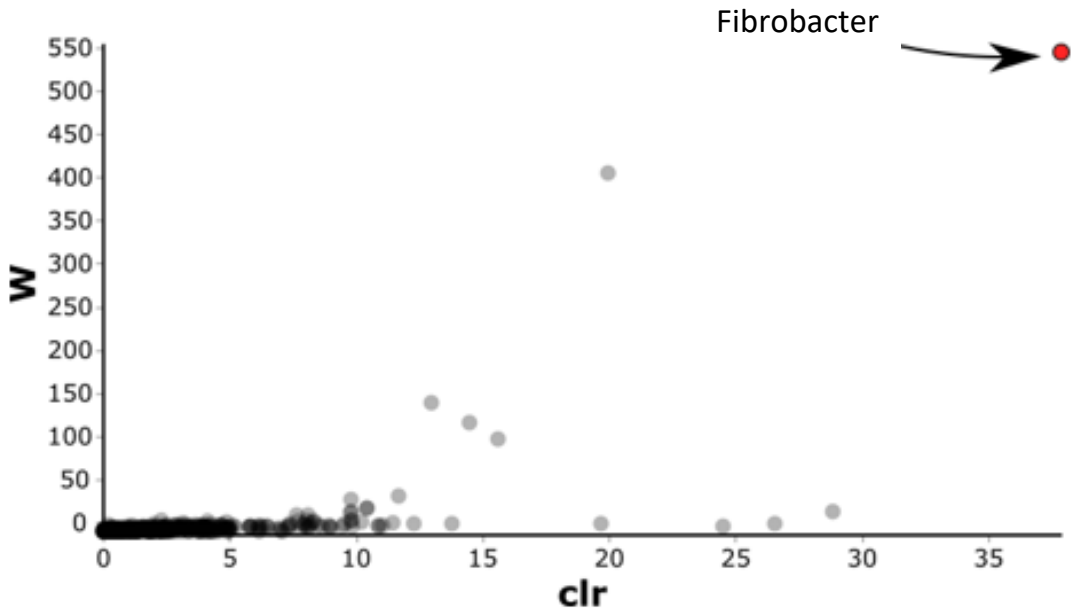


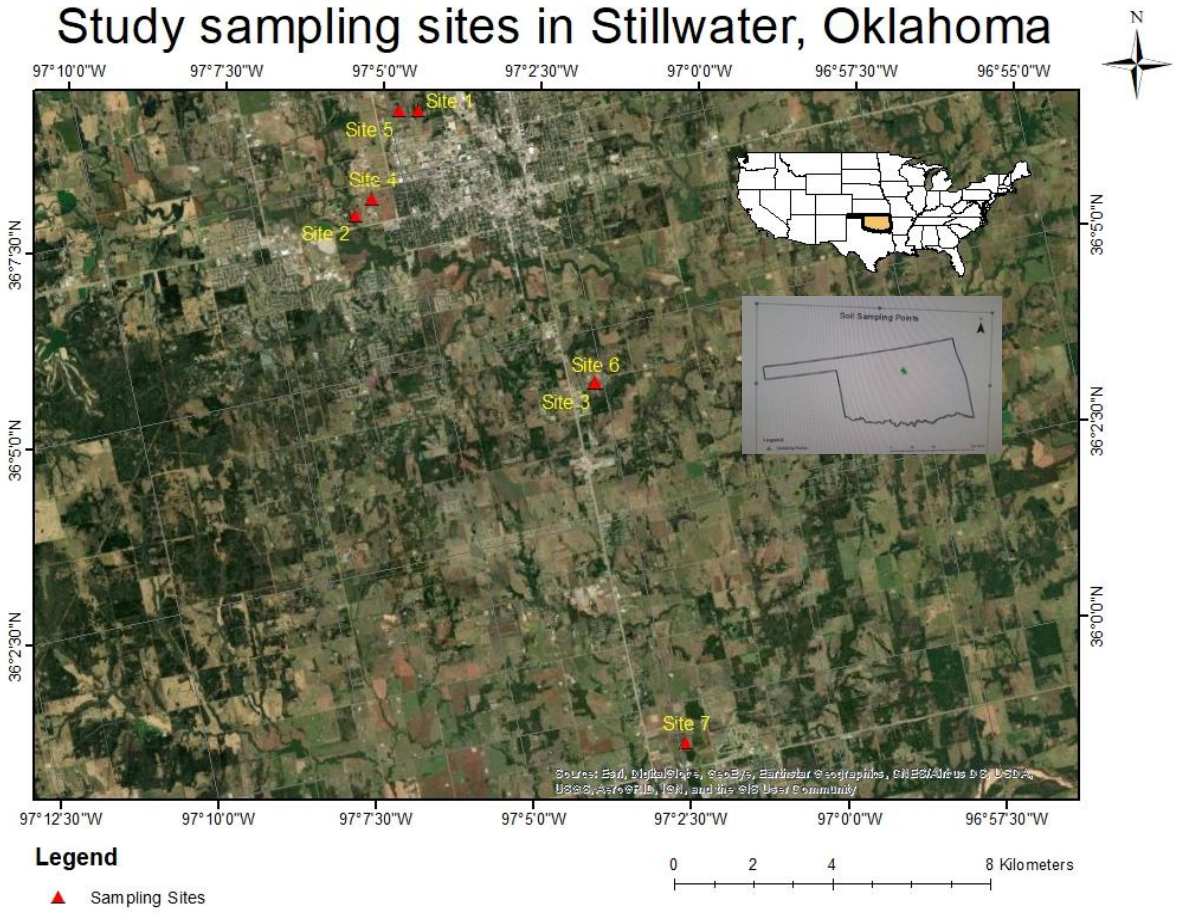
Figure 5: Abundance of microbial 16 S rRNA sequences at the phylum level displayed for different treatments. “Other” describes: Armatimonadetes, WS3, TM6, Chlamydiae, AD3, Chlorobi, TM7, Elusimicrobia, FBP, Fibrobacteres, Cyanobacteria, OD1, Thermi, Spirochaetes, FCPU426, NKB19, BRC1, OP3, Tenericutes, GNO2, WS4, GNO4, MVP-21, WPS-2, WS2.



Percentile abundance of features by group

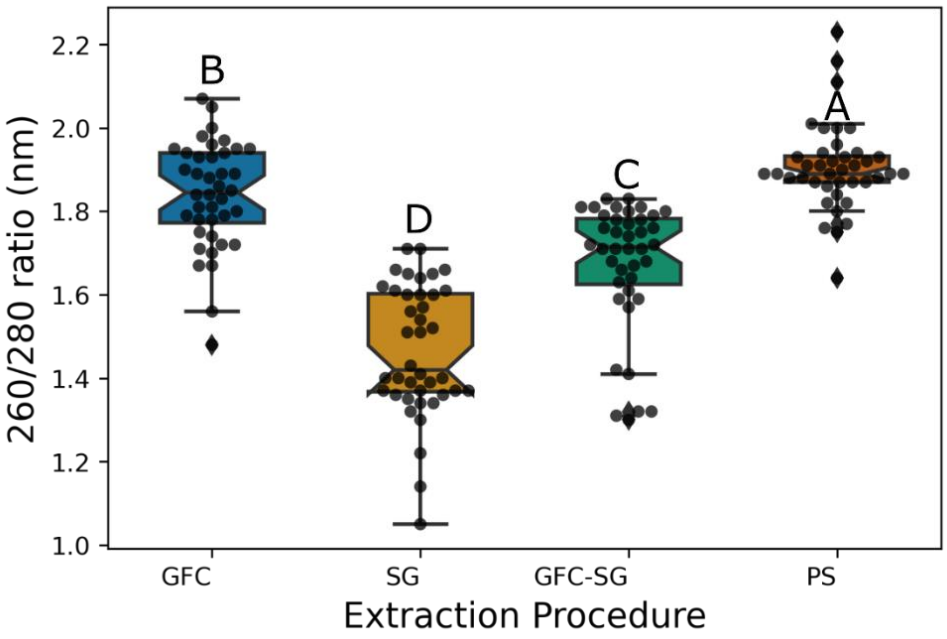
Treatment	Percentile	Fibrobacteria
Blank	100	10
Core	100	27
Shake	100	99

Figure 6: Volcano plot representation of ANCOM statistical analysis showing the genera that is differentially abundant in shake method (Red-filled dot, W > 555).



Site #	1	2	3	4	NA	5	6	7
Soil Name	Clay	Easpur	Garden	Kirtland	Potting-mix	Natural	Rhizosphere	Teller

Figure 7: Map location of different places at Stillwater, Oklahoma where soil samples were collected.



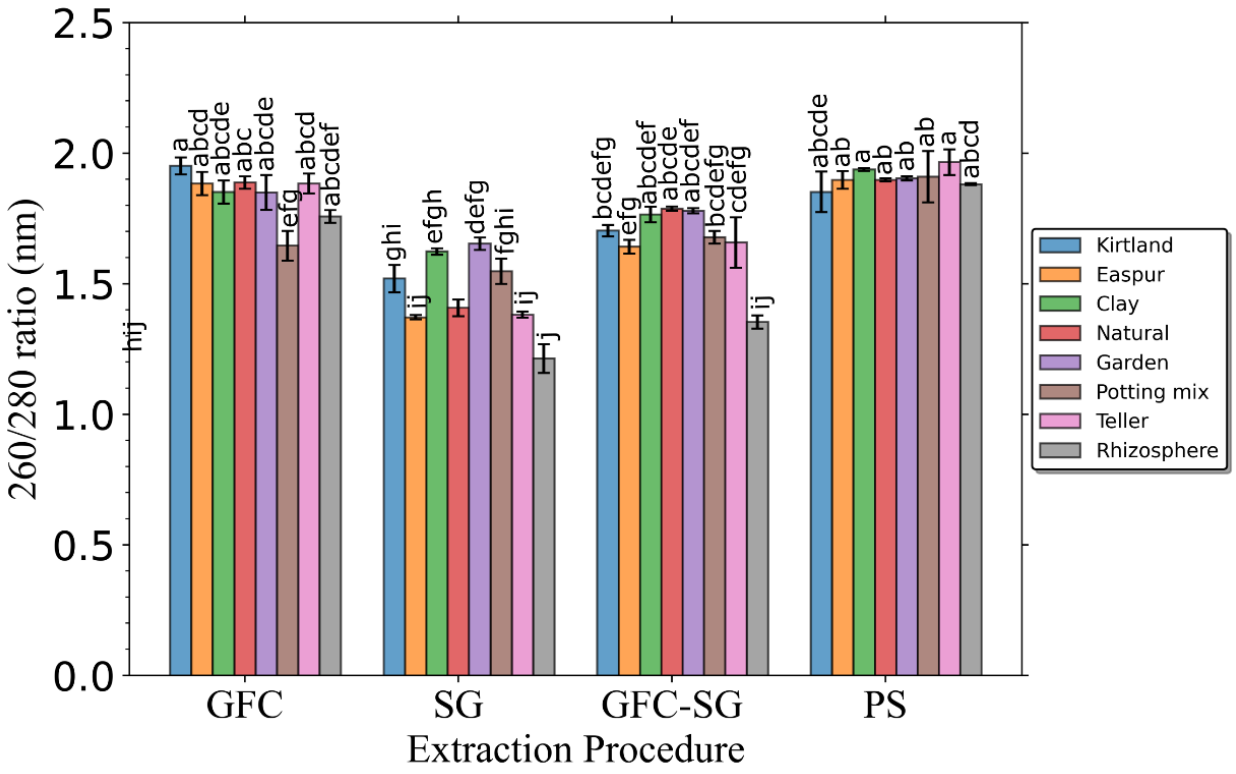


Figure 8: Boxplots showing DNA purity across extraction methods (a), bar graph showing DNA purity for each soil type for each extraction method (b). Different letters on top of bars represent significant differences at $p < 0.0001$ for Tukey's HSD. (GFC = Gel filtration chromatography, SG = Silica gel, GFC-SG = Gel filtration chromatography followed by silica gel, and PS = PowerSoil kit)

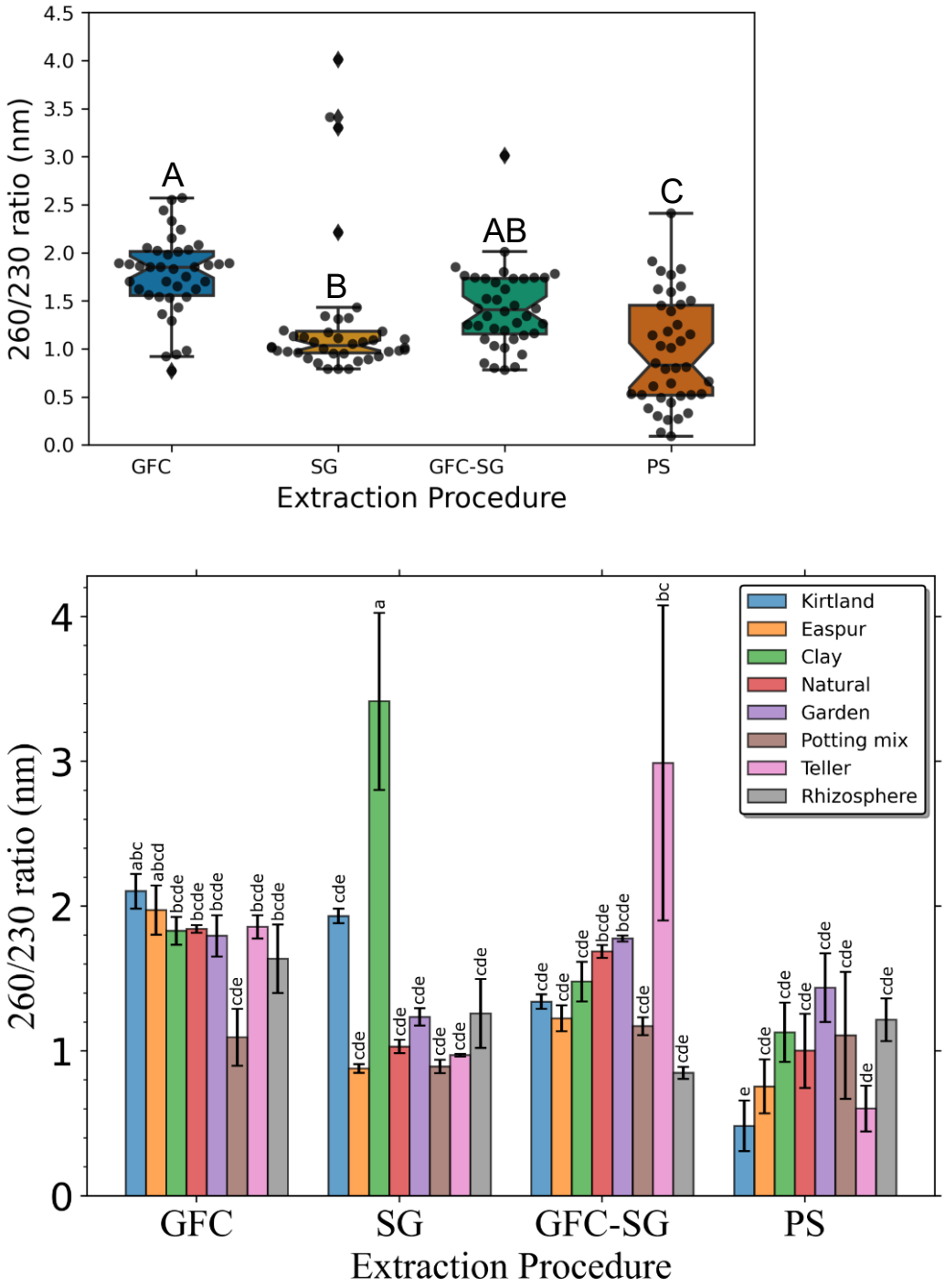


Figure 9: Boxplots showing DNA purity across extraction methods (a), bar graph showing DNA purity for each soil type for each extraction method (b). Different letters on top of bars represent significant differences at $p < 0.0001$ for Tukey's HSD. Errors bars represent ± standard deviation of the mean.

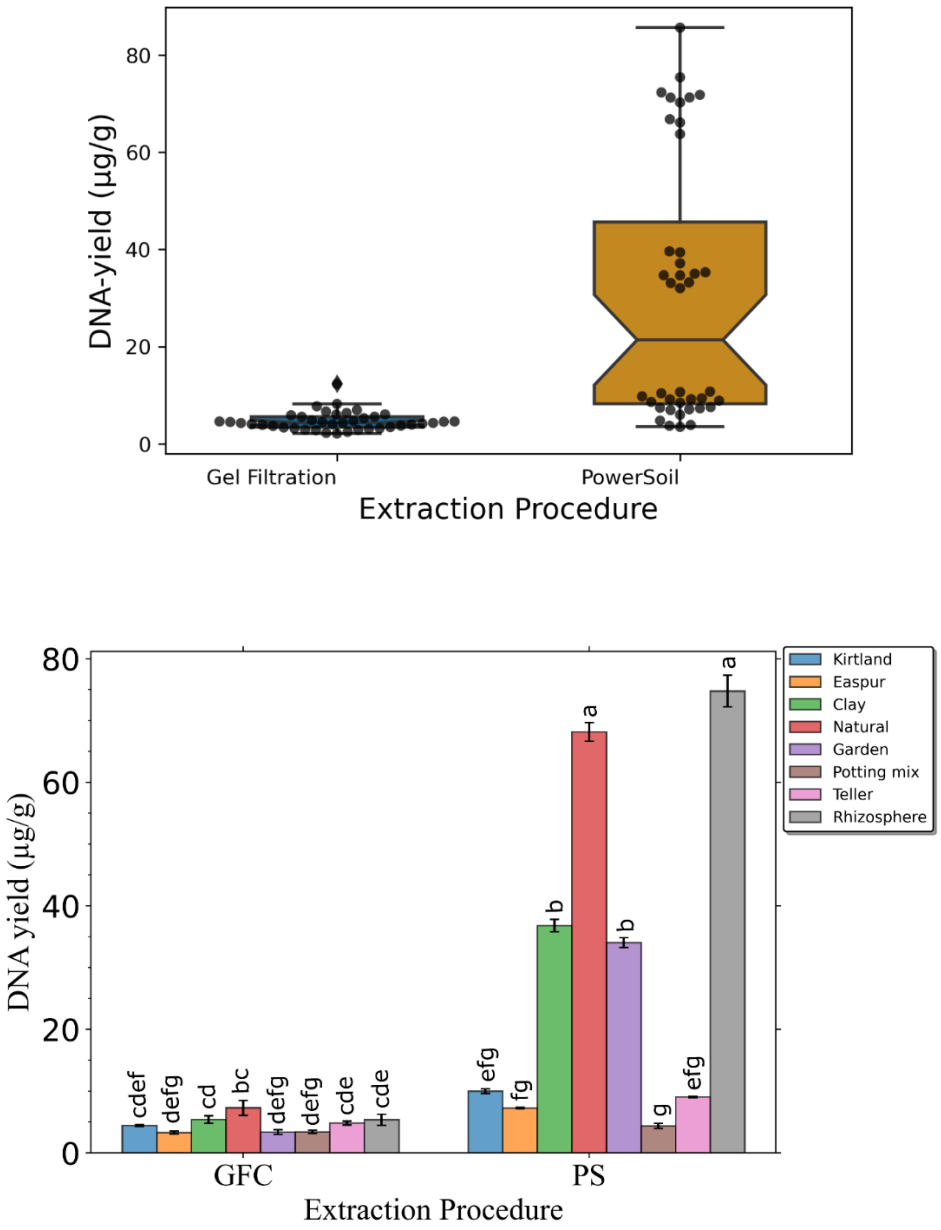
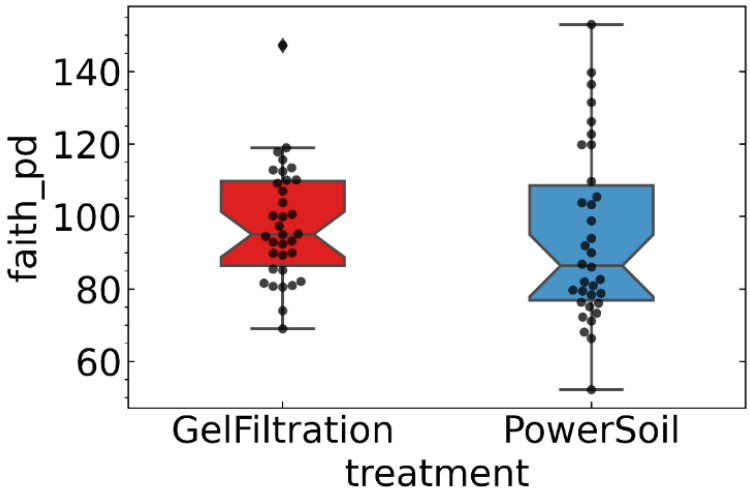


Figure 10: Boxplots showing DNA yield across two extraction methods (a), bar graph showing DNA yield for each soil type in each extraction method (b). Different letters on top of bars represent significant differences at $p < 0.0001$ for Tukey's HSD. Errors bars represent ± standard deviation of the mean.



Treatment	q-value
GFC*PS	0.1734

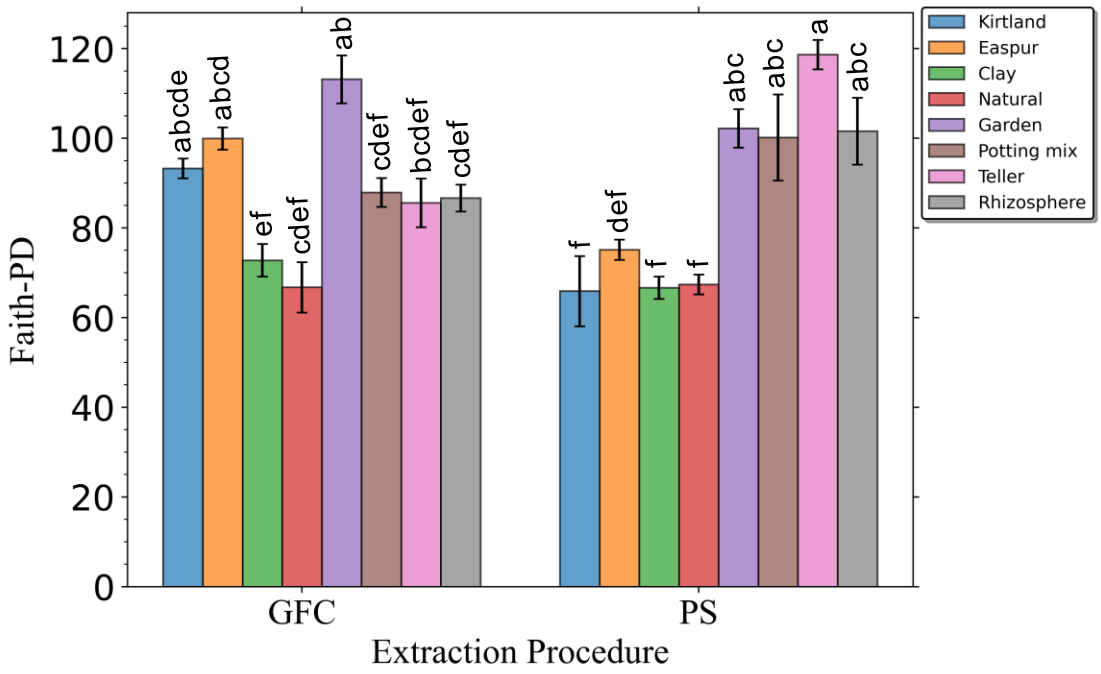
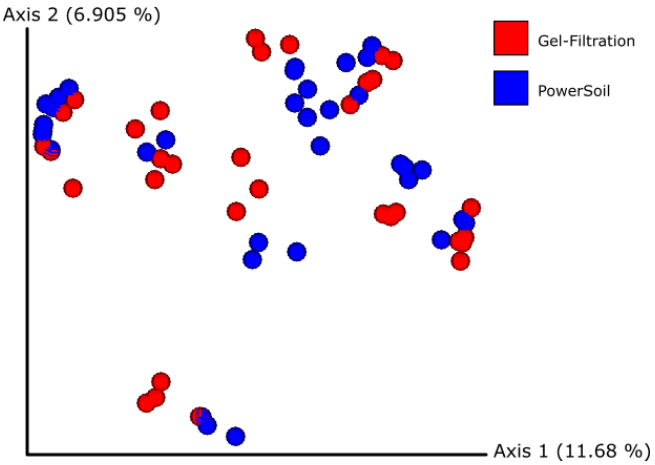


Figure 11: Boxplots displaying Faith Phylogenetic Diversity (PD) index plotted across extraction methods (a), bar graph showing Faith -PD for each soil type in each extraction method (b). Different letters on top of bars represent significant differences at $p < 0.0001$ for Tukey's HSD. Errors bars represent ± standard deviation of the mean. Kruskal-Wallis pairwise comparison table is shown close to figure 11.



Pairwise PERMANOVA comparison

Treatment	q-value
GFC*PS	0.004

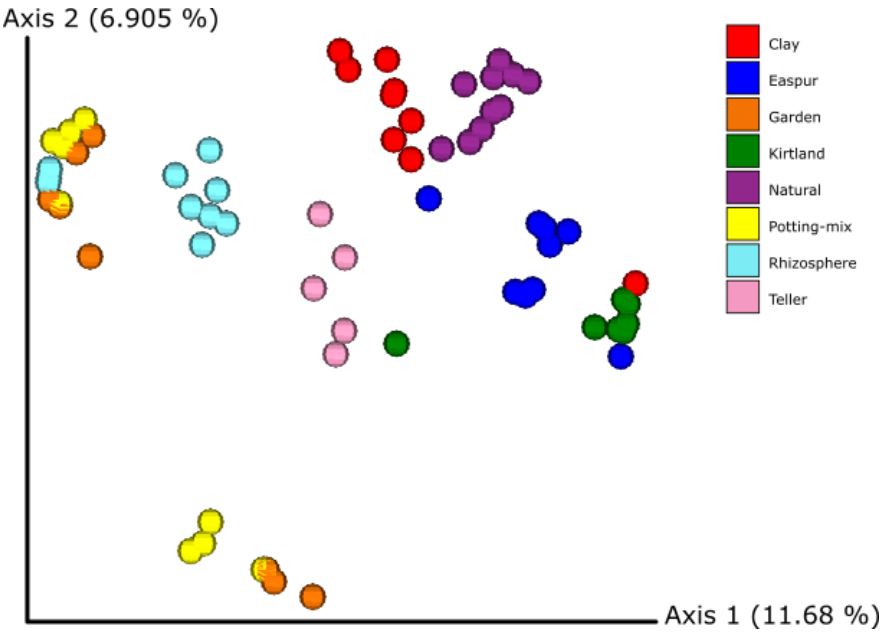


Figure 12. A two-dimensional plot generated from principal coordinate analysis (PCoA) using the unweighted UniFrac Metric showing the bacterial community diversity across the DNA extraction methods (a), across soil types (b). Pairwise PERMANOVA comparison table is shown close to figure 12.

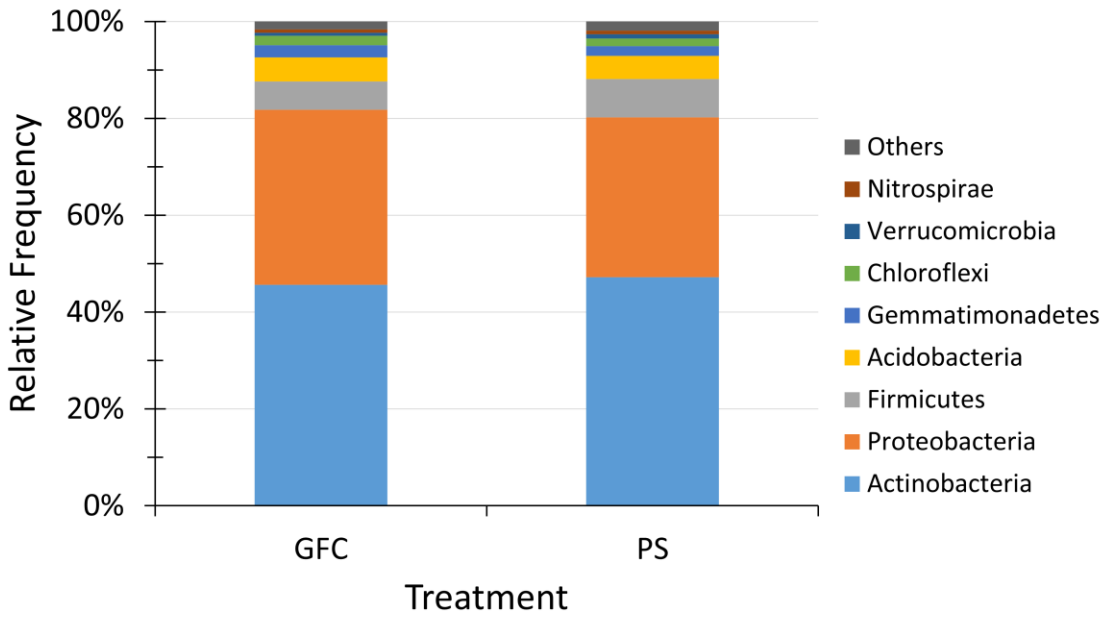
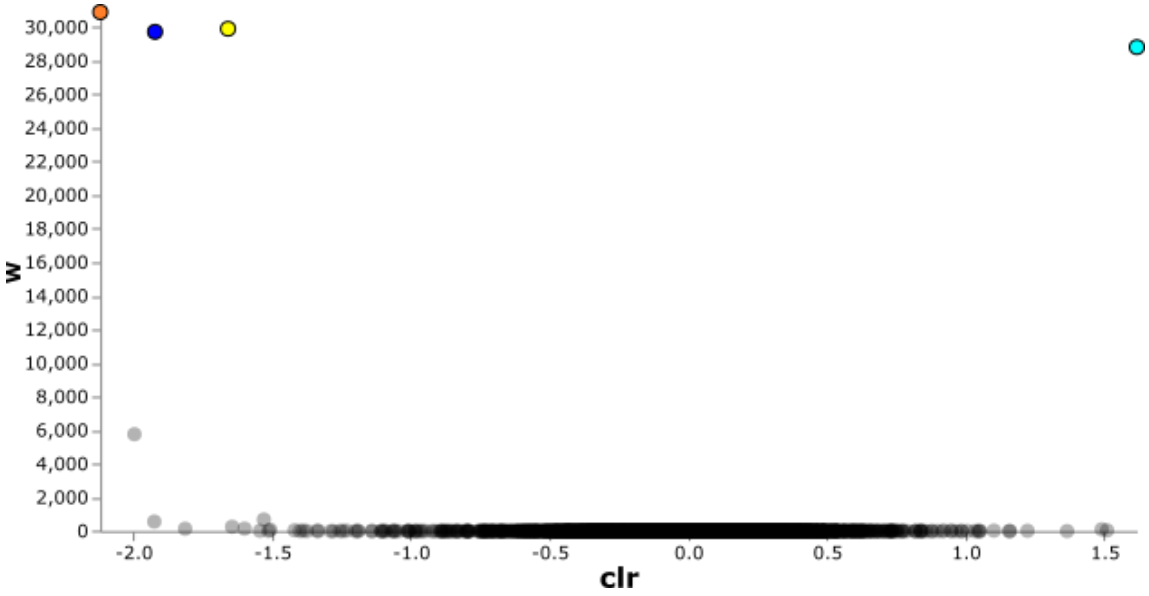


Figure 13. Abundance of microbial 16 S rRNA sequences at the phylum level displayed for DNA extraction method. “Other” describes: Bacteroidetes, Planctomycetes, Armatimonadetes, WS3, TM6, Chlamydiae, AD3, Chlorobi, TM7, Elusimicrobia, FBP, Fibrobacteres, Cyanobacteria, OD1, Thermi, Spirochaetes, FCPU426, NKB19, BRC1, OP3, Tenericutes, GNO2, WS4, GNO4, MVP-21, WPS-2, WS2.



Percentile abundance of features by group

Treatment	Percentile	Rhodococcus	Patulibacter	Arthrobacter	Cryptosporangium
GFC	100	24	1	228	170
PS	100	277	134	3937	18

Figure 14. Volcano plot representation of ANCOM statistical analysis showing the genera that are differentially abundant in PS kit method. The three genera that are found differentially abundant in PS kit method are Rhodococcus (W = 30898) (orange dot), Patulibacter (W = 29907) (blue dot), Arthrobacter (W = 29726) (yellow dot) and the genus that is found differentially abundant in GFC method is Cryptosporangium (W = 28815) (cyan dot).

VITA

URMILA MANANDHAR

Candidate for the Degree of

Master of Science

Thesis: IMPROVEMENTS IN THE SAMPLING METHOD OF THE WHEAT RHIZOSPHERE AND SOIL MICROBIOME

Major Field: Plant and Soil Sciences

Biographical:

Education:

Completed the requirements for the Master of Science in Plant and Soil Sciences at Oklahoma State University, Stillwater, Oklahoma in May, 2022.

Completed the requirements for the Master of Science in Microbiology at Tribhuvan University, Kathmandu, Nepal in 2015.

Experience: Research and Teaching Assistant

Professional Memberships: