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Effects of fertilizer and glyphosate on soil microbial diversity and pathogens in two soil types in Telemark.

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Summary

The occurrence of microorganisms in the soil depends on numerous physical and chemical factors. Microorganisms play a central role in the environment and plant growth. New molecular techniques are currently being used for metagenomic studies which reveal species composition and data output in the form of DNA sequences. This study is an attempt to understand soil microbiome, its analysis and to see the effects of fertilizer and pesticide on rhizospheres' bacteria. We performed an experiment to study the effects of treatment on microbial diversity for a time span of four months. The combination of 16S rRNA amplification and next generation sequencing provided information on microbes and their diversity. Further analysis examines the effects of fertilizer and pesticide on microbial diversity. Functional analysis reveals the effects of various soil treatments on potential pathogens in mixed and sandy soil samples. The sequencing output demonstrates to discussion on advantages and drawbacks of the sequencing method and sheds light on physical properties of the soil.

Keywords: Microbial diversity, rhizosphere, pathogens, metagenomics, glyphosate

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Preface

This project is a part of the Master Thesis Spring 2021 master's in environmental science, USN Bø campus. I am thankful to Prof. Mona Sæbø, thesis guide, for the idea, vision and practical information of the thesis. I express my gratitude to thesis guide Jørn Henrik Sønstebø, who provided all the practical information and training about RStudio programming, analysis of the results and metabarcoding. The abundance of various phyla found in the samples are attached in the annexes. Sofie, Daniel and Howard provided help for experimental design setup, sample processing and functional group analysis. I would like to thank Standard Bio for allowing us to perform the experiment at its premises and provide all the necessary help.

Bø I Telemark, 18/05/2021

Neha Kiran Durugkar

Introduction

The increase in the global per capita food supply and intensive agricultural practices escalates the demand for good quality soil, irrigation, fertilizers and pesticide control (F. A. O., 2010, WHO and UNEP, 1990). Sustainable farming practices provide benefits at no direct cost to the biodiversity, climate change, water, soil and landscape (OECD, 2016). Crop rotation, reduction in use of fertilizers and pesticides, conservation tillage, organic farming practices, rotational grazing and preservation of landscapes are examples of sustainable farming practices (Dessart et al., 2019). For sustainable farming practices, farmers should be aware of biological and agronomic knowledge about agroecosystems, soil types, regions and climate (Tilman et al., 2002). Considering the green revolution and hazardous issues like global warming, soil and water pollution, alternatives to harmful chemicals are being developed (Jongman et al., 2020).

Study of soil microbiology can provide us with a key to unlock the solutions of environmental problems. For example, health hazards due to soil, water and air pollution, improper waste disposal created by humans, farm and industries, and plastics (Medrek and Litsky, 1960, Randall, 1956, Donsel et al., 1967).

In general, the soil consists of water, gas, minerals, living organisms and humus (Garcia and McKay, 1970). Mineral soil can roughly be divided into three classes, namely sand, silt and clay (Needelman, 2013). Soil microbes, which interact with plant communities influence and are influenced by biotic and abiotic ecological factors (Tilman and Pacala, 1993). These plant-associated microorganisms have a huge impact on plant growth and development, nutrition, seed germination, productivity and diseases (Mendes et al., 2013). Numerous direct and indirect interactions between plant roots, rhizosphere microorganisms and soil fauna have been found out experimentally. Mapping changes in root morphology and physiology and changes in the regulation of gene expression by plants are important to understand the signaling pathways. These are the molecular control points of plants (Bonkowski et al., 2009). Whilst there exist many research projects in rhizosphere molecular ecology, it is challenging to identify the molecular (cell-cycle) control points which determine these multiple interactions between soil fauna and plants (Bonkowski et al., 2009). Hiltner in 1904 (the “Einflusssphäre der Wurzel”), first defined the rhizosphere as “the volume of soil around living roots, influenced by root activity” (Hinsinger et al., 2009, Hartmann et al., 2008).

The rhizosphere is considered to be a major juncture for microorganisms, consisting mainly of bacteria, fungi, oomycetes, nematodes, algae, archaea, viruses, protozoa and arthropods (Hinsinger et al., 2009, Lynch, 1994, Raaijmakers et al., 2009, Metting et al., 1993).

The number of microsomal genes in the rhizosphere are more than the number of genes in the plant itself. Here, some of the microbes can be deleterious, leading to multiple interactions and plant defense strategies which in turn can affects the plant's own cycle (Mendes et al., 2013). Mendes et al. (2013) in their study on chemical composition in the rhizosphere and its relation to plant growth discovered that traditional approaches to identify the rhizospheres' microorganisms and their functions have multiple limitations. They concluded that it is necessary to uncover the numerous unknown microorganisms, their functions and traits in the rhizosphere. This can also be used for various applications.

A pathogen is broadly defined as a microbe capable of causing diseases (Hoeprich, 1989, Shulman, 1997). The concept of pathogenicity and virulence has been re-defined several times (Casadevall and Pirofski, 1999). Today, pathogens are defined as "microorganisms capable of producing disease under normal conditions of host resistance and rarely living in close association with a host without producing some level of disease or pathogens can be simply called as microscopic parasites" (Hajek and Shapiro-Ilan, 2018). Microbes can be attenuated in the laboratory and their pathogenicity is regained in the host; hence a neat classification of microbes is complex (Pirofski and Casadevall, 2012). Plants have their own immune system to respond to invading microbes (Thomma et al., 2001, Jones and Dangl, 2006, Cook et al., 2015). Microbial ingress triggers several strategies by plants, like, callose deposition, production of antimicrobial compounds, changes in hormone biosynthesis of the plants, ion influxes, activation of mitogen-activated protein kinases and accumulation of reactive oxygen species (Altenbach and Robatzek, 2007, Boller and Felix, 2009, Bolton, 2009, Macho and Zipfel, 2014). Schnitzer et al. (2011) demonstrated with their grassland biodiversity experiments that increase in soil microbial diversity decreases plant diseases and increases productivity and also concluded that pathogens can be major determinants of the diversity-productivity relationships. Soil pathogens play an important role in determining characteristics of plant community and critically influence the maintenance of diversity (Maron et al., 2011), for example by killing or impairing the growth of young plants (Packer and Clay, 2000, Bell et al., 2006), a process called as negative soil feedback (Bever, 1994).

Presence of pathogenic microorganisms in the soil depends on physical and chemical properties and various other factors, such as pore size, texture, density. These factors are in turn greatly affected by temperature, aeration in the soil, moisture holding and cation adsorbing capacity (Garcia and McKay, 1970, Jones et al., 1948, Waksman, 1945). Continuous cropping can lead to shifts in microbial communities, soil nutrients imbalance and autotoxicity of root exudates (Lin et al., 2012). Pathogens await the opportunity during pre- or post-harvest or while flowering to invade the plant tissues (Jongman et al., 2020, McClellan and Hewitt, 1973). Several disease control measures like fungicides, pesticides, essential oils, and other biocontrol methods are carried out in the fields to save a variety of crops (Feliziani et al., 2014, Bill et al., 2014). The presence of synthetic compounds, such as, pesticides, fertilizers and antibiotics, changes the soil environment, where the microbes coexist and experience antagonistic as well as synergistic effects of these synthetic compounds on their existence (Waksman, 1945).

Conventional techniques for soil analysis limits critical evaluation, isolation and cultivation to account for all types of microbes in various type of soils, as culturing of environmental strains is highly selective and hard to differentiate between different samples. (Garcia and McKay, 1970, Balkwill et al., 1977). Current advances in metagenomics and bioinformatics provide researchers with highly sensitive protocols to detect phytopathogens as compared to the conventional microbiological methods (Carmichael et al., 2018, Singh et al., 2012).

In contrast to the old isolation and identification methods for microorganisms, 16S rRNA gene is used as a common phylogenetic marker for the identification of bacterial communities since last few decades (Garcia and McKay, 1970, Coenye and Vandamme, 2003, Fadrosh et al., 2014). The combination of 16S rRNA gene amplification and high-throughput sequencing has made the identification and classification of bacterial strains/genera possible, allowing metabarcoding of a variety of samples (Venter et al., 2004, Janda and Abbott, 2007). 16S rRNA contains primer specific binding sites and hypervariable regions-species specific signature sequences (Jiang et al., 2006, Pereira et al., 2010). The V1-V9 hypervariable regions of 16S rRNA exhibit different degree of sequence diversity, hence no single hypervariable region is able to characterize all bacteria (Chakravorty et al., 2007). In an experimental research by Chakravorty et al. (2007), V3 region was found to be most suitable for the analysis and classification of pathogenic bacteria.

Illumina sequencing is currently the leading technology for sequencing at the lowest per base cost amongst all next-generation sequencing methods (van Dijk et al., 2018). The other types of NGS used are sequencing by synthesis (SBS), Ion Torrent (Rothberg et al., 2011), PacBio SMRT sequencing, Nanopore-based DNA sequencing (Oxford Nanopore). For example, Illumina MiSeq was used to associate microbial alterations due to post harvest stem-end rot disease in mango fruit (Diskin et al., 2017) and fungal community occurrence in different parts of apple fruit (Abdelfattah et al., 2016). Sequencing of the V4 region of 16S rRNA gene revealed pathogenic bacterial and fungal taxa, which in turn helped to reduce postharvest rots in mango and improve apple fruit storage conditions. Similar knowledge can help to find bacterial populations in other fruits and develop more effective applications against targeted pathogens in order to enhance disease control and crop management (Jongman et al., 2020, Busby et al., 2016).

Soil microbiota influences factors in agro-ecosystems such as soil erosion, biodiversity of organisms, soil fertility, accumulation of agrochemicals and phytopathogens (Babin et al., 2019, Bender et al., 2016). Detail analysis of soil pathogens with targeted soil biological engineering concepts can be of huge interest for agricultural management in regards with crop yield and disease dynamics, sustainability and soil health (Foley, 2005, Grosch et al., 2011, de Vries et al., 2013, Bender et al., 2016).

The aim of this study is to identify the effects of fertilizer and pesticide-based treatment of soil on the microbial community in two soil types, sandy soil and mixed soil. In addition, we also will study the effect of the treatments on the quantity of potential pathogens in the soil. We hypothesize that the treatments will result in changes in the microbial community. We further hypothesize that the effects of the treatments will be different in different soil types and the various treatments will affect the quantity of potentially pathogenic species.

Material and methods

Design of the field experiment at Standard Bio, Bø, Midt Telemark

36 wooden-pallets were filled with three types of soil (clay, sand and mixed soil) and were placed outside Standard Bio, Bø, Telemark, Norway for a time frame of four months, July-October 2020. All soil samples were gathered from Torjus Prestholt's farm in Midt-Telemark, Norway 59°24'58.1"N 9°05'17.8"E. Sandy soil was gathered about 15 cm below ground

surface, and to a depth of 40 cm. The soil has not been treated in the last 20 years. Clay soil was gathered 5- 30 cm below surface and was always and only farmed with horse pasture grass. Mixed soil (mineral soil with higher organic content) was gathered from a mound of earth that had been dug up. All the above-mentioned soil types had not previously been treated with any pesticides or fertilizers.

All soil samples were gathered simultaneously and were all subject to the same experimental conditions. All the three types of soil: sand, clay and mixed were treated with fertilizer, pesticides and fertilizer + pesticides respectively, three replicates of each treatment (the experimental design is explained in Figure 1). Horse pasture was mixed and sown in all 36 experiment pallets before the first treatment. The pallets were watered every other day until grass sprouted. Granular "25-2-6" (25% nitrogen) from Yara was used as a fertilizer and 60 grams was added to each pallet in two rounds. Roundup, a herbicide was used in this experiment and its main ingredient is isopropyl amine salt of glyphosate, which is used against weeds (Duke and Powles, 2008). Roundup solution was prepared by adding water in 1:100 dilution ratio and sprayed on the sprouted grass simultaneously with the fertilizer treatment.

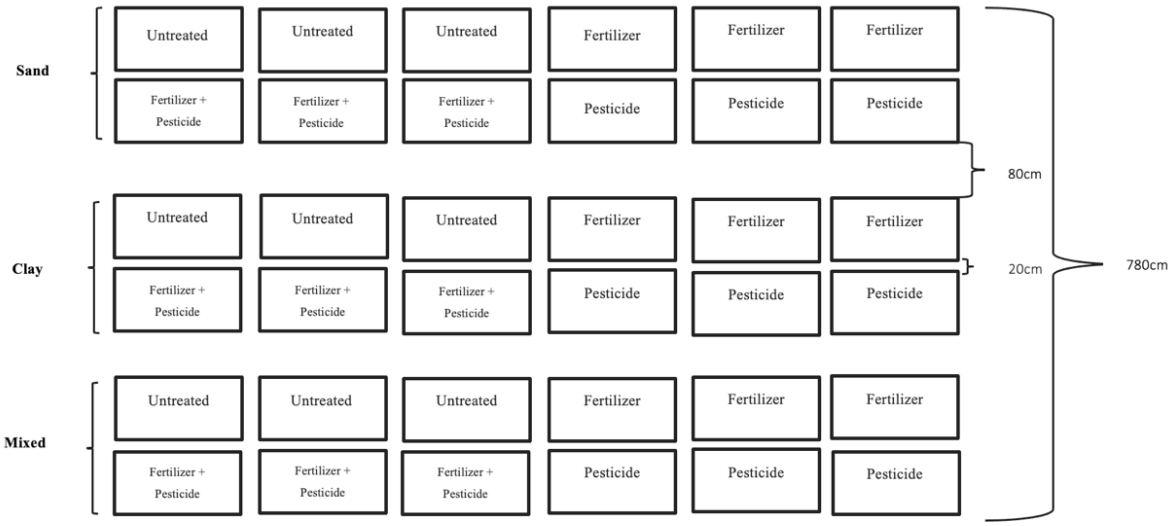

Figure 1 Experimental design of the soil samples with and without treatment. Each box represents a single soil pallet.

Soil collection and DNA extraction

Samples were collected after treatment with fertilizer, pesticide and fertilizer + pesticide from each pallet for DNA analysis. The samples were collected according to the experimental protocol, from the middle of each pallet. While taking samples for DNA analysis from the pallets, one sample (about 10-25 grams) was taken from the middle of the pallets.

Soil samples for DNA analysis were stored in a freezer until DNA extraction. DNA isolation was done from 0.25g of soil (wet mass) using DNeasy® PowerSoil® (QUIAGEN-Gmbh, Hilden, Germany) following the manufactures guidelines. Power Bead Pro Tubes are used for cell lysis in this kit. DNA concentration of the extracted DNA was measured using fluorometer (Qubit-3.0 fluorometer, Invitrogen™, Fisher Scientific UK Ltd). The DNA concentration range of the samples was between 11 to 24 ng/µl. Absorbance ratio and DNA concentration was measured for all the samples using a spectrophotometer (Nanodroplite spectrophotometer, THERMO scientific, Wilmington DE, USA, (10 D = 50 ng/µl) for quality control. The absorbance ratio for all the samples was in the range of 1.7 to 1.9.

Total 68 samples were chosen for sequencing, which included 15 samples treated with fertilizer, 15 samples treated with pesticide, 15 samples treated with fertilizer + pesticide and 21 untreated samples, from mixed and sandy soil (Table 1). The samples from the clay soil were excluded from the further analysis, due to very low quantity of DNA.

Table 1 Number of samples chosen for sequencing from respective soil type and treatment.

Treatments	Sandy	Mixed
Untreated	9	12
Fertilizer	6	9
Pesticide	6	9
Fertilizer + Pesticide	6	9

Illumina sequencing

71 samples were chosen as described in table 1, two blanks, one negative control (H_2O) and one positive control (ZymoBIOMICS Microbial Community DNA standard II, Zymo Research) were prepared for 16S rRNA Illumina sequencing. The samples were sequenced at the Norwegian Sequencing Centre following a protocol from Fadrosh et al. (2014). In short,

the amplicon libraries of V3-V4 hypervariable regions of the 16S rRNA gene were prepared using fusion primers (Fadrosh et al., 2014) and these libraries were sequenced with MiSeq Reagent Kit v3 allowing paired end sequencing of 2 X 300bp. The primers 319F and 806R (Herlemann et al., 2011) were used for the amplification. An Illumina-specific adapter sequence, 12-nucleotide oligo sequence and a heterogeneity spacer were added to the amplicons.

Sequence processing:

Demultiplexing was done and internal barcodes, heterogeneity spacers and primers were removed using an in-house script (https://github.com/nsc-norway/triple_index-demultiplexing/tree/master/src) at the Norwegian Sequencing Center. QIIME 2020.6 software (Bolyen et al., 2019) was used for microbiome data analysis which uses 2 custom formats: qua and qzv, containing the FASTQ sequences. These files can be used in several ways. Samples with no reads were excluded. Data denoising was done using DADA2 (Callahan et al., 2016) algorithm to group similar reads into Amplicon Sequencing Variants (ASVs) for quality performing and error correction and combining paired end reads. These ASVs are analogous to OTUs (Operational Taxonomic Units). DADA2 also removes chimeric sequences. The total number of raw reads were 11737873 and the total usable reads after denoising and chimera removal were 7030106. The ASVs were assigned to a taxonomy using a Naïve Bayes classifier algorithm, trained on data from the SILVA v. 138 database (Quast et al., 2013).

Statistical analysis of ASV richness, rarefaction, diversity measures:

Statistical analysis was carried out with 66 samples excluding one positive control and three negative controls of which two of the negative controls were negative controls from the DNA extraction. The reads from 66 samples were rarefied by adjusting the sample size depending on the sample with the smallest number of reads. The data was further filtered to remove the ASVs with lowest frequency and the ASVs that are found in only one site.

Statistical analysis was done using R studio version 4.0.3 with R package “phyloseq” (McMurdie and Holmes, 2013) and "ggplot2” (Wickham et al., 2016) for diversity plots. Alpha diversity was calculated using observed (Fisher et al., 1943), ACE, Shannon (Shannon, 1948) and InvSimpson (Simpson, 1949) indices. Observed and ACE indices reflect ASV abundance in the samples whereas Shannon and InvSimpson reflects the diversity of OTU in the samples (Chen et al., 2020). Diversity is described as a function of number of species (species richness,

S) and its relative distribution (evenness, *E*) (Pielou, 1984). Bray-Curtis distance is used to assess which covariates have a structuring effect on the communities (Bernard et al., 2017, Bray and Curtis, 1957). Beta diversity (ordination) was calculated and plotted using Bray-Curtis distance. R package "vegan" (Oksanen et al., 2015) was used for redundancy discriminate analysis, which is a version of principal components analysis that is constrained on the explanatory variables, in our case the two different soil types and the treatments (fertilization and glyphosate).

We predicted the function of the taxonomic groups using a taxonomy table made in QIIME2 (Callahan et al., 2016) where the ASVs were picked against the Greengene database (DeSantis et al., 2006). The functional characteristics of the taxonomic groups were assigned using BugBase (Ward et al., 2017) to find probable pathogens.

Results and Discussions

In this thesis a significant difference was found between the microbial diversity in sandy soil and mixed soil. *Actinobacteriota, Chloroflexi and Proteobacteria* were the phyla that occurred most frequently in both mixed and sandy soil. The alpha diversity in the mixed soil changed significantly with glyphosate and fertilizer + glyphosate treatment, whereas alpha diversity in sandy soil showed little change after various treatments. The β diversity was found to be higher in the sandy soil samples than the mixed soil samples, particularly after treatment with fertilizer. Functional group analysis classified a large frequency of the ASVs into potentially pathogenic species, with no significant difference between the different treatments. However, the variation in frequency of pathogens was higher in sandy soil than mixed soil after the treatments.

Microbial diversity showed significant differences in different soil types:

The alpha diversity in mixed soil samples was higher than the sandy soil samples and this was also confirmed by the ANOVA test (Table 2). Scatterplots with observed, ACE and Shannon indices shows similar difference in alpha diversity of mixed and sandy soil samples (Figure 2).

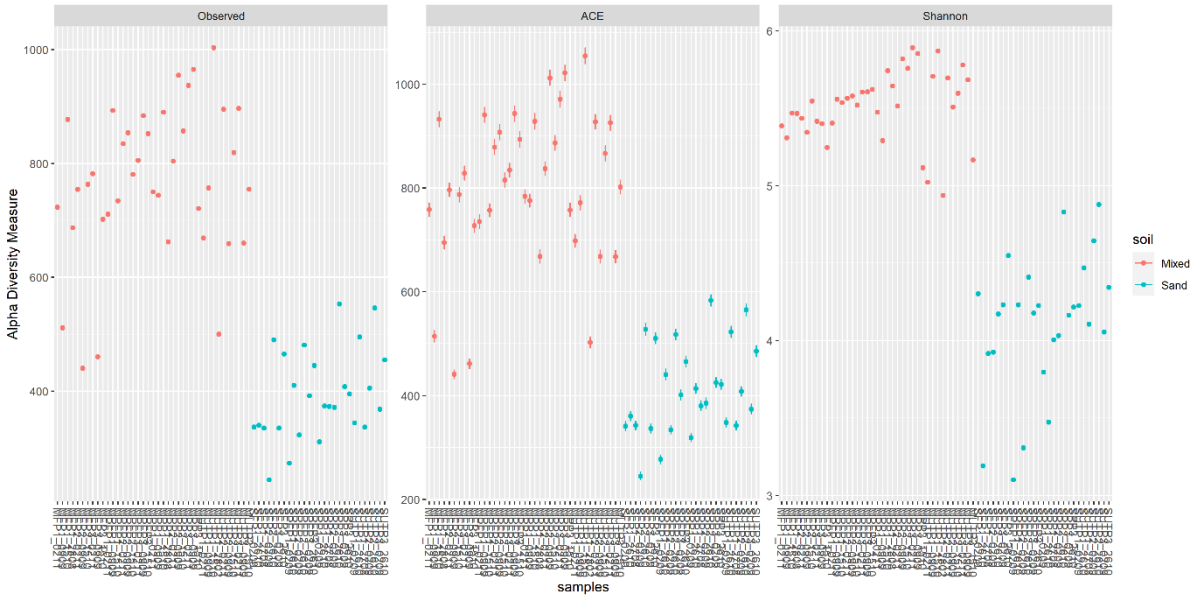

Figure 2 Scatterplot showing alpha diversity in both sandy and mixed soil samples using observed, ACE and Shannon indices. The red circles represent mixed soil samples, and the blue circles describe the sandy soil samples.

Soil type showed significant impact on diversity with 5 times the number of effective species in mixed soil samples as compared to the sandy soil sample in Table 2. This shows that mixed soil properties favor diversity in microbial communities, compared to the sandy soil. The sandy soil has larger particle size than the mixed soil which is an important factor that affects the bacterial community structure significantly (Jocteur Monrozier et al., 1991, Sessitsch et al., 2001). Sessitsch et al. (2001) found that the particle size was more important than the amount of the organic compound input or external factors/ kind of fertilizer applied. Higher microbial diversity was found in clay size particles (Lunsdorf et al., 2000).

Table 2 ANOVA test results for sandy and mixed soil samples using inverse Simpson indices.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Coef Mixed soil	Coef Sandy soil
Soil	2	540124	270062	352.56	< 2.2e-16	116.47752	20.19422
Residuals	64	49024	766				

Effect of fertilizer and glyphosate on alpha diversity of the microbial communities:

Treatment of soil with fertilizer and pesticide significantly changed the microbial diversity in comparison to the untreated samples (for p values see Table 3). The p values indicate

significant difference in alpha diversity in the sandy soil samples. There was a small change in alpha diversity in the sandy soil samples after treatment with fertilizer + pesticide. (Figure 3)

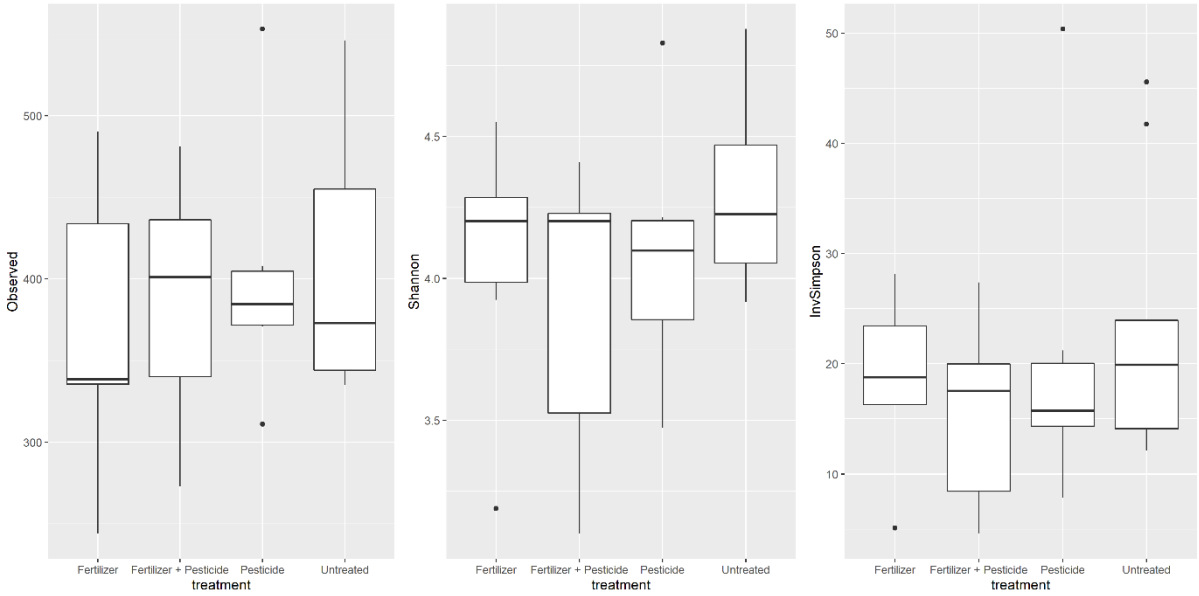

Figure 3 Boxplot of impact of treatments on alpha diversity in sandy soil samples. The boxes denote IQR (Interquartile range), with median as a black line, whiskers and outliers.

The alpha diversity changes with various treatments. The observed species richness increased with two of the treatments (pesticides and fertilizer + pesticides). The species were more evenly distributed after treatment with pesticides and the diversity measure increased with pesticide treatment in the sandy soil. The Shannon indices increased in diversity with fertilizer + pesticide and fertilizer. This indicates fertilizer increases the diversity in the sandy soil. The Shannon and Inverse Simpson indices increased in diversity with fertilizer treatment and decrease with pesticide treatment, suggesting that glyphosate will decrease the alpha diversity in sandy soil. The most striking result was the difference between the two soil types, when it comes to diversity. The sandy soil samples showed a small, but significant little effect of treatment on alpha diversity while the mixed soil samples showed a larger effect. (Figure 3, Figure 4). This was in opposite to the β diversity that showed stronger effects of treatment in the sandy soil compared to the mixed soil (Figure 7).

Inorganic NPK fertilizer treatment to the agricultural soil have been shown to affect the microbial community structure and boosts richness, abundance and diversity of bacterial species (Enebe and Babalola, 2020). In our study we found similar result of increase in microbial diversity with fertilizer (NPK) treatment in sandy and mixed soil samples. A long-

term fertilizer field experiment by Sessitsch et al. (2001) showed green manure and animal manure amended soil had higher microbial diversity and inorganic fertilizer or no fertilizer amended soils had lower microbial diversity.

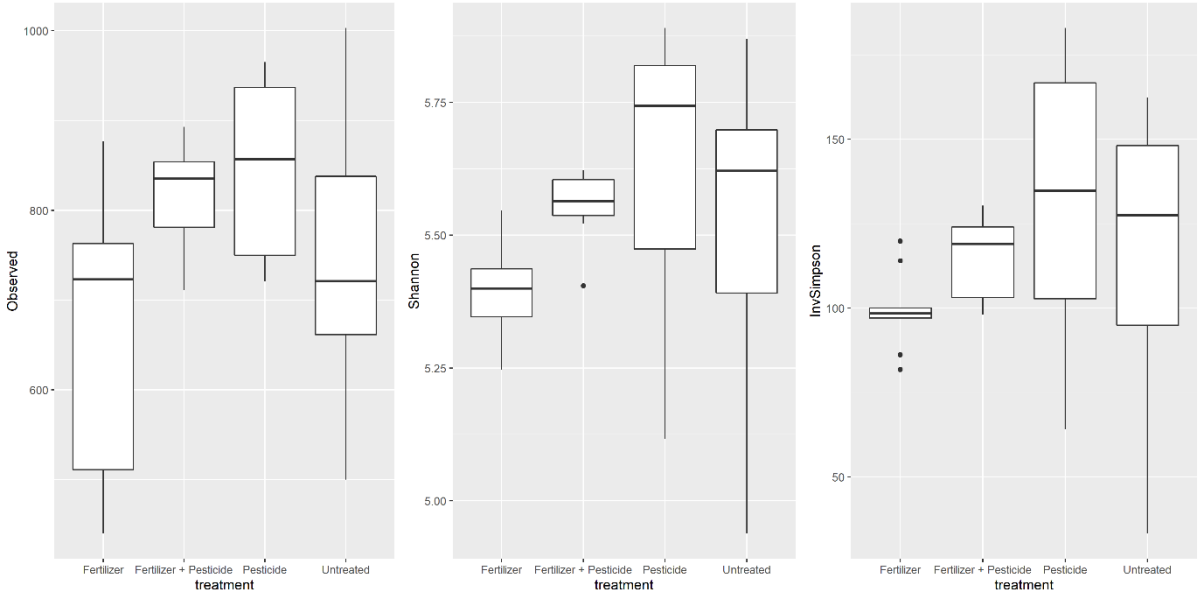

Figure 4 Boxplot of the impact of treatments on alpha diversity in mixed soil samples.

As compared to the sandy soil in Figure 3, Figure 4 shows alpha- diversity of samples in mixed soil. Samples treated with the pesticide glyphosate and fertilizer + pesticide showed increase in alpha- diversity in the observed species richness. Box plots with Shannon and InvSimpson indices showed increased diversity in pesticide treated samples. Diversity decreased with fertilizer treatment in all alpha diversity indices for the mixed soil samples. (Figure 4). The existing scientific literature on glyphosate gives conflicting results about its effects on microbial communities. Farthing et al. (2020) found few but significant changes in soil microbial community structure and diversity which could affect food soil webs and biological processes after long term use of glyphosate. Similar metagenomic studies by Kepler et al. (2020) conclude glyphosate does not change the prokaryotic community diversity and highlights the need to use multiple locations to understand effects of glyphosate on microbial communities. In this experiment, one of the reasons for increase in microbial diversity after pesticide treatment can be washing out of the chemical due to the rainfall. Hence, the effect of pesticide might have been reduced due to its low concentration. Another explanation for increased microbial diversity can be a disturbance in the microbial community structure due to the increase in the dead plant material, which in turn increases the number of decomposing

microbes. It has been studied that glyphosate is used as a substrate by bacterial community, as a readily available source of carbon (Araújo et al., 2003). In some cases, glyphosate stimulates microbial activity and bacterial diversity by increasing carbon and nitrogen mineralization (Haney et al., 2000, Cherni et al., 2015).

Taxonomic composition in sandy and mixed soil samples and effect of treatments on microbial community structure:

When comparing the taxonomic composition of sandy (Figure 5) and mixed soil (Figure 6) we observed a clear difference. In the sandy soil, the most frequent phylum was *Chloroflexi* of which 36.3% (Annex Table 1) of the reads belonged to. In contrast, in the mixed soil, *Chloroflexi* was the fourth most common phylum with 15.0% (Annex Table 2) of the reads, while in the mixed soil *Actinobacteriota* was the most common phylum with 35.6% (Annex Table 2) of the reads. The phylum *Choroflexi* contains many organisms and has been identified from various environments like freshwater (Kadnikov et al., 2012), aquifer (Hug et al., 2013) etc. Although *Cloroflexi* is abundantly found in various environments, it is difficult to relate its lineages with isolated *Cloroflexi* strains. Due to lack of physiological data *Cloroflexi* remains understudied (Krzmarzick et al., 2012).

Actinobacteriota is one of the largest taxonomic phylum in the bacteria domain and contains many beneficial as well as pathogenic species (Ludwig et al., 2012). The population density of *Actinobacteria* depends on its habitat, climate and it needs neutral pH and specific moisture for growth in the soil (Barka et al., 2016). The mixed soil samples showed abundance of *Actiobateriota* phylum implying favorable conditions for its growth. *Actionobacteriota* has been reported in various types of soil environments like sandy soil, black alkaline soil, sandy loam soil, alkaline dessert soil and subtropical dessert soil (Ranjani et al., 2016). In a metagenomic study on *Actinobacteria* in Australian agricultural soils, the abundance of *Actinobacteria* was found to be 32.1 % of the OTUs (Araújo et al., 2003).

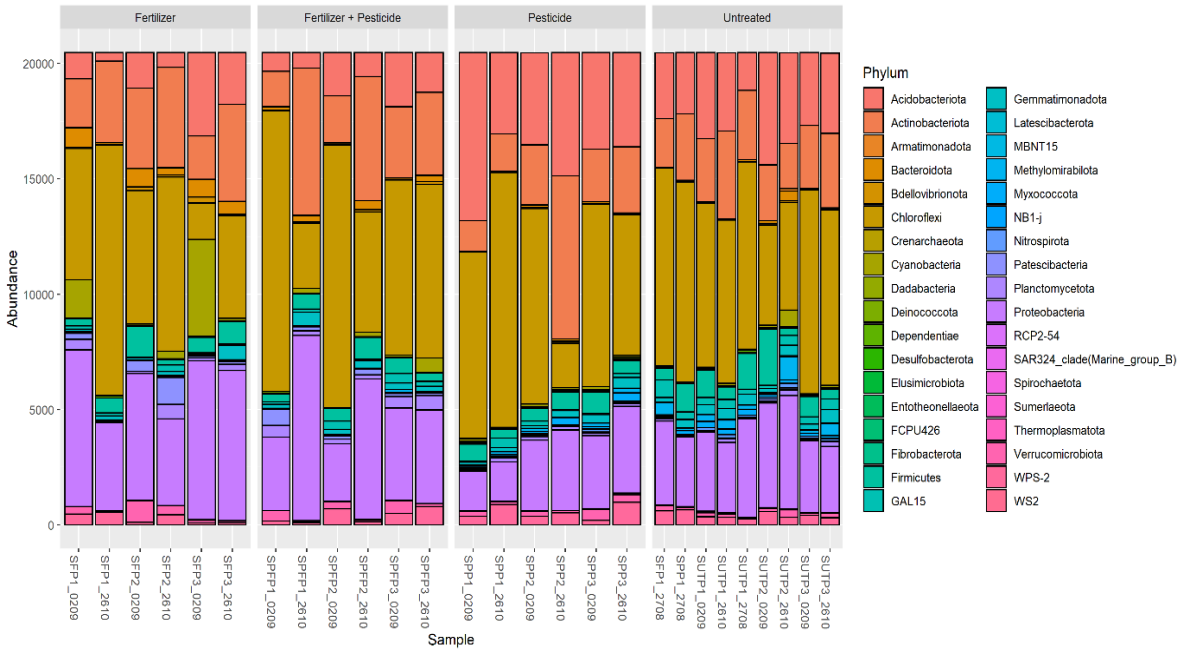

Figure 5 Faceted bar plot of samples with sandy soil with various treatments versus ASV abundance. The abundance values for each ASV are ordered alphabetically, separated by a thin horizontal line. Colors represent the phyla to which each ASV belongs.

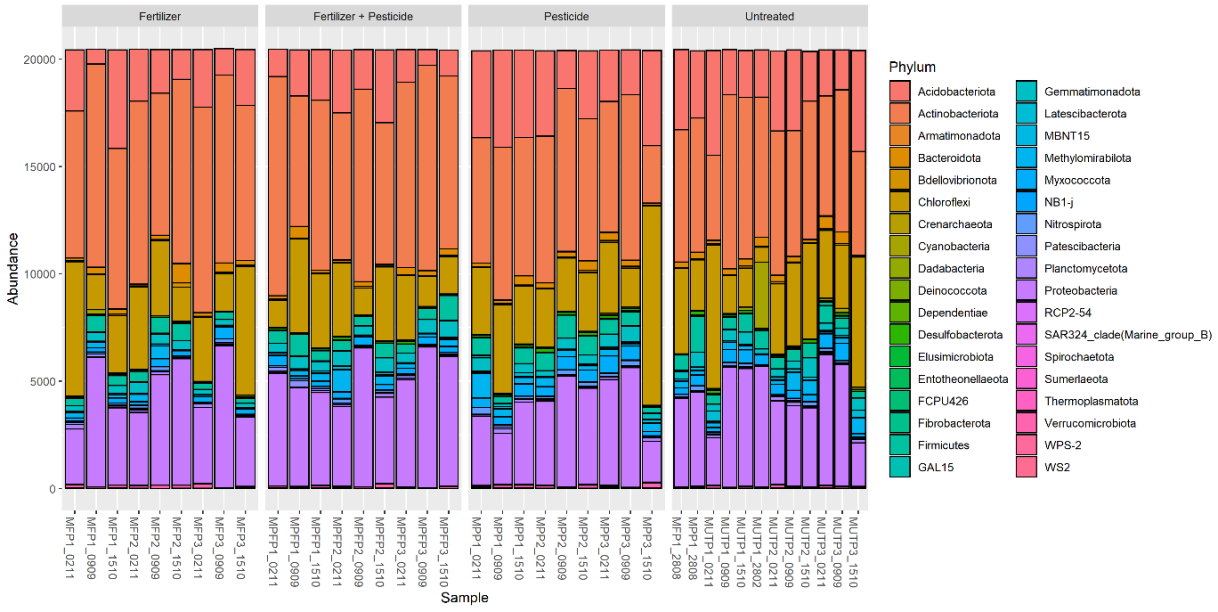

Figure 6 Faceted bar plot of samples with mixed soil with various treatments versus ASV abundance. The abundance values for each ASV are ordered alphabetically, separated by a thin horizontal line. Colors represent the Phylum to which each ASV belongs.

From the above Figures Figure 5 Figure 6 we can see that the common dominant phylum is *Proteobacteria* in both the sandy soil (20.04 %) and mixed soil (21.8 %) types (Annex Table 1, Annex Table 2). Proteobacteria also being one of the largest phyla, known as purple bacteria (Stackebrandt et al., 1988) consists mainly of gram-negative bacteria, most of which are also plant pathogens (Woese, 1987). Research experiment by Lancaster et al. (2010) demonstrated that repeated application of glyphosate affects soil microbial community composition and increases relative abundance of *β-proteobacteria* in the soil (Lancaster et al., 2010). Study on microbial population structure by Sessitsch et al. (2001) demonstrated large particle soil had fewer members of *Acidobacterium* and was dominated by *Proteobacteria*, whereas high microbial diversity of *Acidobacterium* was present in small particle size soil. Sandy soil particles are preferentially colonized by fungi resulting in competition to the bacterial colonies, hence the eukaryotes outnumber the prokaryotes (Kandeler et al., 2000).

Intra-variability between the sandy soil samples was higher than intra-variability in the mixed soil samples:

Beta-diversity measures the variation of microbial communities between samples.

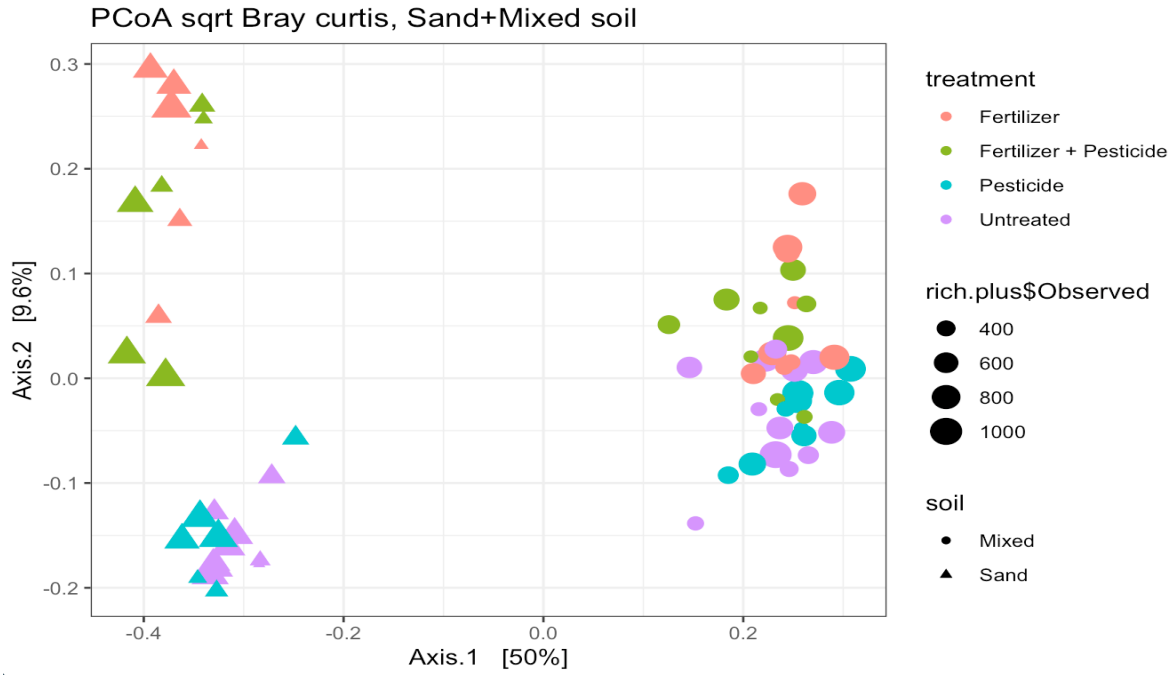

Figure 7 An ordination plot showing beta diversity of all the samples with and without treatment in mixed and sandy soil. The colors represent the various treatments, shape represent the type of soil and size represents the species richness.

Here β diversity shows a clear difference in the microbial composition between the two soil types, which are separated on the first axis in the ordination (Figure 7). The intra-variability between the samples was higher in sandy soil than intra-variability in the mixed soil (Figure 7) and particularly in the treatment with fertilizer. A redundancy analysis was done to identify the significance of the soil type and treatments and how much of the variation in community composition that could be explained by the treatments. The RDA produced PCA ordination summarizes the main pattern of variation in the response matrix; the ASVs are the response variables in this case. The filled circles in Figure 8 are the ASVs (species), and the unfilled circles are the sample (sites). Few filled circles closer to the sand samples are linked and also some of the filled circles are tightly linked to fertilization. First 2 axes were significant and are plotted in Figure 8 (see table 4 for test statistics). The first axis was mostly determined by soil type, while the second axis was related to fertilization indicating that the change in β diversity is significant with the soil type and on treatment with fertilizer.

In a study by Smit et al. (2001) the authors explains how soil structure influences microbial interactions by causing spatial isolation and demonstrated that high spatial isolation shows high microbial diversity and vice versa (Smit et al., 2001). Environmental factors like water content, nutrient availability and temperature influences qualitative variation in the microbial community composition (Smit et al., 2001). This study focusses on external chemical treatment with fertilizer and glyphosate and the physical and chemical properties of the soil are not taken into consideration. By including a study of soil properties, a thorough analysis of the microbiome would be possible, and it would be possible to understand the effects of internal and external factors on soil microbes. It will also be interesting to look at the variability between samples with same type of treatment which will allow us to understand the changes in microbial communities due to fertilizer and glyphosate treatment.

Table 3 ANOVA test results for treated and untreated samples in mixed and sandy soil.

Treated Vs Untreated	Df	F value	Pr(>F)	untreated (coef)	treated (coef)
Sandy soil samples					
Fertilizer Vs Untreated	2	43.92	6.584e-09	22.66434	17.10656
Pesticide Vs Untreated	2	41.684	1.091e-08	21.67707	18.34064
Fertilizer+Pesticide Vs Untreated	2	43.163	7.795e-09	21.50561	15.60434
Mixed soil samples					
Fertilizer Vs Untreated	2	227.29	< 2.2e-16	123.6885	108.0647
Pesticide Vs Untreated	2	225.17	< 2.2e-16	109.8622	124.1954
Fertilizer+Pesticide Vs Untreated	2	214.54	< 2.2e-16	116.4746	116.4872

Research by Kamaa et al. (2011) on the effects of fertilization on soil bacterial and fungal microbial diversity demonstrate that long term use of combination of organic and inorganic fertilizers boosts microbial community growth and promotes diversity (Kamaa et al., 2011). Whereas Kibunja et al. (2010) found in their study that long term, continuous application of inorganic fertilizer drops soil organic matter and pH which in turn change the bacterial communities in the soil (Kibunja et al., 2010). In the current study, fertilizer treatment changed the microbial community diversity in sandy and mixed soil samples, and it is supported by previous studies (Kibunja et al., 2010, Kamaa et al., 2011). Further, microbial diversity analysis after repetitive fertilizer application should be studied to see the long-term effects of soil fertilization.

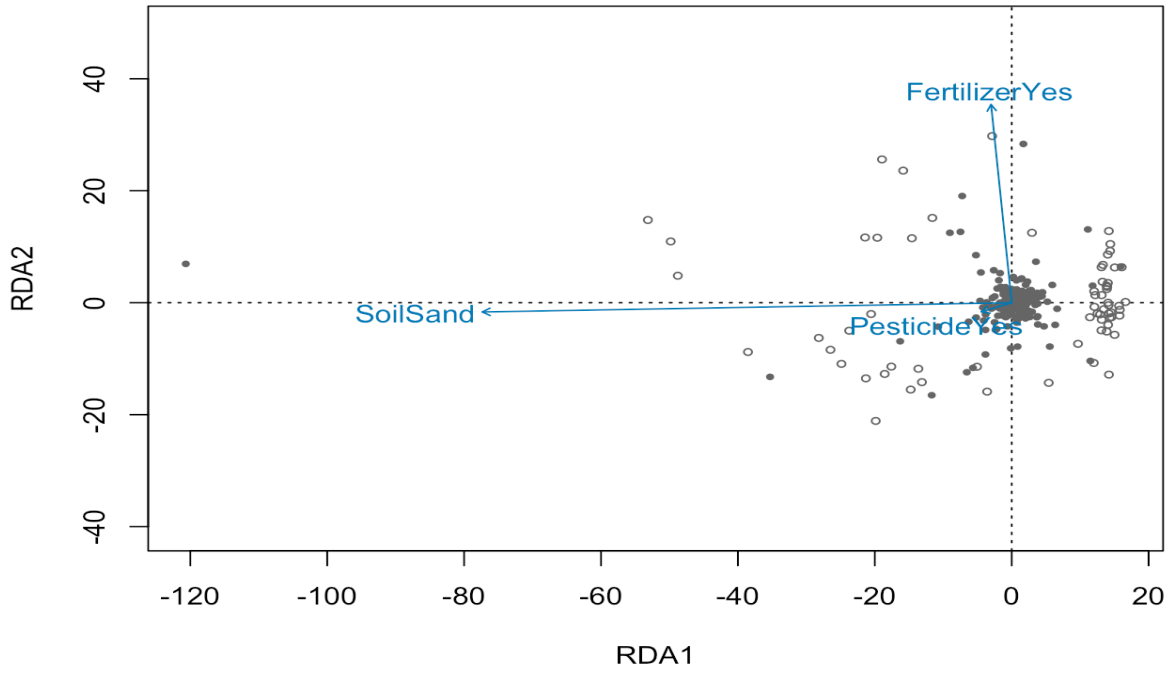

Figure 8 An ordination plot of Redundancy analysis. The RDA plot was constructed using ASVs as response variable and fertilizer, pesticide and soil type as explanatory variables.

Table 4 ANOVA test results showing significance of axes and the eigenvalues for the constrained axes.

Axes	**Df**	**Variance**	**F**	**Pr (>F)**	**Eigenvalues**
RDA1	1	5152546	84.6315	0.001 ***	5152546
RDA2	1	223481	3.6707	0.028 *	223481
RDA3	1	37253	0.6119	0.679	37253
Residual	61	3774693			

More ASVs were classified as pathogens:

A relatively large amount of the ASVs were classified as pathogens after the functional analysis in Bugbase (Figure 9).

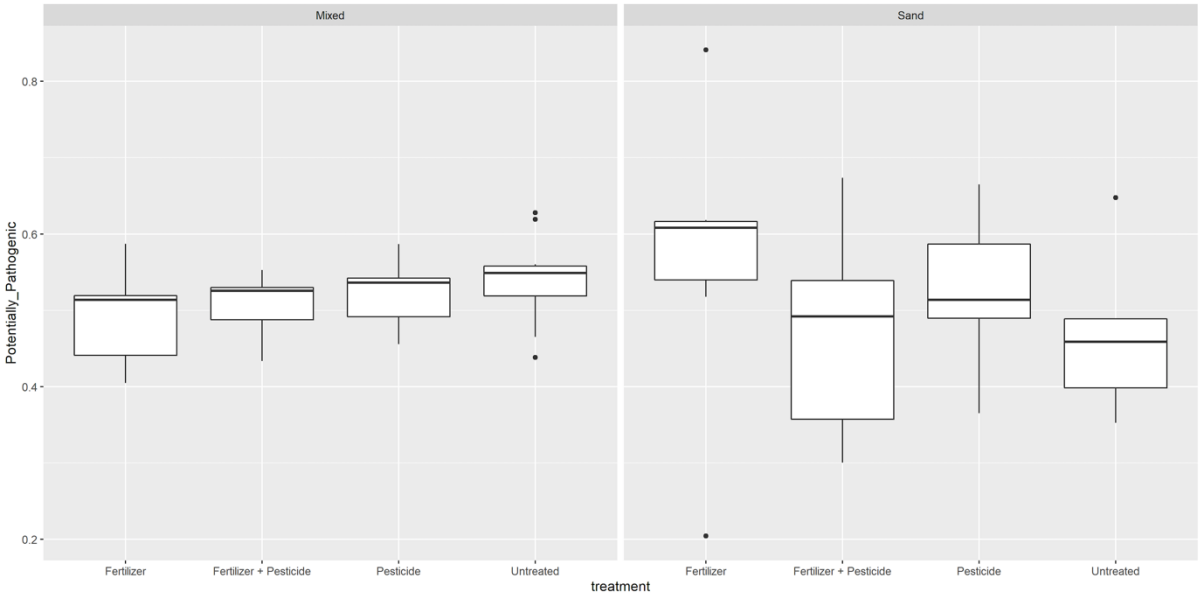

Figure 9 Box plot showing abundance of potentially pathogenic species in treated and untreated mixed and sandy soil samples.

In the mixed soil there was very little variation in the number of predicted pathogens among the different treatments, while in the sandy soil particularly in the treatment with pesticide and fertilization, an increase in the number of predicted pathogens was observed. In a previous study it is discovered that glyphosate reduces the functional diversity of soil bacteria and can affect food soil webs (Lupwayi et al., 2009).

No significant difference was observed between the various treated samples, untreated samples and samples from different sand types after pairwise Mann-Whitney-Wilcoxon Tests. The data with less frequency may be the reason for having no significant difference between different functional groups. In one of the sandy soil samples treated with fertilizer, 85 % of the reads were classified as pathogens, which is less likely. The Bugbase platform has been extensively used to identify phenotypes in clinical data, for example to assess gut microbiota associated with chronic pancreatitis (Han et al., 2019). Bugbase phenotype predictions are particularly useful for analysis of clinical data (Ward et al., 2017). A large amount of Bugbase reference database is based on clinical or human studies. In soil samples, the dominant species, for example *Choloflexi* are difficult to culture in the lab (Krzmarzick et al., 2012), hence indirect

estimation of functional group has to be done for soil analysis and identification of functional groups.

More functional study is needed for soil and agriculture functional group analysis. Metagenomics, using whole genome sequencing might provide more accurate information about the function of the members of the community as compared to the 16 S rRNA V3, V4 region sequencing. Amplification of full-length 16S rRNA using high-throughput sequencing generates operational taxonomic units (OTUs) with higher taxonomic resolution than 16S rRNA variable regions with short-read sequencing platforms and is capable of identifying bacteria at a species-level variation (Johnson et al., 2019). Shotgun metagenomic sequencing provides better resolution of sequencing data at species level as compared to amplicon sequencing of 16S rRNA (Mohiuddin et al., 2017). This new sequencing method will give better understanding of analysis of sequencing data, structural and functional diversity of microbial communities (Mohiuddin et al., 2017).

Conclusion

Physical and chemical properties of the soil, especially the particle size contributes largely to the microbial diversity. Chemical treatment with glyphosate and combination of fertilizer and glyphosate increased the microbial diversity in mixed soil samples. However, in sandy soil samples, fertilizer treatment had a larger effect on the increased microbial diversity. Soil environment is diverse, and it is complicated to analyze it, making functional analyses difficult. More reference data generated from soil microbiome will improve the functional group predictions for soil bacteria.

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Annexes

Annex Table 1 ASV abundance and frequency of all the phyla found in sandy soil samples.

Phylum	**Abundance**	**Frequency**
Chloroflexi	191208	34.59%
Proteobacteria	110755	20.04%
Actinobacteriota	84202	15.23%
Acidobacteriota	76159	13.78%
Firmicutes	20748	3.75%
WPS-2	11286	2.04%
Cyanobacteria	9161	1.66%
Gemmatimonadota	7913	1.43%
Verrucomicrobiota	6320	1.14%
GAL15	6187	1.12%
Bacteroidota	5767	1.04%
Planctomycetota	5538	1%
Patescibacteria	4392	0.79%
Methylomirabilota	4183	0.76%
Myxococcota	3003	0.54%
Bdellovibrionota	1418	0.26%
Nitrospirota	1253	0.23%
Dependentiae	933	0.17%
RCP2-54	784	0.14%
Elusimicrobiota	287	0.05%
Armatimonadota	271	0.05%
Dadabacteria	236	0.04%
MBNT15	210	0.04%
Deinococcota	207	0.04%
Desulfobacterota	105	0.02%
FCPU426	69	0.01%
Entotheonellaeota	42	0.01%
Latescibacterota	40	0.01%
Crenarchaeota	27	0%
Fibrobacterota	21	0%
WS2	11	0%
NB1-j	5	0%
SAR324_clade (Marine_group_B)	2	0%
Spirochaetota	0	0%
Sumerlaeota	0	0%
Thermoplasmatota	0	0%

Annex Table 2 ASV abundance and frequency of all the phyla found in mixed soil samples.

Phylum	**Abundance**	**Frequency**
Actinobacteriota	277274	34.82%
Proteobacteria	173617	21.8%
Chloroflexi	126792	15.92%
Acidobacteriota	104909	13.17%
Firmicutes	24652	3.1%
Gemmatimonadota	17707	2.22%
Myxococcota	15120	1.9%
Methylomirabilota	12155	1.53%
Bacteroidota	10734	1.35%
Cyanobacteria	4981	0.63%
Nitrospirota	4540	0.57%
Verrucomicrobiota	3584	0.45%
Planctomycetota	3518	0.44%
Latescibacterota	3280	0.41%
Desulfobacterota	3084	0.39%
Patescibacteria	2328	0.29%
Bdellovibrionota	2207	0.28%
MBNT15	1002	0.13%
Fibrobacterota	755	0.09%
RCP2-54	752	0.09%
GAL15	599	0.08%
Dependentiae	500	0.06%
WS2	438	0.06%
NB1-j	367	0.05%
WPS-2	345	0.04%
Elusimicrobiota	329	0.04%
Armatimonadota	283	0.04%
Dadabacteria	112	0.01%
SAR324_clade (Marine_group_B)	94	0.01%
Thermoplasmatota	70	0.01%
Entotheonellaeota	57	0.01%
Sumerlaeota	48	0.01%
FCPU426	28	0%
Spirochaetota	20	0%
Crenarchaeota	0	0%
Deinococcota	0	0%