The use of digestate from animal wastes and its impacts on the soil microbiome and nutrient dynamics in agricultural soils

（家畜糞尿由来消化液の利用が農地土壌微生物叢と栄養素ダイナミクスに及ぼす影響の評価）

北海道大学　大学院農学院

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Yvonne Musavi Madegwa

Abstract

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Digestate is the semi liquid byproduct of the biogas production process. Due to its basic pH and high nutrient content (i.e inorganic nitrogen, phosphorous, organic carbon), digestate use as a fertilizer has been increasing. Additionally, to improve its efficiency as a fertilizer, digestate can be separated into solid and liquid fractions using a flocculant. However, the effect of digestate and its separated fractions on the soil microbiome and nutrient dynamics in agricultural soils is not clear. Therefore, this study, involving fieldwork and incubation experiments was conducted to address this research need. The field work involved sampling soils from farmers' fields in Kamishihoro town in Hokkaido to determine the effect of land use, seasons and fertilizer application on the soil microbiome and related functions. The second experiment was an incubation to determine the effect of soil pH on microbial stability against the application of digestate from dairy wastes. The third experiment was an incubation to analyze the effect of solid-liquid separation of digestate on soil nutrient dynamics and Japanese mustard spinach yields in different soils.

The field study was conducted in an Andosol (volcanic soil) dominated agricultural region in cool temperate climate to determine the effect of land use (cropland, grassland), season (spring, summer) and fertilizer (digestate) on soil microorganisms and related functions. Soils were sampled from farmers' fields, DNA extracted and sequenced targeting 16S rRNA region. In result, land use had a significant effect on beta diversity and evenness with higher values recorded in cropland than grassland. However, grassland had a higher number of unique operational taxonomic units (OTUs) (10303) compared to cropland (5112). In cropland, season had a significant effect on beta diversity, evenness, OTU numbers and Shannon index with higher values recorded in summer compared to spring. Based on predicted soil functions, nitrogenase (*nifH*) had significantly higher values in cropland-summer while nitrite reductase (*nirK*) and ammonia

monooxygenase (*amoA*) were significantly higher in cropland-spring. In grassland, season had a significant effect on beta diversity only. These results indicate that grassland microorganisms were stable and more resistant to seasonal changes than cropland, suggesting that conventional tillage practices have a negative effect on soil microbial stability. Additionally, grassland-spring (7059) had a higher number of unique OTUs than grassland-summer (2597). Based on predicted soil functions, *nifH* was significantly higher in grassland-spring while *nirK* and *amoA* were significantly higher in grassland-summer. These results indicate that the impact of seasons on soil microorganisms' distribution and abundance in cropland and grassland may directly affect soil functions.

The first incubation experiment was performed to understand the effect of lime application (pH = 6.5 and 5.5 for the soils with and without lime, respectively) and fertilizer (digestate, urea and control) on the soil microbial community structures, stability and gene functions. Soils were sampled weekly after the application of fertilizers for a month. For microbial community analysis, DNA was extracted and sequenced targeting 16S rRNA region. For gene abundances (i.e 16S rRNA, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), nitrous oxide reductase (*nosZ*) and nitrite reductase (*nirS*), quantitative PCR was conducted. In result, the relative abundance of *Actinobacteria* was influenced more strongly by digestate in lime soils, while *Alphaproteobacteria* was influenced more strongly by digestate in the no lime soil. In no lime treatments, digestate had a significant effect on more operational taxonomic units (146) compared to lime (127), indicating that lime application increased soil microbial community's stability. Liming and fertilizer had a significant effect on 16S rRNA gene copy numbers with the highest values observed in lime plus digestate treatments. Soil pH had a significant on AOA, *nosZ* and *nirS* gene copy numbers with the highest values observed in lime treatments. In the lime treatments digestate application had a positive impact on AOB gene copy numbers but this was not the case for soils without liming treatments. These results indicate that soil pH and fertilizer type should be taken into consideration for the management of functional gene abundance in agricultural soils.

The second incubation experiment was setup to analyze the effect of soil types (Kamishihoro and Arakida) and fertilizers (digestate, solid digestate, liquid digestate, chemical and control) on soil and plant (Japanese mustard spinach) nutrients, gene abundance and nitrous oxide (N_2O)

emissions. Based on the results, soil type significantly influenced the above ground plant biomass and Nitrogen (N) content with higher values observed in Kamishihoro compared to Arakida. Kamishihoro had significantly higher soil inorganic N (NO_3^- and NH_4^+) content compared to Arakida soil. Fertilizer had a significant effect on soil NO_3^- (solid-Kamishihoro, digestate-Arakida) and NH_4^+ (liquid-Kamishihoro, chemical-Arakida) content. The results indicate that digestate and its derived fertilizers (solid digestate, liquid digestate) can be used to influence soil inorganic N content in agricultural soils. Digestate derived fertilizers influenced soil pH with highest values observed in liquid in both Kamishihoro and Arakida soils. Arakida soil had significantly higher 16S rRNA and ITS gene copy numbers compared to Kamishihoro. Regarding soil N_2O emissions, Kamishihoro soil had significantly higher emissions compared to Arakida. Additionally, digestate and solid fertilizer had significantly higher N_2O emissions compared to liquid in both Kamishihoro and Arakida soils. The results indicate the importance of soil type and digestate derived fertilizers in modulating soil gene abundance and N_2O emissions in agricultural soils. Furthermore, the separation of digestate into solid and liquid fractions can be considered a reliable method to regulate aspects of soil nutrient dynamics in agricultural soils.

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Chapter 1 Introduction

1.1 Background

Soil microbes are one of the largest pools of biodiversity on Earth (Bhattacharyya, 2012). Soil microbes are important towards the maintenance of soil health, which is defined as the "continuous capacity of soil to function as a vital living system" (Sathya et al., 2016). These microbes maintain soil health through the provision of ecosystem services (Singh et al., 2014; Yuliar et al., 2015). Some of these services include (Davison, 1998; Marque et al., 2014) 1) nutrient cycling such as carbon, nitrogen 2) production of plant growth hormones 3) reduced plant pathogen activity 4) enhanced soil structure 5) mineralization of pollutants. The maintenance of soil health has been identified as a central part in the establishment and maintenance of sustainable agricultural practices (Sathya et al., 2016). To ensure sustainable establishment of ecosystem services soil microbes should be diverse.

Microbial diversity is defined as the different types of microbes that are present in a specific habitat (Hendrick et al., 2000). Microbial diversity measurements include three main aspects; richness (microbes present in each habitat), evenness (relative abundance of microbes in each habitat) and community composition (the identity of the microbes in each habitat) (Hooper et al., 2005). High microbial diversity has been associated with sustainable provision of ecosystem services, increased soil health and microbial stability (Bell et al., 2005; Loreau, 2010). In contrast, low microbial diversity has been associated with the loss of soil health, microbial stability and related ecosystem services (Bell et al., 2005; Trivedi et al., 2016). Essentially for the sustainable creating and maintenance of soil health and related ecosystem service, high microbial diversity should be established. Despite the significance effect of microbial diversity to the provision soil health and ecosystem services, there is lack of adequate research and information on the effect of agricultural management practices on different aspects of the soil microbial diversity.

To address this gap in research, the current research sought to understand the effect of different agricultural management practices (land use, seasonality, fertilizers, soil pH) on soil microbial properties (abundance, composition, diversity), functions and stability.

1.2 Research Objectives

The objectives of this research were to

Determine the effect of land use, seasonality and fertilizer application on soil microbial communities and related functions in agricultural soils; Chapter 3.

Determine the effect of soil pH on microbial stability against the application of anaerobic digestate from dairy wastes; Chapter 4.

Determine the impact of separating digestate on soil and plant nutrients, nitrogen use efficiency, Japanese spinach yields, N_2O emissions and microbial abundance in agricultural soils; Chapter 5.

Thesis Structure

The thesis is divided into 6 chapters.

Chapter 1 This chapter introduces the importance of soil microbes in the agricultural environment. The chapter also highlights the current gap in research that will be addressed by the current research.

Chapter 2 This chapter is a literature review that describe the current available information on soil microbes and their importance in agriculture with emphasis on the nitrogen cycle. The chapter also highlights the importance of microbial diversity and factors that may influence microbial diversity within the agricultural environment.

Chapter 3 This chapter reports on the results of soil sampling that was conducted in farmers' fields in Kamishihoro, Hokkaido, Japan. Sampling was conducted to determine the effect of land use, seasonality and fertilizer application on soil microbial communities and related functions in agricultural soils.

Chapter 4 This chapter reports on the results of an incubation experiment that was designed to determine the effect of soil pH on microbial stability against the application of anaerobic digestate from dairy wastes. Two levels of soil pH were established during the experiment i.e., 6.5 (with lime) and 5.5 (without lime).

Chapter 5 This chapter reports on the results of an incubation experiment that was designed to determine the impact of separating anaerobic digestate on soil nutrients, crop yields and microbial abundance in agricultural soils. Anaerobic digestate was separated into solid and liquid portions using a flocculant.

Chapter 6 This chapter summarizes major findings from the research and recommendations for future research.

Chapter 2 Literature Review

2.1 Role of soil microorganisms in agriculture

The agricultural sector is one of the largest engineered ecosystems, consisting of a third of the global land area (Zhang et al., 2007). Agriculture is an essential component for food, shelter and fiber production on Earth (Muhamad et al., 2020). There is an expected increase in demand of agricultural products due to the rapid increase in human population, with projections showing that the world population is likely to reach 9.7 billion people by 2050 (Roser, 2014). Soils are the most essential part of the agricultural production system, as the main source of plant nutrients (Tahat et al., 2020). Establishment and maintenance of soil health is significant towards provision of soil nutrients to plants. Soil health is defined as "the capacity of a soil to function as a vital living system within ecosystem and land use boundaries to sustain plant and animal production, maintain or enhance water and air quality, and promote plant and animal wellbeing" (Doran and Zeiss, 2000). Various researchers have identified that soil microorganisms play vital roles in the establishment and maintenance of soil health (Muller et al., 2016). Soil microorganisms directly influence soil health through provision of ecosystem services that offer many beneficial services within the agricultural environment (Singh et al., 2014).

Ecosystem services have been defined as "the conditions and processes through which natural ecosystems, and the species that make them up, sustain and fulfill human life" (Daily, 1977). Within the agricultural environment, ecosystem services are determined by the soil health, which is established and maintained by soil microorganisms (Kibblewhite et al., 2007). Ecosystem services provided by soil microorganisms include provisioning and regulatory services (Table 2.1).

Table 2.1: Role of soil microbes in provisioning and regulating services provided by soil ecosystems (adapted from Dominati et al., 2010).

Soil service	Role of soil microbes
Provisioning services – products obtained from the agricultural ecosystems	
Physical support	Organic matter decomposition, nutrient cycling, and improved soil aggregation.
Plant growth medium	Soil microbes mobilize nutrients from insoluble minerals to support plant growth
Regulating services – provide a stable and healthy environment	
Buffering water flows	Soil macropores are formed by soil biota which depend on microbes for food and fuel.
Detoxification and waste cycling	Achieved through microbial mineralization an immobilization of wastes and toxic substances. These microbes depend on soil nutrient availability, which rely on soil microbes
Filtering contaminants	Soil microbes influence soil properties such as hydrophobicity and wettability which effect ability of soil to filter contaminants
Biological control of pests, weeds and pathogens	Soil microbes such as bacteria increase nutrient availability to plants and outcompeting invading pathogens.
Carbon storage and regulation of greenhouse gas emissions	Methane producing and consuming microbes and denitrifying bacteria regulate soil nitrous oxide (N_2O) and methane (CH_4) emissions.
Nutrient cycling	Soil microbes drive the Carbon and Nitrogen nutrient cycles.

2.2 Soil microorganisms and the nitrogen cycle

Nitrogen is an important element in agricultural production, constituting the main component of plants organic compounds (Stark and Richards, 2008). Although 78% of the atmosphere consists of N gas, plants cannot take up the nutrient in this form (Hirsch and Mauchline, 2015). A significant part of N occurs in organic forms and it must be converted to inorganic forms i.e. NH4+ (NH_4^+) and nitrate (NO_3^-) for plant uptake (Boyle et al., 2008). Through the N cycle organic N is converted to inorganic forms, availing N for plants and removing excess amounts from the system (Dixon and Kahn, 2004). Soil microorganisms play important roles in all processes of the N cycle thus regulating plant N availability (Yao et al., 2011). The N cycle involves different process which include N_2 fixation (N_2 to NH_4^+), nitrification (NH_4^+ to NO_3^-), denitrification (NO_3^- to N_2) and ammonification (Figure 2.1) (Dueri et al., 2007).

N Fixation

Nitrogen fixation involves the conversion of N_2 gas from the atmosphere to ammonia (NH_3) Fixation is carried out by free living and symbiotic bacteria and archaea that contain the nitrogenase enzyme (Franche et al., 2009). Some of the bacteria involved in the process are *Azotobacter*, *Bacillus Pseudomonas* among others (Kennedy et al., 1997; Li et al., 1992; Rozycki et al., 1999).

Nitrification

Nitrification is the process through which NH_3 is then converted to NH_4^+ and then NO_3^- in a twostep process (Thomson et al., 2012) (Figure 2.1). The first step of the process, which involves conversion of NH_4^+ to nitrites (NO_2^-) is considered the most significant and rate limiting step. The process is driven by NH_3-oxidizing microorganisms which include NH_3-oxidizing bacteria (AOB) and NH_3-oxidizing archaea (AOA) that contain NH_3 monooxygenase, an enzyme that converts NH_4^+ to NO_2^- (Prosser and Nicol, 2008; 2012). The AOB microbes include *Nitrosomonas*, *Proteobacteria*, *Nitrosospira* among others (Norton, 2014). The AOA microbes include *Euryarchaeota* and *Crenarchaeota* among others (Zhou et al., 2015). The second step of the nitrification process (nitration) involves the conversion of NO_2^- to NO_3^-, which is driven by nitrobacter bacteria (Moreira and Siqueira, 2006). The NO_3^- form of N is readily taken up by plants and is very mobile in the soil and easily lost through leaching. This process of the N cycle

is responsible for NO_3^- water pollution and related consequences such as reduced crop yields, agricultural overapplication of N fertilizers, soil acidification and eutrophication (Davidson and Hackler, 1994).

Denitrification

Denitrification is the stepwise conversion of NO_3^- or NO_2^- through nitric oxide (NO) to N_2O or N_2 gas (Figure 2.1). The process is driven by different types of microbes including bacteria, archaea and eukaryotes (Shoun et al., 2012). The first step involves the conversion of NO_3^- to NO_2^- which is controlled by bacteria and fungi that contain the membrane-bound NO_3^- reductase (*Nar*) or the periplasmic NO_3^- reductase (*Nap*) (González et al., 2006). This is followed by conversion of NO_2^- to NO^- driven by bacteria that encode for the nitrite reductase (*Nir*) enzyme. More specifically, there are two different types of the *Nir* enzyme have been found among denitrifies. The cupper containing (Cu-Nir) *nirK* gene and heme *c* and heme d_1 (cd_1-Nir) *nirS* gene. The two are not functionally different and do not occur in the same cell (Priemé and Tiedje, 2002). Microbes involved in the process include *Proteobacteria, Firmicutes, Bacteroidetes,* among others (Heylen et al., 2006). The produced nitric oxide (NO) is a very reactive gas and therefore soil accumulation should be prevented. The process by which NO is converted to N_2O gas (during incomplete denitrification) is facilitated by prokaryotes which encode for nitric oxide reductase (*Nor*) (Figure 2.1) (Braker and Tiedje, 2006). Three different types of the *Nor* gene have been characterized; *cNor*, *qNor* and *qCuANor* (Vries S and Pouvreau, 2007; Spanning 2011). Some microbes involved in the process include *Paracoccus halodenitrificans, Pseudomonas, Alcaligenes faecalis among others (Sakurai and Sakurai, 1998; Zumft et al., 1994). This step in the denitrification process has received significant attention as most of the* N_2O gas produced from agricultural soil has been attributed to denitrification (Signor and Cerri, 2013). N_2O is a greenhouse gas with a global warming potential of 298 (Signor and Cerri, 2013). Complete denitrification will convert NO to N_2 gas, resultantly removing N from the soil without the environmentally harmful N_2O gas emission (Butterbach-Bahl et al. 2013).This is a process that is driven by bacteria and archaea which encode for the nitrous oxide reductase (*nosZ*) enzyme (Burger and Matiasek 2009). Some of the microbes that encode for the gene are *alpha, beta* and *gama proteobacteria* among others (Orellana et al., 2014).

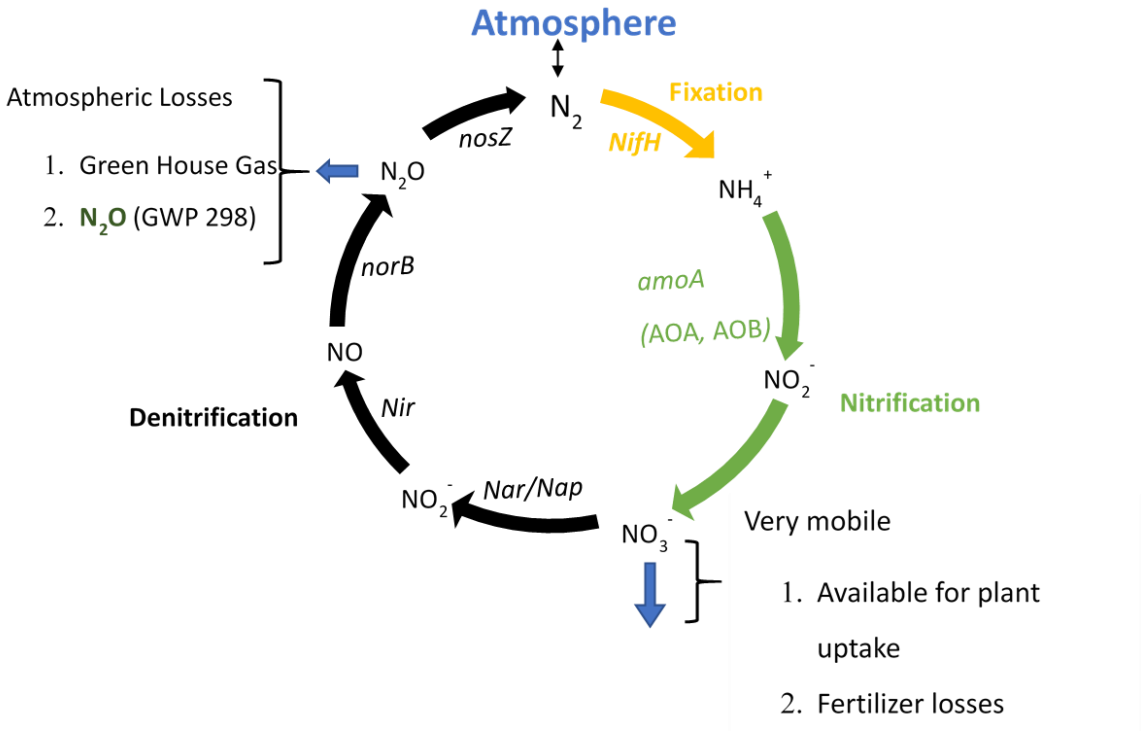

Figure 2.1: Partial soil nitrogen cycling processes with emphasis on nitrification, denitrification and related biomarker genes

2.3 Soil microbial diversity and stability

2.3.1 Diversity

Microbial diversity is defined as the number of different microbes in a given habitat (Hendrick et al., 2000). Components of microbial diversity include richness (number of microbes present in each habitat), evenness (relative abundance of microbes in a given habitat), community composition (identity of microbes present in a given habitat) (Hooper et al., 2005). Microbial diversity analysis includes the specific number of different microbes present and how even they are distributed. For example, a community with a high number of different microbial species is more diverse. However, a community with high evenness is considered more diverse than one that is less even with the same number of microbial species (Hendrick et al., 2000).

2.3.1.1 Components of microbial diversity

2.3.1.1.1 Richness

High microbial species richness has a positive effect on overall ecosystem functioning. Positive species interactions have been observed in habitats that have high species richness (Chapin et al., 1994). High richness leads to establishment of microbes with different ecological niches and feeding preferences, which causes efficient use of resources and higher productivity (Chapin et al., 1994). Jiang (2007) and Langenherder (2010) reported that changes in microbial richness could have a direct effect on ecosystem services. Bell et al. (2005) conducted research on the effect of bacterial richness on ecosystem services and their results showed that increasing bacterial richness enhanced rates of respiration. These results demonstrate the importance of microbial richness within the agricultural environment

2.3.1.1.2 Evenness

Microbial evenness has a significant impact on provision of agricultural ecosystem services. Research by Wittebole et al. (2009) showed that evenness played a significant role in establishing and maintaining functional stability in denitrifier microbes. Hillebrand et al. (2008) similarly found that microbial evenness and stability were directly related. High evenness enhanced microbial stability and related ecosystem services compared to low evenness. Specifically, low evenness values indicate that a specific habitat is dominated by few microbes, which reduces microbial stability. The resistance to external disturbances in such conditions would be achieved only if the dominant species are tolerant to disturbance (Hillebrand et al., 2008).

2.3.1.1.3 Microbial community composition

Microbial community composition has a direct effect on provision of agricultural related ecosystem services. A report by Strickland et al. (2009) found that biogeochemical processes and microbial stability were influenced by microbial community composition. Specifically, Bell et al. (2005) and Salles et al (2012) reported that if changes in microbial community composition caused the loss or introduction of functional groups such as denitrifies, related ecosystem services would be affected. Similarly, Hallin et al. (2009) found a positive correlation between the soil's bacterial community composition and the N cycle related soil functions such as nitrification. For sustainable provision of ecosystem services, management practices that reduce negative changes in microbial community composition should be established.

In summary high microbial diversity ensures the continued provision of agricultural ecosystem services. Additionally, high diversity increases the systems tolerance to disturbances such as land use, seasonal changes, pH and fertilizer application (Griffiths and Philippot, 2013; Loreau, 2010). Decreased diversity would reduce the systems stability and cause a loss of related ecosystem services (Trivedi et al., 2016).

2.3.2 Stability

Stability is defined as soil microbe's response to disturbances. Stability is usually measured based on two main concepts: resistance and resilience. Resistance is the degree to which microbes withstand a disturbance and resilience is the rate at which the community returns to its original state after a disturbance (Allison and Martiny, 2008; Shade et al., 2012). High resistance and resilience is accompanied with increased microbial stability and vice versa (Griffiths et al., 2001). Bearing in mind the significant role microbes play in providing agricultural ecosystem services, the effects of disturbances on their stability an associated function should be considered.

Research show that microbial diversity modulates stability. For instance, higher diversity values have been found to increase microbial stability and vice versa (Wertz et al., 2007). Girvan et al. (2005) conducted research on the effect of different toxins (copper sulphate and benzene) on the stability of soils with different natural diversity levels. Based on their results there was a positive relationship between microbial diversity and stability. On the contrary research by Wertz et al. (2007) found that a decline in diversity did not influence the resistance and resilience of key soil microbial groups following a disturbance. Therefore, research needs to be carried out to clarify the relationship between microbial stability and diversity.

2.4 Factors that affect soil microbial properties

2.4.1 Land use

Agricultural lands make up one third of the global land area (Ellis and Ramankutty, 2008; Ramankutty et al., 2008). Some of the common agricultural land use types include croplands for yields and grassland for animal feed (Yu et al., 2017). Cropland and grassland are characterized by specific management practices which impact soil microbial diversity and stability (Ramankutty et al., 2018).

Cropland conventional tillage practices are usually focused on increasing crop yields at the cost of soil health (Tilston et al., 2010). Common practices associated with conventional tillage practices include inorganic chemical fertilizers use and continuous tillage and monoculture (Zingore et al., 2005). However, grassland management systems are characterized by very little if any inorganic fertilizer use, reduced tillage and high plant diversity (Bissett et al., 2014; Lauber et al., 2009).

2.4.2 Agricultural conventional tillage practices

Research show that conventional tillage practices such as inorganic chemical fertilizer use, continuous tillage and monoculture have a negative effect on soil physical, chemical and microbial properties (Zingore et al., 2005).

Inorganic fertilizer use changes the soil nutrient content and decreases soil pH, subsequently affecting soil microbial abundance and diversity (Bünemann et al., 2006). There have been reported positive and negative effects of inorganic N fertilizers within the agroecosystem. Apart from increasing crop yields, inorganic N fertilizer use has been associated with increased abundance of some functional genes in the soil. Zhang et al. (2016) reported that inorganic N fertilizer increased the abundance of AOB. However, there is extensive research showing the negative effects of chemical fertilizers on soil health and functional gene abundance. Tiston et al. (2010) reported that inorganic N fertilizers were the main cause of soil acidification on agricultural soils. Research show that high levels of acidity negatively affect soil nutrient immobilization and mineralization (Fageria and Baligar, 2008). Acidification increases loss of microbial biomass, essential plant nutrients (Lucas et al., 2011), increased NO_3^- leaching (Otto et al., 2016) and agricultural N_2O emissions (Clough et al., 2003). Additionally, inorganic N fertilizers have a negative effect on some functional gene abundance in agricultural soils. Sterngen et al. (2015) and Yaying et al. (2013) reported that fertilizers reduced the abundance of NH_3 oxidizing archaea and nitrous oxide reductase, which are related to the N cycle.

Soil tillage practices affect soil physical, chemical and biological properties, subsequently impacting soil health and productivity (Mathew et al., 2012). Cropland related conventional tillage practices disrupt soil aggregates and have a negative effect on soil physiochemical properties (Alam et al., 2016). Various scientists have reported that conventional tillage practices have a negative effect on soil bulk density, soil aggregate distribution and water holding capacity (Alam

et al., 2016; Trajan and Linden et al., 1998; Wander et al., 1998). These changes create shifts in soil microbial habitats and community structures (Alguacil et al., 2008; Feng et al., 2003; Kandeler et al., 1999).

Continuous monocropping is a common conventional agricultural management, practiced to ensure maximum crop yield production. However, research shows that the practice has a negative effect on soil physiochemical properties and microbial habitat (Zhou et al., 2012). Fu et al. (2016) conducted research on the effect of continuous monoculture on soil microbial properties and enzyme activities. Based on their results monocropping decreased most quality indicators which included substrate richness, Shannon diversity, soil urease, available N and potassium. Gu et al. (2012) similarly reported that continuous monocropping reduced soil microbial diversity.

2.4.3 Effect of agricultural management practices on microbial diversity and stability

Conventional tillage practices such as inorganic N fertilizer use, frequent tillage and monoculture have been linked to decreased microbial diversity and stability (Zingore et al., 2005).

Inorganic N fertilizer use has been associated with reduced soil diversity compared to use of organic fertilizers such as anaerobic digestate. Wu et al. (2020) conducted research on the effect of organic fertilizer application and reduced chemical N fertilizers on soil properties and bacterial community of an agricultural soil. Their results showed that organic fertilizer treatments significantly increased bacterial diversity compared to chemical N fertilizer treatments. Similar results were reported by Sun et al. (2015) and Yuan et al. (2018).

Various researchers (Ceja-Navaro et al., 2010; Ovreas and Torsvik, 1998) have reported that increased tillage intensity in croplands reduced microbial stability and diversity compared to reduced or no tillage that was practiced in grasslands. In agreement, Quadros et al. (2012) conducted research on the effect of tillage on soil microbial composition and diversity. Based on their results, frequent tillage had significantly lower Shannon diversity index values, compared to treatments with reduced tillage.

The practice of monoculture has been found to have negative effects on soil microbial stability and diversity. Zhao et al. (2018) conducted research on the long term effect of monoculture on soil and microbial properties. Based on their results, continuous cropping decreased soil bacterial alpha

diversity. In agreement, Liu et al. (2014) conducted research on microbial community diversities and taxa abundances in soils with potato monoculture. They found that increased monocropping reduced microbial diversity. In addition, Tiemann et al. (2015) found that increased above ground diversity, commonly observed in grasslands stimulated microbial functionality in the soil. These results show that although conventional tillage practices may be effective in increasing crop yields, they may not be sustainable for maintenance of soil health and microbial diversity.

2.4.4 Seasonality

Seasonal changes are usually accompanied with shifts in rainfall, temperature and soil nutrient content which affect soil microbial properties (Kim et al., 2016; Luo et al., 2019). Additionally, seasonal changes are related to farmer management practices such as planting, fertilizer application schedules and harvesting that affect the soil physical, chemical and microbial properties (Bevivino et al., 2014). These seasonal changes have been identified as some of the main factors that drive changes in soil microbial structure and diversity (Shigyo et al., 2019).

Previous reports have identified the effect of seasonal changes on the soil microbial community within the agricultural environment (Bevivino et al., 2014; Lacerda-Junior et al., 2019; Li et al., 2019). However due to differences in soil physical, chemical and microbial properties, there have been contrasting results on the effects of seasonal treatments on specific soil microbes. Li et al. (2019) conducted research on the effect of seasonal changes i.e., summer, autumn and spring in microbial diversity and its relationship with soil chemical properties. Their results showed that there was a significant increase in microbial properties in summer due to the high rainfall and temperatures experienced during the season. Bevivino et al. (2014) conducted research in the soil bacterial community response to differences in agricultural management and seasonal changes in the Mediterranean region. Their results showed that seasonal variations affected soil chemical and structural fractions of the soil organic matter. Additionally, soil bacterial communities were affected by seasonal changes. For example, *Bacilli* and *Actinobacteria* were identified as the dominant microbes in spring while *Actinobacteria* and *Proteobacteria* were the dominant microbes in summer. Lacerda-Junior et al. (2019) conducted research on the effect of land use and seasonal effects on the soil microbiome. Their results showed a significant increase in

Actinobacteria and *Cyanobacteria* during the dry season, while *Proteobacteria, Acidobacteria* and *Bacteroidetes* significantly increased during the rainy season.

Therefore, research needs to be conducted to clarify the effect of seasonal variations on specific soil microbes within the agricultural environment, to develop sustainable management practices that are specific to each season (Shigyo et al., 2019).

2.4.5 Fertilizers

Fertilizers are organic or inorganic based compounds that add nutrients to the soil (Maximum Yield Inc. 2019). Agricultural fertilizer use has been credited with increased soil fertility and crop yields (Vitousek et al., 2002). The 'Green Revolution' is credited for the significant increase in inorganic fertilizers in agriculture (Erisman et al., 2008). The term 'Green Revolution' was developed by William S. Gaud in 1968 and related to the introduction of various technologies and policies in developing countries with assistance from developed nations to increase agricultural food production (Conway, 1997). One of the main methods to achieve this objective was the use of chemical fertilizers to improve soil nutrient levels and subsequently crop yields (Parayil, 1992). The use of inorganic fertilizer involves animal excreta and plant residues. Farmers process animal excreta and use it in different forms within the agricultural environment. Some of the common processes and forms used include composting (the anaerobic fermentation of animal manure), anaerobic digestate (by product of the biogas production process) (Bernal et al. 2009; Yabe 2013) among others.

Agricultural fertilizer use is an important factor in ensuring global food security (Roberts, 1999). According to Roser (2014) the world global population is expected to reach 9.7 billion by 2050. As expected, this will increase global food demand by 70%, increasing dependency on fertilizers (Dawson and Hilton, 2011; FAO, 2012).

2.4.5.1 Inorganic nitrogen fertilizers

Inorganic N fertilizers were first generated during the 20th century using the Harber-Bosch process developed by Fritz Haber (Erisman et al., 2008). Considering the rise in human population, the current society would not exist with the use of inorganic N fertilizers, to ensure food security (Erisman et al., 2008). The N is one of the nutrients that are essential for plant growth and development (Sharma and Bali, 2017). The nutrient is involved critical plant processes such as leaf

expansion, plant growth and biomass production. More specifically, plant molecules such as chlorophyl, amino acids and nucleic acids contain N and are necessary for biological processes such as photosynthesis, carbon metabolism, among others (Crawford et al., 2002; Frink et al., 1999). In addition, N application improves root growth and length, plant nutrient uptake and dry mass production (Diaz et al., 2006; Stitt and Krapp, 1999). Therefore, N is considered one of the main limiting nutrients for plant development in agriculture (Rütting et al., 2018).

The quantity of N fertilizer in agricultural soils has been increasing over the last 50 years (Zahoor et al., 2014). This practice as led to excessive inorganic N fertilizer application, which had led to various environmental and ecological problems (Ahmad et al., 2017). Some of these include eutrophication, greenhouse gas emissions (N_2O), soil acidification among others (Bouwman et al., 2005; Guo et al., 2010).

2.4.5.2 Organic anaerobic digestate fertilizer

Due to the negative effects of inorganic N fertilizer use on the environment and agricultural production sustainability, farmers have been turning to organic fertilizers. The biogas production process is a technology that is gaining popularity where organic matter is converted to biogas and the nutrient rich byproduct anaerobic digestate (Risberg et al., 2016). More specifically, biogas production is a biological process involving organic matter decomposition and stabilization under anaerobic conditions. One of the main benefits of anaerobic digestate is that various organic materials can be used as feed stock for the process. Some of the organic materials used as feedstock include animal wastes, energy crops, plant biomass, food industry and agricultural wastes (Appels et al., 2011; Yan et al., 2008). The microbial based process produces biogas (methane and carbon dioxide) and anaerobic digestate. Biogas is used as a source of energy for producing electricity, heat and vehicle fuel (Sun et al., 2016). Anaerobic digestate is considered a fertilizer with the potential of reducing the use of fossil fuel dependent inorganic N fertilizers (Holm-Nielsen et al., 2009; Walsh et al., 2012).

2.5.5.2.1 Biogas production process

The biogas production process consists of four phases: enzymatic hydrolysis, acidogenesism and methanogenesis (Odwuge et al., 2020).

During the enzymatic hydrolysis process, large polymers are broken down by facultative and obligate anaerobic bacteria (*Streptococcus* and *Enterococcus*). Polysaccharides are degraded to

oligosaccharides and monosaccharides, proteins are degraded to peptides and amino acids, lipids are degraded to glycerol and fatty acids (Paritosh et al., 2017).

During acidogenesis, hydrolysis products are fermented to volatile fatty acids by facultative anaerobic bacteria (*Ruminococcus, Paenibacillus, Clostridium*). The volatile fatty acids from the process include acetate, valerate, butyrate, isobutyrate and propionate. Furthermore, NH_3, hydrogen and carbon dioxide gas are produced along with fatty acids (Paritosh et al., 2017).

In the methanogenesis phase, methane is produced through reduction of carbon dioxide and fermentation of acetic acid. Methane is produced from acetic acid by acetolactic methanogens archaea. Additionally, methane is produced through the reduction of carbon dioxide by hydrogen by hydrogenotrophic methanogens. About 70% of the methane is produced from acetic acid fermentation while 30% is produced from carbon dioxide reduction (Karakashev et al., 2005). Anaerobic digestate is produced as a byproduct of the process.

2.4.5.2.2 Anaerobic digestate characteristics and utilization

Anaerobic digestate is composed of a mixture of water, organic, inorganic substrates and various nutrients (Möller et al., 2015). Anaerobic digestate fertilizer contains higher mineralized nutrients than untreated feedstocks (Arthurson, 2009). During the biogas production process, nutrient transformations of the organic substrate occurs. Easily decomposable carbon compounds are removed, leaving behind compounds that are more difficult to decompose (Glowacka et al., 2020). The N in the substrate is transformed to NH_4^+. Gemmeke et al. (2009) reported that the anaerobic digestate NH_4^+ content could increase by a factor of three depending on substrates used. Essentially the NH_4^+ content of anaerobic digestate is generally higher than the substrates, allowing the nutrient to be immediately available for plant uptake (Arthurson, 2009). Additionally, pathogenetic microbes such as parasites, viruses and bacteria are destroyed. Anaerobic digestate has an increase in vitamin and amino acid content, decrease in C/N ratio and no changes in macro and micronutrients (Glowacka et al., 2020).

Various researchers have reported increased soil nutrient and yield content due to soil anaerobic digestate application. Tampio et al. (2015) reported that anaerobic digestate fertilizer had a high NH_4^+ content (4.07 mg N g^{-1}). Šimon et al. (2015) similarly reported that anaerobic digestate application resulted in higher wheat yields due to increased soil NH_4^+ content. Risberg et al. (2019) conducted research on characterization of anaerobic digestate and its effects on soil microbial

activity. Based on their results, anaerobic digestate increased the soil organic carbon and N content. Other researchers have found that anaerobic digestate soil application has been linked with increased soil pH, phosphorous, potassium, magnesium, soil carbon sequestration and increased water holding capacity (Pivato et al., 2016; Pranagal et al., 2019; Smith et al., 2014). Therefore, the application of anaerobic digestate is considered a practical substitute for reducing use of inorganic N fertilizers in the agro ecosystem (Risberg et al., 2016; Weiland, 2009).

2.4.5.2.3 Solid Liquid Separation of Anaerobic digestate

The use of anaerobic digestate as an organic fertilizer has been increasing in recent years. However there have been some challenges associated with anaerobic digestate use as a fertilizer. One of the main challenges that has been observed with its use as a fertilizer is the high costs related to its transport and storage, compared to chemical fertilizers (Drosg et al., 2015). The high costs are caused by anaerobic digestates' low nutrient and large volume compared to chemical fertilizers. Bojesen et al. (2014) reported that transportation of substrates and anaerobic digestate are responsible for approximately 33% of the biogas production process cost. Another challenge is that due to anaerobic digestates' high moisture content, there is a risk of temporary flooding associated with its use during the rainy season especially in humid environments. In addition, farmers are interested in specific nutrient concentrations of anaerobic digestate so that they can apply the required amounts of nutrients based on soil requirements (Drosg et al., 2015). To address these challenges, anaerobic digestate can be separated to remove the solid and liquid fractions before field application (Heviánková et al., 2015). Research shows that separated fractions have substantially lower transportation costs due to reduced water contents, especially for the solid fraction. Additionally, the solid-liquid separation results in changes in nutrient distribution between solid and liquid fractions allowing for more precise nutrient applications based on soil requirements (Figure 2.2).

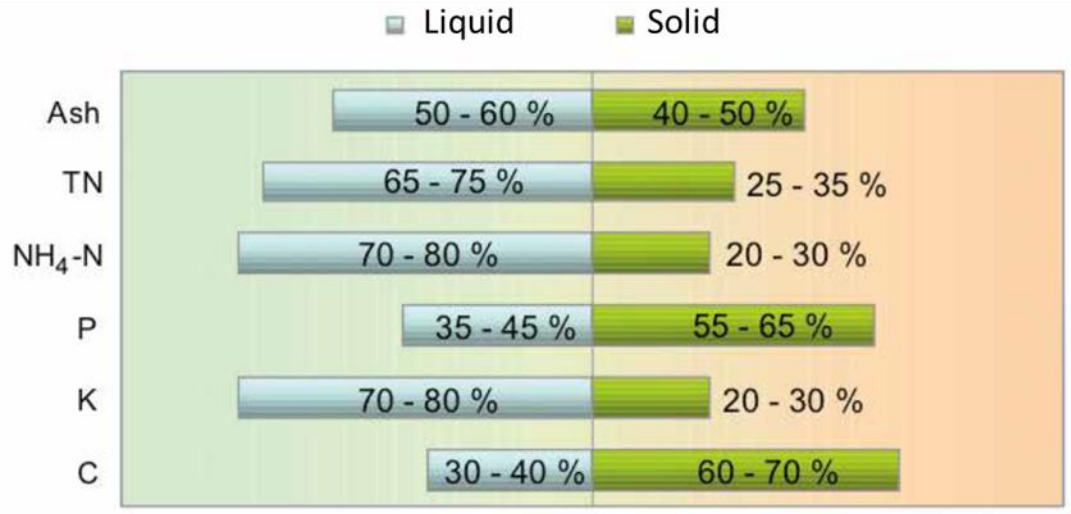

Figure 2.2: Distribution of the principal constituents after solid–liquid separation (data based on own investigations and various references. Abbreviations; TN-Total Nitrogen, P-Phosphorous, K-Potassium, C-Carbon (Adapted form Bauer et al., 2009*)*

2.4.5.2.4 Anaerobic digestate Flocculation

Flocculation is a chemical treatment used to separate anaerobic digestate into solid and liquid fractions (Nowostawska et al., 2005). Anaerobic digestate particles have negative charges that tend to repel each other and therefore do not sediment. This characteristic makes the process of anaerobic digestate solid liquid fraction a bit more complicated (Fuchs et al., 2010). To enhance aggregation of the solid fraction, multivalent cations that cause coagulation are added in addition to polymers that enhance flocculation (Hjorth et al., 2010).

Anaerobic digestate flocculation involves two steps. In the first step, the flocculant, a water soluble metal salt such as iron-III-chloride, is mixed with anaerobic digestate. Cations formed from the metal salt react with the negatively charged anaerobic digestate particles to form bigger particles (Figure 2.3). A flocculant aid polymer is usually added to increase the size of the solid particles for separation. The flocculant aid polymer is a long negatively charged chain where metal cations from the polymer connect to form a high particle for more efficient solid-liquid separation (Fechter and Kraume, 2016).

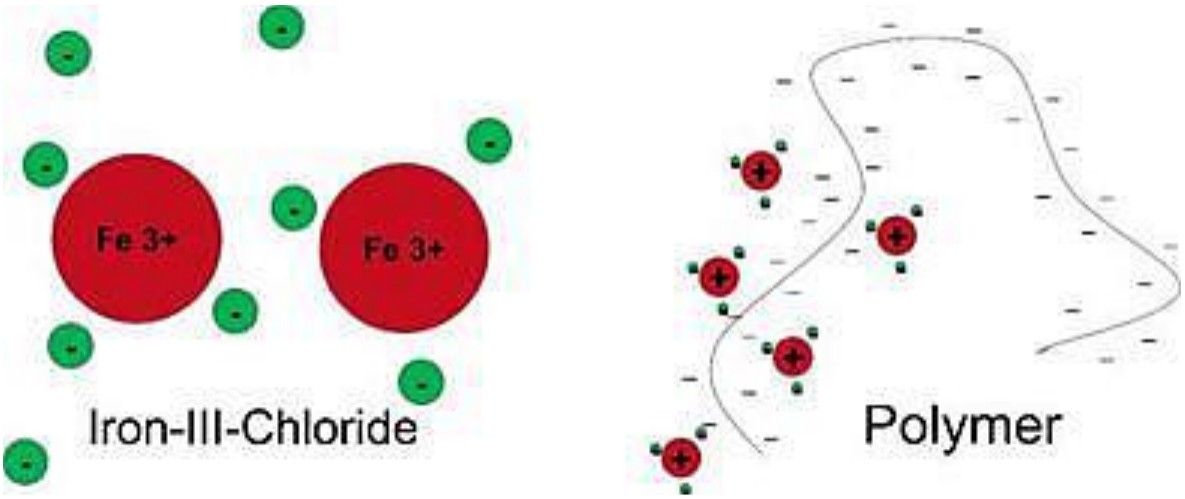

Figure 2.3: Principle of flocculation (Adapted form Fechter and Kraume, 2016)

2.4.5.3 Effect of fertilizers on microbial abundance and stability

The use of organic and inorganic N fertilizers has a direct and indirect effect on soil physical and chemical properties (Dong et al., 2014; González-Chávez et al., 2010). Changes in soil physical and chemical properties have been identified as the main drivers in changes in microbial abundance and stability in agricultural soils (Martin et al., 2015. Thus, the use of fertilizers influences soil microbial abundance and stability (Bell et al., 2015).

The use of inorganic N fertilizers has been associated with reduced microbial abundance and stability. One of the main reasons is the effect of inorganic N fertilizers on soil pH. Inorganic N fertilizer use has been associated with agricultural soil acidification, which has a negative effect on microbial abundance and diversity (Guo et al., 2010; Sun et al., 2015). Brady and Weil (1996) and Wang et al. (2012) identified pH as one of the main factors affecting the diversity and microbial community composition. Soil pH influences other soil factors such as soil nutrient availability and cations solubility which influence the soil microbial community. Fierer and Jackson (2006) conducted research on factors that influence the diversity and biogeography of soil bacterial communities. Based on their results, soil pH was one of the main factors influencing the microbial abundance and diversity. Diversity was highest in soils with near neutral pH and lowest in acidic soils.

Organic fertilizers have a high impact on the soil microbial community than chemical fertilizers (Wei et al., 2017). This can be attributed to organic fertilizers improving different aspects of soil

fertility, organic carbon content, soil aggregate stability and microbial biomass which cause shifts in soil microbial community (Darwish et al., 1995; Lazcano, 2013; Marschner, 2003). Sapp et al. (2015), Hartman et al. (2014) and Coelho et al. (2020) conducted research on the effect of anaerobic digestate as one of the treatments on the soil microbial community. Based on their results it can be concluded that anaerobic digestate fertilizers were a major driver of microbial diversity. Specifically, anaerobic digestate increased various aspects of diversity including community composition, richness and evenness. Although there are few studies that have analyzed the effect of organic fertilizers on microbial stability, research shows that high microbial diversity is associated with enhanced microbial stability (McCann, 2002). Therefore, the increased diversity with organic fertilizer use is expected to result in enhanced microbial stability.

2.4.6 Soil pH

Soil pH is defined as the negative logarithm to the base of $_{10}$ (p) of the soil's hydrogen ion (H^+) concentration i.e., $pH = -\log_{10}[H^+]$ (Shukla et al., 2014; Zhou, 2008). Therefore, a change in pH denotes a tenfold change in H^+ concentration. The pH scale, which ranges from 0 to 14, is used to show the level of acidity (<7) or alkalinity (>7) (Blackson, 1989). Soil pH is defined as a master soil variable due to the significant effect it has on the soil physical, chemical and biological properties (Figure 2.4) (Minasny et al., 2016; Neina et al., 2019).

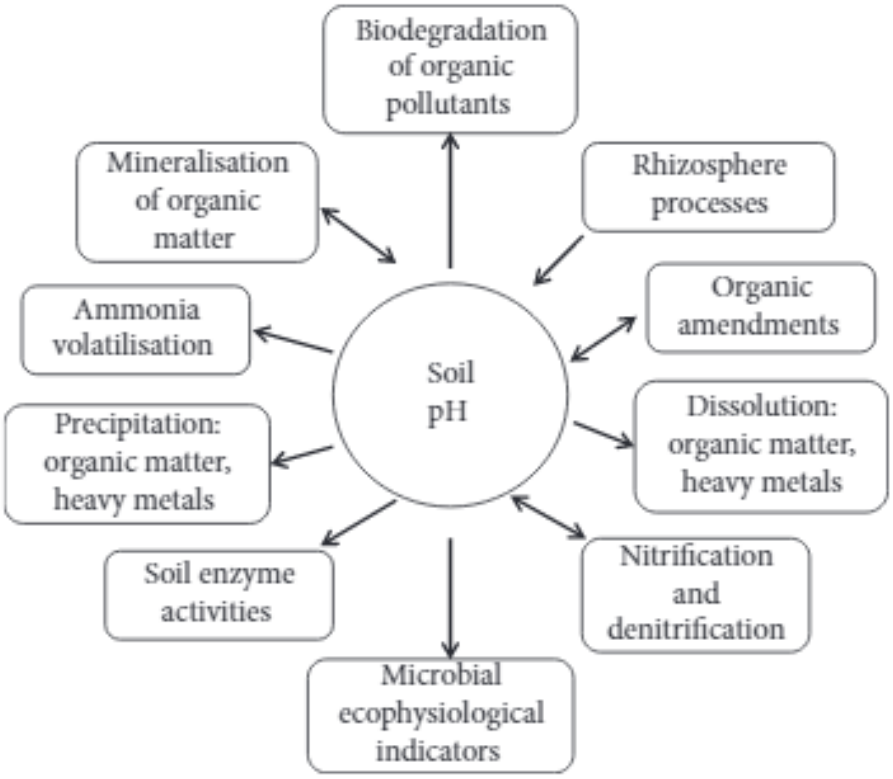

Figure 2.4: Soil biochemical processes and their relations to soil pH (Adopted from Neina, 2019)

2.4.6.1 Factors that affect agricultural soil pH

2.4.6.1.1 Inorganic nitrogen fertilizers

Inorganic N fertilizer use is considered one of the most efficient methods to increase the soil N content and subsequent crop yields (Chen et al., 2017). However, the increased use of inorganic N fertilizers has caused the acidification of agricultural soils, which is negatively affecting the soil physical, chemical and microbial properties (Schroder et al., 2011). About 50% of the global agricultural lands have been negatively affected by soil acidification due to the use of inorganic N fertilizers (Dai et al.2017). Soil acidification has been identified as one of the most significant yield limiting factors in agricultural soils (Sumner and Noble, 2003). The N fertilizers themselves are not acidic, but their additions to soils are acid forming (Schroder et al., 2011). The N fertilizers cause soil acidification through the nitrification process through which NH_4^+ to NO_3^-, producing H^+ which acidify the soil (Kariuki et al., 2007; Tang et al., 2002).

Schroder et al. (2011) conducted research on the effect of long term use of N fertilizers on soil acidification. Their results showed that the long term use of inorganic N fertilizers significantly decreases soil pH levels, enhancing soil acidity. These results agree with various researchers who have reported that the continuous use of inorganic N fertilizers caused soil acidification in agricultural soils (Bouman et al., 1995; Chien et al., 2008).

2.4.6.1.2 Liming

Liming is the most common and effective method to reduce increasing soil acidification in agricultural soils (Mkhonza et al., 2020). Most of the liming materials contain Calcium which are effective in reducing soil acidity (Rasnake et al., 2002). Adding lime to the soil reduces soil acidity by neutralizing H^+ in the soil (Equation 1) (Bolan et al., 2003; Buni, 2014).

$CaCO_3$ (s) + $6H^+$ (aq) → $3Ca^{2+}$(aq) + $3CO_2$ (g) + $3H_2O$ (l)

$3Ca^{2+}$(aq) + $2AlX_3$ (ex) → $3CaX_2$ (ex) + $2Al^{3+}$(aq)

$2Al^{3+}$(aq) + $3H_2O$(l) → 2Al $(OH)_3$ (s) + 6H+(aq) ……Equation 1

It has previously been reported that liming agricultural soils reduces soil acidity (Alemayehu and Taffesse, 1999). Buni (2014) conducted research on the effect of liming acidic soils on soil properties and yields of Haricot bean. Based on their results, soil pH increased with increasing lime application. In their experiment, soil pH values increased from 5.03 (soils without lime) to 6.72 (soils with lime-3750 kg $CaCO_3$ ha^{-1}). Additionally, Temesgen, et al. (2016) conducted research on the effect of lime and phosphorous fertilizer on acidic soils in the central highlands of Ethiopia. They reported that liming at the rate of 0.55, 1.1, 1.65 and 2.2 t ha^{-1} increased soil pH by 0.48, 0.71, 0.85 and 1.1 units, respectively.

2.4.6.2 Effect of soil pH on soil microbial community

Soil pH is one of the most significant factors affecting the soil microbial community in agricultural soils (Singh, 2018). Soil pH values that are near neutral to basic tend to increase soil microbial abundance compared to acidic pH values. Msimbira and Smith (2020) reported that the ideal pH for most soil microbes in the agricultural environment was from 5.5 to 6.5. Shen et al. (2010) conducted research on the effect of long-term fertilizer application on the abundance and composition of soil bacterial communities. Their results showed that less acidic soil conditions had higher bacterial populations compared to acidic conditions. Rousk et al. (2009) conducted

research on the effect of soil pH on fungal and bacterial growth. Their findings showed that decreasing soil pH levels from 8.3 to 4.0 caused a five-fold reduction in soil bacterial growth.

Soil pH levels significantly influence the soil N cycles nitrifier and denitrifier community (Zhang et al., 2016). Various studies have showed that soil pH levels influence the niche separation of AOA and AOB. More specifically, AOA prefer acidic environments of pH values of <5.5 while AOB prefer basic soil pH (He et al., 2012; Zhang et al., 2016). Additionally, soil pH has been found to influence the denitrification *nosZ* and *nirS* abundance. Čuhel et al. (2010) and Herold et al. (2018) reported that increasing the soil pH values through liming, enhanced the abundance of *nosZ* and *nirS* genes. Considering the significance of soil pH on different aspects of the microbial community and related soil functions, consideration should be taken on the effect of farmer management practices on soil pH values.

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Chapter 3 Land use and season drive changes in soil microbial communities and related functions in agricultural soils

3.1 Abstract

Soil microorganisms are important for maintenance of soil health and related functions. Agricultural management practices such as land use, season and fertilizer affect soil microbial community structures. However, the effect of these management practices on soil microorganisms and related functions, influenced by regionally different soil types, is still not clear. Hence, the study was conducted in an Andosol (volcanic soil) dominated agricultural region in cool temperate climate to determine the effect of land use (cropland, grassland), season (spring, summer) and fertilizer (anaerobic digestate) on soil microorganisms and related functions. Soils were sampled from farmers' fields, DNA extracted and sequenced targeting 16S rRNA region. In result, land use had a significant effect on beta diversity and evenness with higher values recorded in cropland than grassland. However, grassland had a higher number of unique operational taxonomic units (OTUs) (10303) compared to cropland (5112). In cropland, season had a significant effect on beta diversity, evenness, OTU numbers and Shannon index with higher values recorded in summer compared to spring. Based on predicted soil functions, nitrogenase (*nifH*) had significantly higher values in cropland-summer while nitrite reductase (*nirK*) and ammonia monooxygenase (*amoA*) were significantly higher in cropland-spring. In grassland, season had a significant effect on beta diversity only. These results indicate that grassland microorganisms were stable and more resistant to seasonal changes than cropland, suggesting that conventional tillage practices have a negative effect on soil microbial stability. Additionally, grassland-spring (7059) had a higher number of unique OTUs than grassland-summer (2597). Based on predicted soil functions, *nifH* was significantly higher in grassland-spring while *nirK* and *amoA* were significantly higher in grassland-summer. These results indicate that the impact of seasons on soil microorganisms' distribution and abundance in cropland and grassland may directly affect soil functions.

3.2 Introduction

Soil microorganisms are significant to the maintenance of soil health by providing ecosystem services (Chaparro et al., 2012; Singh et al., 2014), such as decomposition of organic matter and nutrient cycling (Marques et al., 2014; Yuliar et al., 2015). Microbial diversity is an essential attribute of the soil microbial community (Bender et al., 2016). High microbial diversity enhances soil ecosystem services and tolerance to disturbances such as land use, seasonal changes and fertilizer application (Griffiths and Philippot, 2013; Loreau, 2010). On the contrary, a decrease in diversity would lead to potential loss of ecosystem services reducing the systems stability (Trivedi et al., 2016). Thus, soil microorganisms should be diverse to ensure sustainable provision of ecosystem services.

Agricultural soil disturbances are defined as factors that cause changes in the composition and structure of the soil (Thompson et al., 2002). Soil disturbances can be caused by farmer management, such as physical and chemical soil disturbances. Physical soil disturbances are usually caused by tillage practices, which are determined by the land use management system (Chen et al., 2011; Liu et al., 2019). Chemical soil disturbances are usually related to agricultural fertilizer application (Ogundijo et al., 2014). Additionally, soil disturbances can also occur naturally by shifts in rainfall and temperature, caused by seasonal changes, which induce soil disturbances (Luo et al., 2019). Although there are many other factors that can cause soil disturbance within the agricultural environment, site visits identified land use, fertilizer application and seasonality as some of the main factors that may have caused soil disturbance in the study site. Nonetheless, the influences of these factors, i.e., land use, seasonal changes and fertilizer application on soil microbial diversity and related soil functions in the study region are still not fully explored.

Although there are considerable research efforts made towards examining the effect of disturbances on soil microbial community composition on a global scale (Crowther et al., 2014; Johnson and Curtis, 2001), there is a lack of research on the same at a regional scale, focusing on the disturbance factors as stated above (i.e. land use, seasonality and fertilizer inputs). In addition, specific analyses of field-based observation of soil microbial diversity at a regional scale, on the same soil type, are essential because basic soil characteristics determine soil microorganisms' structures and related functions (Pan et al., 2014; Wang et al., 2019). Therefore, research needs to

be conducted to clarify the interaction of land use, seasonality and fertilizer application on soil microorganisms and related functions at a regional scale in agricultural soils.

Land use is one of the factors that drives soil microorganisms and functions (Degens et al., 2000; Pabst et al., 2013). Various studies have documented that land use types affect soil properties (soil pH, organic carbon, inorganic nitrogen, soil water content), which are intimately connected to microbial community structure, diversity and functions (Pabst et al., 2013; Sengupta et al., 2020; Bissett et al., 2014). Common land use types in agriculture involve grassland for animal feed and cropland for yields (Yu et al., 2017). Previous research shows that conventional cropland management practices such as intensive tillage, monoculture and inorganic fertilizer use, have been associated with a decrease in soil microbial diversity and related soil functions (Wardle, 1992; Zingore et al., 2005). However, due to differences in experimental locations, setups and soil properties among various studies, there have been contrasting effects of cropland and grassland management on specific microorganisms. For instance, Szoboszlay et al. (2017) reported that abundance of *Verrumicrobiota* increased in cropland and decreased in grassland in European soils. On the other hand, Sengupta et al. (2020) reported that the abundance of *Acidobacteria* and *Gemmatimonadetes* increased in cropland and decreased in grassland soils.

In addition to the effects of land use, seasons drive changes in soil microbial diversity and functions (Bowles et al., 2014; Castro et al., 2009). Seasonal changes are usually accompanied with variations in rainfall, temperature and soil nutrient content (Smith et al., 2010). Seasons with high soil moisture and temperature like summer stimulate soil microbial activity and nutrient cycling compared to seasons with low soil moisture and temperature like spring (Galazka et al., 2017; Zifčáková., 2015). Considering cultivated agricultural soils, Li et al. (2019) observed that the relative abundance of *Acidobacteria* significantly increased in summer while *Actinobacteria* were significantly reduced in both spring and summer. However, research by Li et al. (2020) found that *Chloroflexi* and *Actnibacteria* were significantly higher in summer while *Acidobacteria* were significantly higher in spring. Therefore, research needs to be conducted to clarify the effects of season on specific soil microorganisms in agricultural soils.

Another factor controlling soil microbial diversity is fertilizer types, including animal waste based organic materials that have been found to increase microbial species richness in *Alphaproteobacteria*, *Betaproteobacteria*, and *Firmicutes* (Faisal et al., 2018). These microbes are

involved in decomposition of complex organic compounds (Buresova et al., 2019). As the price of chemical fertilizer soars (Ricker-Gilbert et al., 2014), not only livestock farmers but also crop farmers may utilize animal waste-based fertilizers in future, at the community level. To understand the effect of anaerobic digestate application on soil microbes, community level studies must be carried out. In some cases, animal wastes are collected from multiple farms and centralized to improve the efficiency of the effluent management. Community level field studies are critically important because interactions among soil types, the ratio between crop farmers and livestock farmers, sources of animal wastes and many other factors are unique to each community.

To evaluate the soils' microbial structure and potential changes in diversity, next generation sequencing (NGS) platforms will be used. Traditionally, researchers relied on culture dependent methods, which involved growing microbes in the laboratory. However, the main limitation of these methods is underestimation of diversity as some microorganisms do not grow well under laboratory culture conditions or as monocultures (Jo et al., 2016). To address these challenges, culture independent methods such as NGS platforms were developed. Through NGS platforms, soil microbial research has become cheaper, faster and convenient (Doolittle and Zhaxybayeva, 2010; Ghazanfar et al., 2009). To determine bacterial taxonomic classification and phylogeny, 16S rRNA gene is the most common genetic marker used (Janda and Abbott, 2007). This is because its present in almost all bacteria, its functions have not changed over time and the gene is large enough for informatics (Janda and Abbott, 2007).

Thus, this study aimed to address the following key questions; 1) In a regional scale, which of the imposed treatments (land use, season or fertilizer) is the biggest factor controlling soil microbes? and 2). Is it possible to identify the key microbes and related functions impacted by each imposed treatment? We therefore conducted research on soils from cropland and grassland applied with biogas slurry, mainly made of dairy wastes, during summer and spring. We hypothesized that land use would be the biggest factor controlling soil microbiome in Kamishihoro. More specifically, grassland-summer with anaerobic digestate application would enhance microbial diversity and related functions compared to other treatments.

3.3 Materials and methods

3.3.1 Study site and treatments

Soils were sampled in 2018 from Kamishihoro (43°14′N, 143°18′E), located in Tokachi town, Hokkaido prefecture, Japan. Soils in the region are classified as Andosol (FAO/UNESCO). Treatments included two land use regimes; cropland and grassland (Figure 3.1), two seasons; spring (April to June) and summer (August to October) and fertilizer inputs; anaerobic digestate (AD) , ammonium sulphate (AS) and control

Regarding land use, common management practices for cropland included crop rotation for 4 years involving potatoes, sorghum, wheat and beets. Beets (the focus of this study) were planted in April and August and harvested in July and November, respectively. The soil was tilled 4 times a year before seeding at a depth of 30 cm, using tractors. Fertilizers (Nitrogen-N, Phosphorous-P and Potassium-K) were applied at the rate of 40 kg N ha^{-1}, 340 kg P ha^{-1} and 158 kg K ha^{-1} at planting. For grasslands farmers were practicing no till system and anaerobic digestate fertilizer was applied at the rate of 60 kg N ha^{-1}. Grasslands and croplands were fertilized at the same time.

During the experimental period spring (April to June) had average temperature of 10.6°C and cumulative rainfall of 252 mm, while summer (July to October) had average temperature of 14°C and cumulative rainfall of 474 mm (Japan Meteorological Agency).

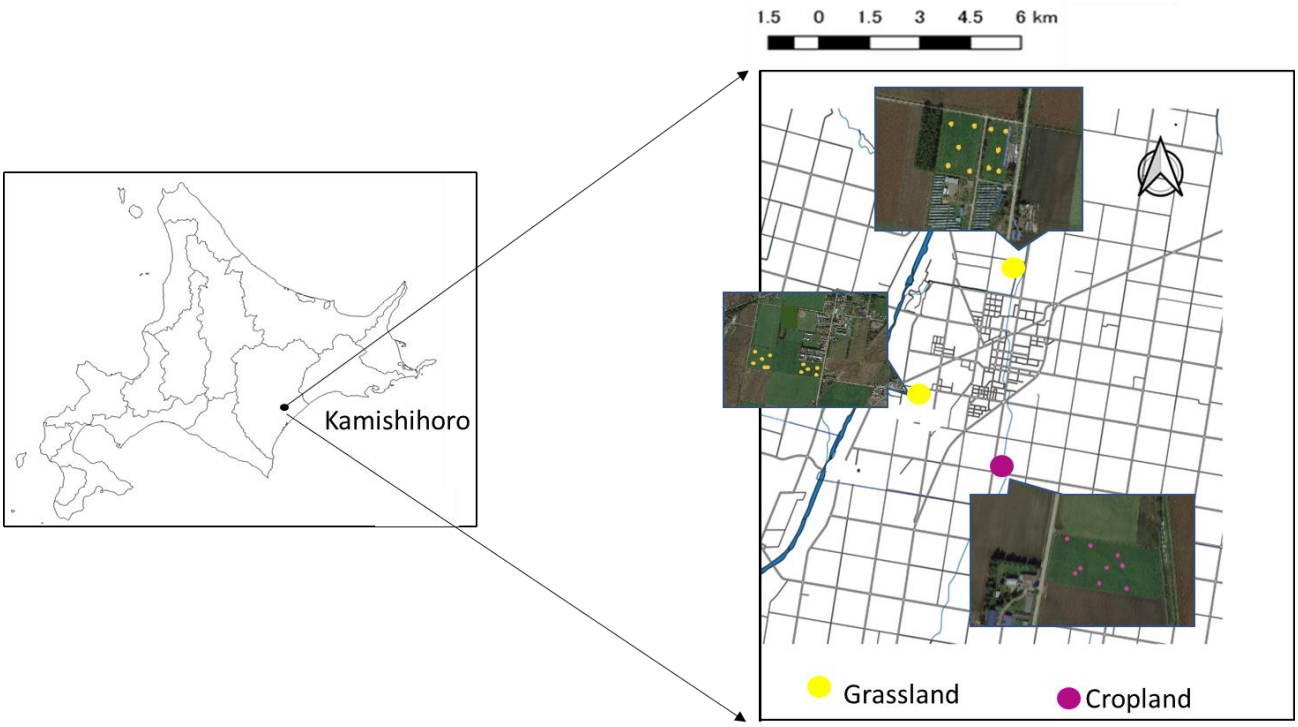

Figure 3.1:Kamishihoro soil sampling sites

3.3.2 Soil sampling

To avoid any contamination, all equipment and sampling procedures followed recommendations provided by Taberlet et al. (2012). For soil physical (bulk density-BD) analysis, soils were sampled from each fertilizer treatment using cores (100 cm^3). For soil microbial and chemical analysis (pH, N, K, magnesium-Mg, cation exchange capacity-CEC and humus), 500 g of soil samples were sampled from each plot and fertilizer treatment using a shovel. For microbial analysis, samples were stored at −30°C before DNA extraction.

We took a total of 72 soils samples at a depth of 0-15 cm on 30th April 2018 (spring) and 30th August 2018 (summer). To benefit from periods of optimum plant growth and enhanced microbial activity, soils were sampled during seasons of high rainfall and temperature (spring and summer). Cropland samples were from a beet (*Beta vulgaris*) farm (36 samples) and two fertilizer treatments; 30 t ha^{-1} AD (12 samples), 30 t ha^{-1} AD + 100 kg ha^{-1} AS (ADAS-12 samples) and control (12 samples). Grasslands were sampled from 4 plots with timothy (*Phleum pretense*) and alfalfa (*Medicago sativa*) grass species. Grassland fertilizer treatments were AD (18 samples) applied at

30 t ha^{-1} and control (18 samples). To reduce risks and prevent biased results, each treatment was sampled at least three times during the experimental period.

3.3.3 Measurement of soil physical and chemical properties

Analysis for soil physical (pH, BD), and chemical (N, K, Mg, CEC and humus) properties from cropland and grassland was conducted by Tokachi Federation of Agricultural Cooperatives and data means provided as sample values (Table S3.1 and S3.2) using methods as described below. Soil pH was measured with a grass electrode pH meter. Three phase distribution was calculated according to the methods by (Dexter, 2004) with a sand plate and a pressure plate extractor, then BD was calculated. Total N was measured with CN coder (N.C-Analyzer, Sumika Chemical Analysis Service, Ltd). The K, Mg, CEC were measured according to the method by Scollenberger and Simon (1945) with an atomic absorption spectrophotometer. Soil humus was visually estimated with a soil color book based on the Mansell System.

3.3.4 Soil DNA extraction

Soil DNA was extracted by phenol-chloroform extraction method (Sagova-Mareckova et al., 2008). A 0.5 g of soil sample was mixed with 0.2 g of skim milk to reduce DNA attachment to Andosols (Hoshino and Matsumoto, 2005). Afterwards the sodium phosphate extraction buffer (600 µl) was added, and the mixture shaken for 10 minutes. Phenol-chloroform-isoamyl alcohol (300 µl) and chloroform-isoamyl alcohol (250 µl) were then added to separate the soil solution into an organic phase (lipids and cellular debris) and an aqueous phase (Deoxyribonucleic acid-DNA). Isopropanol (300 ul) and ethanol (1000 µl) were used to precipitate DNA from solution. Nuclease free water (30 µl) was then added to store DNA in solution. An extraction control with all the chemical reagents (without soil) was prepared to check for possible contamination during extraction. The DNA was quantified with Qubit ds DNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) based on manufacturer's protocol.

3.3.5 16S rRNA Gene Amplicon Library Preparation and Sequencing

A metabarcoding approach was used for library preparation and sequencing. Polymerase Chain Reaction (PCR) was conducted targeting V4 region of 16S bacterial gene using 515F (5’-

GTGCCAGCMGCCGCGGTAA-3') and 806R (5'–GGACTACHVGGGTWTCTAAT-3') primer sets. Each PCR sample contained 10 µl of Amplitaq Gold® 360 Master Mix DNA polymerase, 0.4 of forward primer-F515, 0.4 µl of reverse primer-R806 (Caporaso et al., 2010), 8.2 µl of nuclease free water and 1 µl of DNA template, making up a final volume of 20 µl. Positive and negative controls were used during the amplification. The positive control was used to confirm that primers had attached to the DNA while the negative control was used to check for possible contamination during amplification. The controls contained the same mixture of polymerase, primers and water as the samples. However instead of 1 µl of DNA, the positive control contained 1 µl of Escherichia coli (Funakoshi Frontiers in Life Sciences, Bunkyo-ku, Tokyo, Japan) while the negative control contained 1 µl of nuclease free water.

PCR was conducted under the following conditions: 95°C for 600 sec and thereafter 30 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec with a final extension of 72°C for 60 sec. Second PCR was conducted to attach Ion P1 adaptor (Ion Torrent; Life Technologies), specific to each sample. Second PCR samples each contained 10 µl of Amplitaq Gold® 360 Master Mix DNA polymerase, 7.2 µl of nuclease free water, 0.4 µl of reverse primer (P1-R806), 0.4 µl of forward primer (Ion A-Barcode) and 2 µl of DNA template, making up a final volume of 20 µl. PCR was conducted under the following conditions: 95°C for 600 sec and thereafter 5 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec with a final extension of 72°C for 60 sec. Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Palo Alto, CA, USA) was used to determine final library concentration and length, based on manufacturer's protocol. Libraries were then diluted to 50 pM using Low TE (Tris-EDTA; 19 mM Tris base, 0.1 mM EDTA, Ion Torrent; Life Technologies). Sequencing was done with Next Generation Sequencer (Ion PGM, Life Technologies). Ion Chef was used to load libraries to Ion 318 chip (Ion Torrent; Life Technologies) and thereafter sequencing performed with the Ion PGM™ Sequencer (Thermo Fisher Scientific K.K., Japan) using Ion PGM™ Hi-Q™ View Sequencing Kit.

3.3.6 Sequence processing and data analysis

Raw sequenced data was processed using QIIME 2 software package version 2019.7 (Boylen et al., 2009). DADA2 was used to cut primer sequences, denoise and select representative sequences (Callahan et al., 2016). Classify-sklearn classifier, which uses naïve brayes classifier to set

taxonomy, was used to classify representative sequences. Closed reference OTU picking was based on Greengenes 13_8 database and clustered at 99% identity. The Greengenes database is used to classify archaea and bacteria. The classification is based on *denovo* automatic tree construction (McDonald et al., 2011). The Greengenes database has been used successfully in various research projects identify taxa accurately (Boers et al., 2019; McDonald et al., 2011). However, the database is incomplete and contains some unidentified and poorly interpreted sequences (Boers et al., 2019). To avoid bias due to different sampling depth, samples were rarefied to an even sampling depth, based on the sample with the lowest reads.

3.3.7 Soil microbial and chemical analysis

Alpha diversity, based on Pielou evenness, OTU counts and Shannon index, were generated in QIIME 2. Pielou evenness, which includes species abundance measurements, is considered a reliable measure of microbial community diversity analysis (Ehsani et al., 2019). As most alpha diversity analysis metrics are based on OTU data we analyzed microbial richness based on OTU counts (Chao, 1984; Chiu et al., 2014). The Shannon diversity index, which considers microbial richness and evenness, was used for alpha diversity analysis. Due to its reliability, Shannon index is one of the most reliable and widely used indices for microbial alpha diversity analysis (Strong, 2016). Weighted unifrac using Principal coordinate analysis (PCoA) was used for beta diversity analysis. Unlike most beta diversity measures, the Weighted unifrac measure takes into account species phylogeny and species relative abundance (Lozupone, 2011; Lozupone and Knight, 2005). To identify community composition variation between treatments, permutational multivariate analysis of variance (PERMANOVA) multivariate analysis using 999 permutations was performed in QIIME 2. Venn diagrams were constructed using phyloseq package (McMurdie and Holmes, 2013) in R software version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria) to visualize shared and unique OTUs between treatments. To determine the amount of variation in community composition explained by soil properties, Non-metric multidimensional scaling (NMDS) was conducted using vegan package (Dickson, 2003) in R software.

Linear Discriminant Analysis (LDA) with LEfSe was used to characterize microbial communities under imposed treatments (Segata et al., 2011). Significant ($p < .05$) alphas were based on Kruskal–Wallis test.

Tax4Fun (Aßhauer et al., 2015) package was used to predict functional profiles from 16S rRNA database. Genes encoding key enzymes were identified using their KEGG orthologs. However, as some of the soil microbes are uncultured or unknown, not all sequenced microbes can be identified to KEGG organisms. Nonetheless, Tax4Fun is considered a reliable bioinformatics tool for predicting microbial genes, due to its high correlation with metagenomic sequencing of functional profiles (AßHauer et al., 2015). Tax4Fun provides a more practical procedure to predict functions compared to shotgun and quantitative polymerase chain reaction (qPCR), which are significantly expensive and prone to experimental variability, respectively (Smith and Osborn, 2009; Kaiser et al., 2016; AßHauer et al., 2015).

All statistical analyses for soil physical and chemical properties were performed using R software. In all analysis, $p < 0.05$ were considered significant. Two-way and three-way analysis of variance (ANOVA) was performed to determine the effect of land use, season and fertilizer. For significant interactions, Tukey's HSD test was performed.

3.4 Results

3.4.1 Evenness, richness and microbial diversity

Land use had a significant ($p < .05$) effect on soil microbial evenness with higher values observed in cropland than grassland (Figure 3.2 A). In cropland, season had a significant ($p < .05$) effect on microbial evenness indices (Figure 3.2 B), with higher values in cropland-summer than cropland-spring. Fertilizer treatments did not have a significant effect in both cropland and grassland (data not shown).

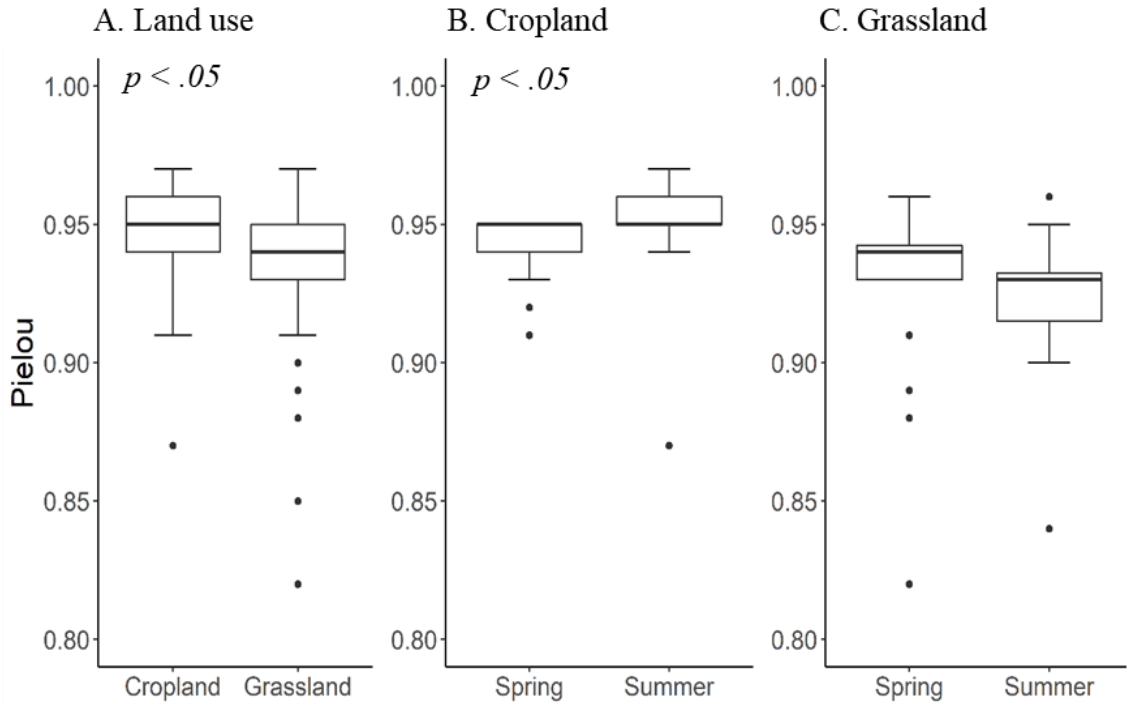

Figure 3.2: Box plots illustrating pielou evenness at OTU level based on land use and season in cropland and grassland. The boxes denote interquartile range with median as a black line and whiskers extending to the most extreme points. p values calculated based on Kruskal-Wallis test.

Land use did not have a significant effect on OTU numbers (Figure 3.3 A). In cropland, season had a significant ($p < .05$) effect on observed OTU numbers (Figure 3.3 B), with more OTUs observed in cropland-summer than cropland-spring. In grassland although season was not significant (Figure 3.3 C), grassland-spring had more OTUs than grassland-summer. Fertilizer treatments did not have a significant effect in both cropland and grassland (data not shown).

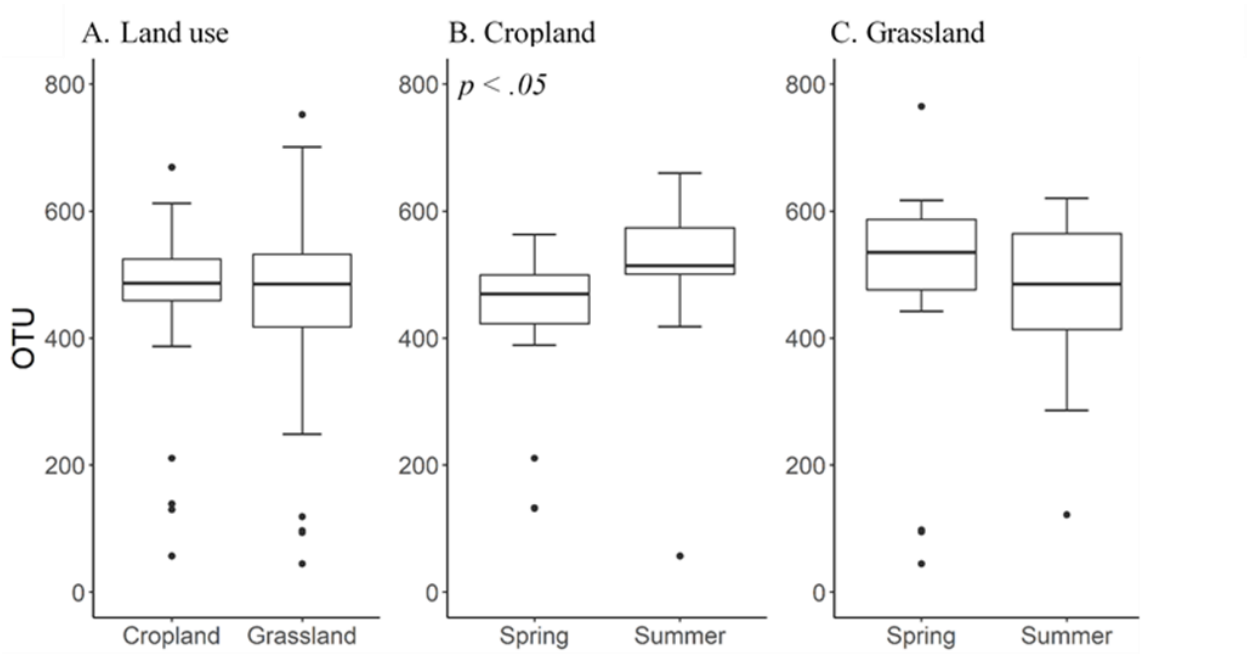

Figure 3.3: Box plots illustrating observed OTU numbers based on land use and season in cropland and grassland. The boxes denote interquartile range with median as a black line and whiskers extending to the most extreme points. p values calculated based on Kruskal-Wallis test.

Regarding Shannon Indices, land use did not have significant differences between cropland and grassland (Figure 3.4 A). In cropland, season had a significant ($p < .05$) effect on diversity, with cropland-summer microorganisms being more diverse than cropland-spring (Figure 3.4 B). In grassland although season was not significant, grassland-spring microorganisms were more diverse than grassland-summer (Figure 3.4 C). Fertilizer treatments did not have a significant effect in both cropland and grassland (data not shown).

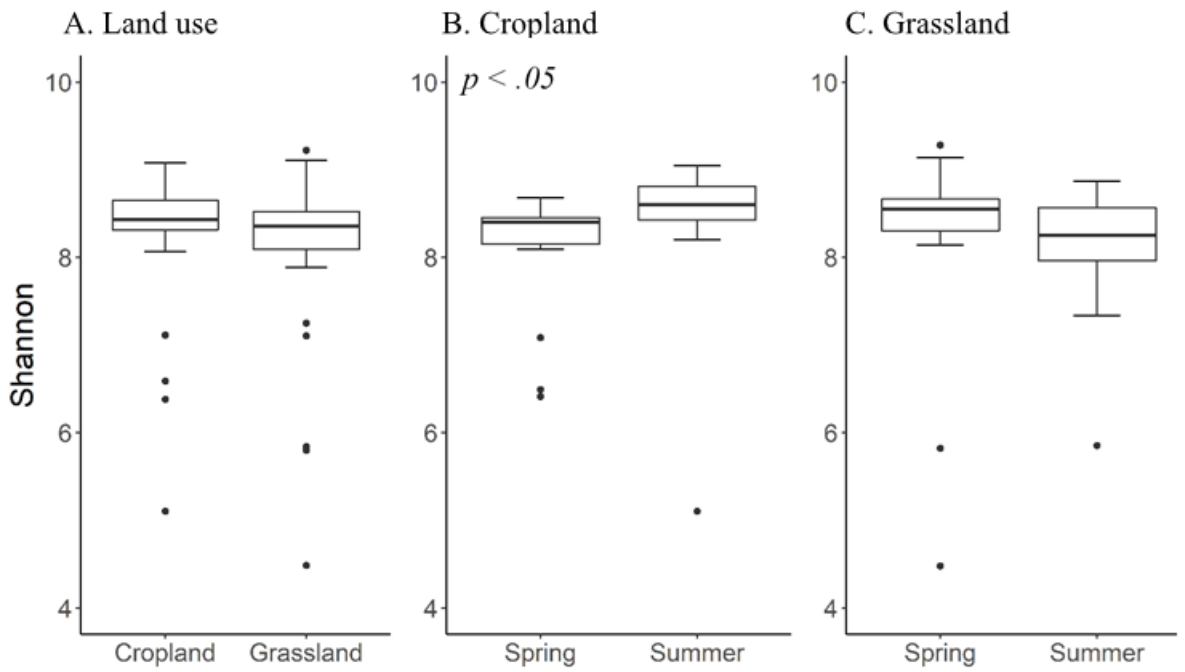

Figure 3.4: Box plots illustrating Shannon diversity index at OTU level based on land use and season in cropland and grassland. The boxes denote interquartile range with median as a black line and whiskers extending to the most extreme points. p values calculated based on Kruskal-Wallis test.

Regarding the soil beta diversity (weighted unifrac), samples from the same land use shared a high degree of similarity with significant ($p < .001$) distinct clustering observed in cropland and grassland treatments (Figure 3.5 A). In addition, seasonal treatments shared a significant ($p < .05$) high degree of similarity in both cropland (Figure 3.5 B) and grassland (Figure 3.5 C) treatments.

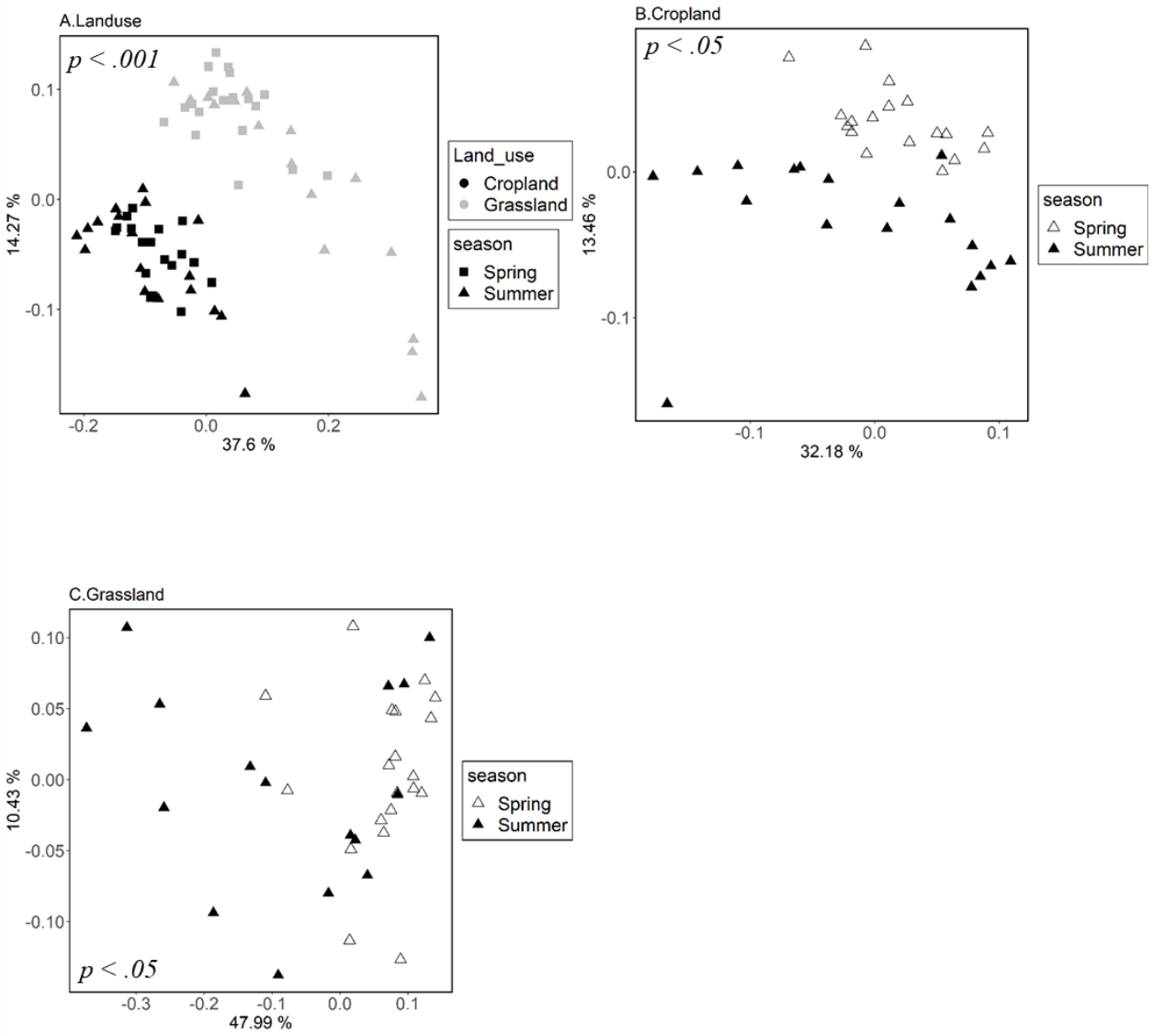

Figure 3.5: Principal coordinate analysis (PCoA) by weighted unifrac based on land use and season in cropland and grassland. Axes represent the two dimensions explaining greatest proportion of variances in microbial communities. p values calculated based on permutational multivariate analysis of variance.

3.4.2 Distribution of OTUs under imposed treatments

3.4.2.1 Unique OTUs

Grassland had a higher number of unique OTUs than cropland (Figure 3.6 A). At phylum level, unique OTUs in cropland were dominated by *Protobacteria* (23.6%, 1156 OTUs), *Planctomycetes* (17.1%, 830 OTUs), *Acidobacteria* (12.9%, 356 OTUs), *Actinobacteria* (12.6%, 343 OTUs) and

*Chloroflex*i (11.5%, 556 OTUs). Unique OTUs in grassland were dominated by *Planctomycetes* (23%, 2006 OTUs), *Proteobacteria* (22.2%, 1855 OTUs), *Acidobacteria* (19.3%, 934 OTUs), *Chloroflexi* (10.2%, 1222 OTUs) and *Nitrospirae* (4.9%, 127 OTUs).

The effect of season varied based on land use. Cropland-summer had a higher number of unique OTUs than cropland-spring (Figure 3.6 B) while grassland-spring had a higher number of unique OTUs than grassland-summer (Figure 3.6 C). At phylum level, the unique OTUs in cropland-summer were dominated by *Planctomycetes* (26.3%, 575 OTUs), *Proteobacteria* (23.8%, 744 OTUs), *Acidobacteria* (10%, 296 OTUs), *Chloroflexi* (8.6%, 371 OTUs) and *Verrumicrobia* (8%, 165 OTUs). In cropland-spring unique OTUs were dominated by *Proteobacteria* (30.9%, 404 OTUs), *Acidobacteria* (13.1%, 117 OTUs), *Actinobacteria* (10.3%, 125 OTUs), *Planctomycetes* (9.8%,195 OTUs) and *Chloroflexi* (7.4%, 180 OTUs). At phylum level, grassland-summer unique OTUs were dominated by *Planctomycetes* (22.2%, 485 OTUs), *Proteobacteria* (19.1%, 450 OTUs), *Acidobacteria* (14.4%, 212 OTUs), *Chloroflexi* (11.8%, 306 OTUs) and *Actinobacteria* (5.9%,122 OTUs). Grassland-spring unique OTUs were dominated by *Proteobacteria* (25.8%, 1290 OTUs), *Planctomycetes* (24.1%, 1294 OTUs), *Acidobacteria* (16.3%, 604 OTUs), *Chloroflexi* (9.2%, 893 OTUs) and *Actinobacteria* (6.1%, 341 OTUs).

In land use treatments all unique phyla in cropland were present in grassland. However, grassland treatment had 22 unique OTUs at phylum level that were not present in cropland. These unique OTUs were dominated by *Spirochaetes* (49 OTUs), *NKB19* (17 OTUs), *WS5* (13 OTUs), *WS1* (10 OTUs), *BHI80-139* (11 OTUs) and *OP8* (12 OTUs). In cropland seasonal treatments *GN04* (1 OTU) and *WWE1*(1 OTU) were found exclusively in cropland-spring while *Thermi* (5 OTUs), *Spirochaetes* (3 OTUs), *MVP-21* (2 OTUs), *BHI80-139* (1 OTU) and *NKB19* (1 OTU) were found exclusively in cropland-summer. In grassland seasonal treatments all unique phyla in grassland-summer were present in grassland-spring. However, grassland-spring treatments had 19 phyla that were not present in grassland-summer. These unique OTUs were dominated by *NKB19* (16 OTUs), *WS5* (13 OTUs), *WS1* (12 OTUs) and *OP8* (10 OTUs).

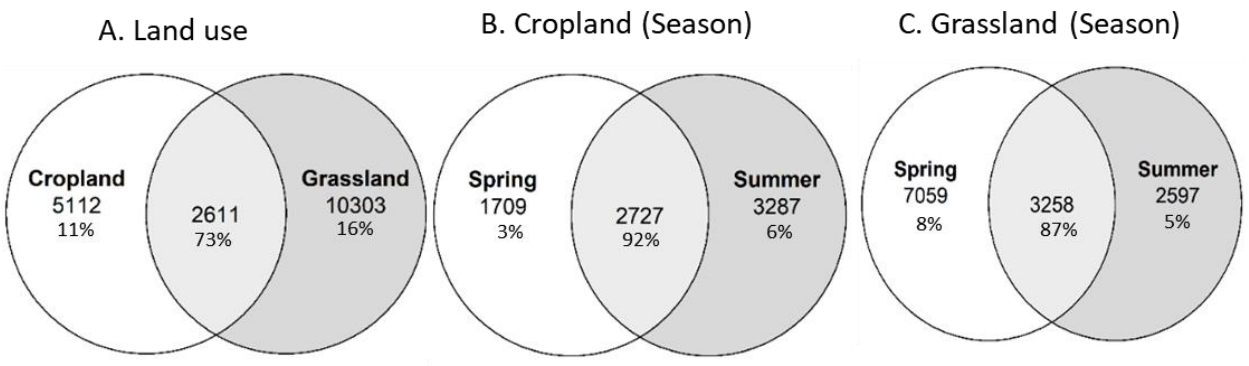

Figure 3.6: Venn diagrams showing the number of shared and unique OTUs based on land use and season. Percentage values represent relative abundance of OTUs in each section.

3.4.2.2 Shared OTUs

Shared OTUs at phylum level based on land use and seasonal treatments in cropland and grassland were dominated by *Protobacteria Planctomycetes, Chloroflex*i, *Acidobacteria* and *Actinobacteria.* Considering the shared microorganisms (2611 OTUs) in land use treatments (Figure 3.6 A), *Actinobacteria, Firmicutes* and *Gemmatimonadetes* were significantly ($p < .05$) higher in cropland. In grassland *Euryarchaeota, Bacteriodetes, Nitrospirae* and *WS3* were significantly ($p < .05$) higher (Figure S3.1A). Of the shared microorganisms (2727 OTUs) in cropland seasonal treatments (Figure 3.6 A). *Crenarchaeota* were significantly ($p < .05$) higher in cropland-spring while *Actinobacteria* and *Cyanobacteria* were significantly higher in cropland-summer (Figure S3.1B). Of the shared microorganisms (3258 OTUs) in grassland seasonal treatments (Figure 3.6 C) *Bacteroidetes* were significantly ($p < .05$) higher in grassland-spring (Figure S3.1C).

3.4.3 Effect of imposed treatments on soil functions

In cropland seasonal treatments, cropland-spring was significantly ($p < .05$) enriched with predicted functions related to *amoA* and *nirK* while cropland-summer had significantly ($p < .05$) enriched with *nifH* (Figure 3.7 A). In grassland season treatments, grassland-spring was significantly ($p < .05$) enriched with predicted functions related to *nifH* while grassland-summer was significantly ($p < .05$) enriched with *amoA* and *nirK* (Figure 3.7 B).

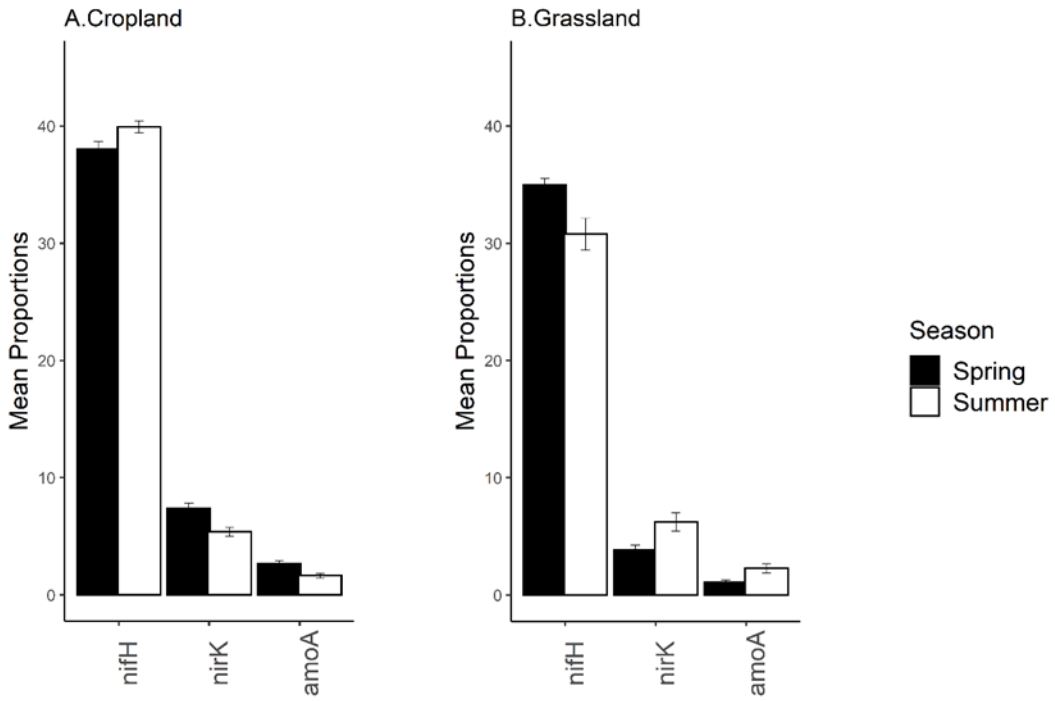

Figure 3.7: The effect of season on predicted soil functions in cropland and grassland. Error bars show statistical differences of selected 16S rRNA gene-predicted functional profiles. Abbreviations ; nifH-nitrogenase, nirK-nitrite reductase, amoA-ammonia monooxygenase.

3.4.4 Relationship between microbial community structure and soil properties

Soil variables were fitted to the non-metric multidimensional scaling (NMDS) plot and distinct clustering was observed in cropland seasonal treatments (Figure 3.8 A). Significant ($p < .05$) correlations between cropland community composition and soil properties were found for pH, K and Mg (Figure 3.8 B). In grassland, significant ($p < .05$) correlations between grassland community composition and soil properties were found for K and BD (Figure 3.8 B).

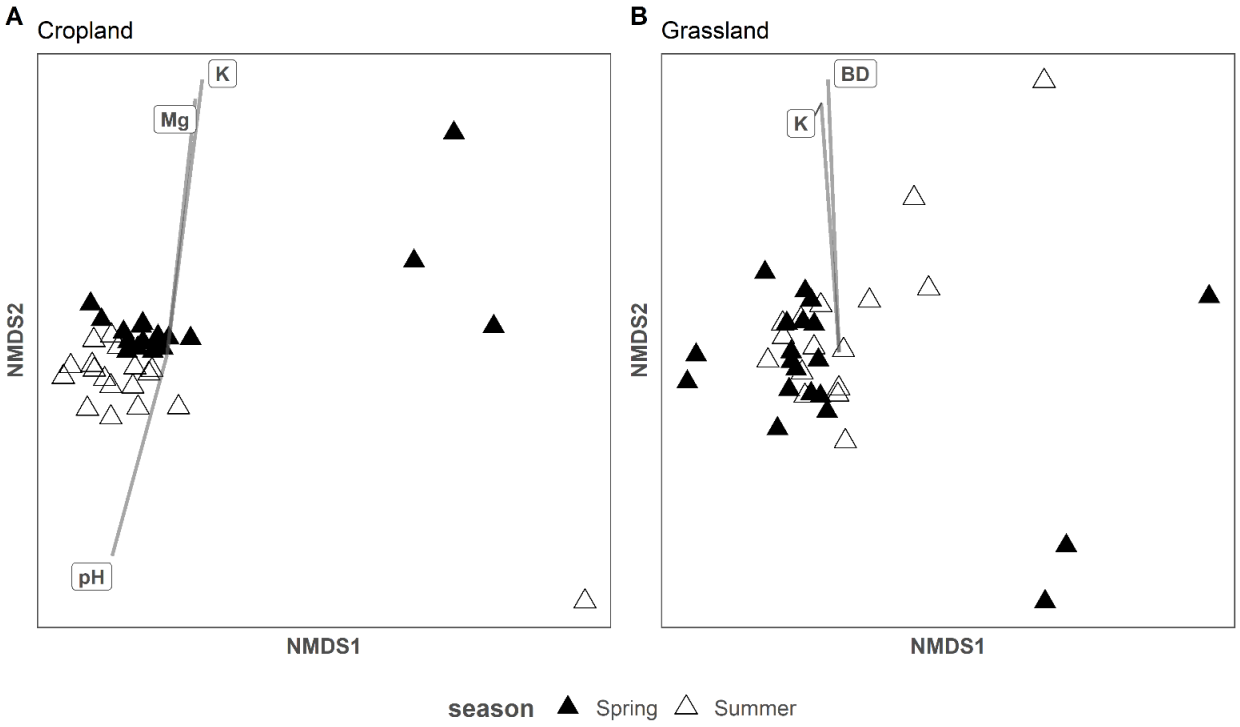

Figure 3.8: Nonmetric dimensional scaling plot based on bray curtis dissimilarity of microbial community structure in soils among seasonal treatments in cropland and grassland. Arrows show direction of significant ($p < .05$) environmental parameters obtained by fitting environmental factors in the sample ordination space.

3.5 Discussion

3.5.1 Effect of land use on the soil microbial community

The land use influenced soil alpha (pielou evenness) and beta (weighted unifrac) diversity. Regarding the alpha diversity, significantly ($p < .05$) higher soil microbial evenness was observed in cropland compared to grassland (Figure 3.2 A). Tillage practices mix the microbial habitat, unifying the microbial community structures and enhancing microorganism's evenness (Lienhard et al., 2013). Thus, our result may have been due to increased tillage intensity in cropland compared to grassland. Considering beta diversity, there was strong clustering of cropland and grassland on PCoA plot based on weighted unifrac distance metrics (Figure 3.5 A). This may have also been caused by differences in management practices between the two land use regimes. Specific to our

study, grassland management involved anaerobic digestate application and minimal soil disturbance, while cropland was characterized by relatively larger frequency of tillage and the addition of relatively larger amounts of inorganic fertilizers. Our results agree with Bissett et al. (2014) and Lauber et al. (2009) who reported that microbial community differences in cropland and grassland could be related to management practices that influenced soil physical and chemical properties, initiating changes in soil microorganisms' structure. Our results also indicate that management practices related to agricultural land use are critical in shaping microbial community structures in agricultural soils, by their influence on alpha (evenness) and beta (weighted unifrac) diversity.

Higher number of unique OTUs were observed in grassland compared to cropland (Figure 3.6 A). This may be due to cropland having lower soil pH (5.23) compared to grassland (5.90). Low soil pH in cropland may have been caused by continuous use of ammonium sulphate, an inorganic nitrogen fertilizer. Inorganic nitrogen fertilizer use lowers soil pH by enhancing oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) which produces hydrogen ions (H^+) (Garvin and Carver, 2003). Additionally, Msimbira and Smith (2020) found that the ideal pH for most soil microorganisms in agricultural soils was from 5.5 to 6.5, therefore their growth would be enhanced in grassland (pH 5.90) compared to cropland (pH 5.23). Furthermore, cropland management systems in Kamishihoro, that involved relatively stronger disturbances to the soils compared to the grasslands could be related to the decreased number of OTUs and microbial stability. Ovreas and Torsvik (1998) conducted research on the effect of soil management practices on microbial diversity and community structure in agricultural soils. They found that increased tillage intensity in cropland reduced soil microbial stability and diversity, in line with our results. Furthermore, a previous study (Atlas, 1984) reported that reduced bacterial stability was characterized by fewer soil microorganisms and low microbial stability, which was observed in cropland in our study. Our results suggest that management practices that directly or indirectly influence soil pH (such as fertilizer additions) and tillage influence the stability of microbial communities in agricultural soils.

The relative abundance of shared and unique OTUs in cropland and grassland were dominated by similar phyla. A similar trend was observed in seasonal treatments in cropland and grassland. The OTUs were dominated by microorganisms belonging to phyla *Proteobacteria*, *Planctomycetes*,

Chloroflexi, Actinobacteria and *Acidobacteria*. This may be explained in part by the phyla being identified as the dominant soil bacteria taxa in soil 16S rRNA studies (Janssen, 2006; Miyashita, 2015). Phyla *Proteobacteria, Planctomycetes, Choroflexi, Actinobacteria* and *Acidobacteria* dominating both shared unique OTUs in different treatments (land use and season) suggests that they may play important roles in shaping the soil microorganism's community structure in agricultural soils.

Considering shared microbes at phylum level based on land use (Figure 3.6 A), *Actinobacteria, Firmicutes* and *Gemmatimonadetes* were significantly ($p < .05$) higher in cropland compared to grassland (Figure S3.1A). The significantly ($p < .01$) higher N content of cropland (0.51%) compared to grassland soils (0.39%) may have caused the significant increase of *Actinobacteria, Firmicutes* and *Gemmatimonadetes* in cropland treatments (Table S3.1). In addition, their abundance had a positive correlation with N suggesting that their growth is closely associated with increased soil N content (Table S3). Other authors (Dai et al., 2018; Madigan et al., 2009) have previously reported increased relative abundance of *Actinobacteria, Firmicutes* and *Gemmatimonadetes* in N fertilized cropland soils. Our results may be due to *Actinobacteria, Firmicutes* and *Gemmatimonadetes* being classified as copiotrophic. Microorganisms classified as copiotrophic have relatively faster growth rates with research showing that they rapidly increase under N rich conditions in agricultural soils (Fierer et al., 2011; Ho et al., 2017). Of the shared microbes, the significantly ($p < .05$) higher relative abundance of *Euryarchaeota, Nitrospirae* and *WS3* in grassland compared to cropland (Figure S3.1A) may be attributed to higher pH in grassland (5.90) compared to cropland (5.23) (Table S3.1). Soil pH influences soil microbial growth and abundance by changing pH homeostasis or regulating soil nutrient availability (Zhalnina et al., 2014). Based on previous studies, *Euryarchaeota, Nitrospirae* and *WS3* were positively correlated and relatively more abundant as soil pH increased, in agreement with our research findings (Lauber et al., 2009; Rousk et al., 2010; Zhang et al., 2017). Additionally, the significantly ($p < .05$) higher relative abundance of *Bacteroidetes* in grassland may be attributed to the higher anaerobic digestate application of grasslands compared to croplands. In our study *Bacteroidetes* was identified as one of the main phyla in anaerobic digestate fertilizer (Figure S3.2). Jiang et al. (2019) and Walter et al. (2018) similarly identified that *Bacteroidetes* was one of the major phyla in sequenced anaerobic digestate fertilizer samples. Grassland management in the study site involved 4 times annual application of anaerobic digestate while croplands involved chemical fertilizer

application before seeding. We therefore infer that the high *Bacteroidetes* content of anaerobic digestate fertilizer may have increased the phylum abundance in grassland compared to cropland.

Our results showed that cropland management practices involving beet crop influenced the crop microbial community. Huang et al.(2019) and Cerecetto et al. (2021) conducted research on the effects of continuous tillage of beet crops on the soil microbial community. Their results showed that conventional tillage practices reduced some aspects of microbial diversity, in agreement with our findings. Nonetheless, a limitation of our study is that we considered only one crop (beet) although research (Lange et al., 2015; Yang et al., 2020; Ugrinovic et al., 2014) shows that different crops influence soil properties, microbial structure and diversity in distinct ways. Further studies are therefore needed to determine the influence of different crops on soil microbial diversity.

3.5.2 Effect of season on soil microbial community

Significantly ($p < .05$) higher evenness (Figure 3.2 B), richness (Figure 3.3 B) and diversity indices (Fig. 3.4 B and 3.5 B) were recorded in cropland-summer compared to cropland-spring. The same trend was observed in Venn diagrams (Figure 3.6 B) with more unique OTUs recorded in cropland-summer, than cropland-spring. Our findings may be attributed to higher rainfall experienced during cropland-summer compared to cropland-spring, that increased soil moisture content and subsequently microbial diversity. Soil moisture influences microbial structure by driving changes in soil temperature, pH, nutrient distribution, microbes cell metabolism and bacterial movements (Wang et al., 2014). Luo et al. (2019) conducted research on the effect of seasonal changes (summer, spring and autumn) on soil microbial diversity in agricultural soils. Based on their results, due to higher rainfall summer recorded higher diversity compared to spring in agreement with our findings.

In grassland, season did not have a significant ($p < .05$) effect on microbial diversity. These results suggest that grassland had a relatively stable microbial community that could withstand the effect of seasonal changes. Jangrid et al. (2008) conducted research on the impact of land use, management intensity and fertilization on soil microbial community structure in agricultural systems. Based on their results, the absence of seasonal changes on microbial diversity in grassland

could be due to increased bacterial stability which enabled microbes to withstand seasonal changes, in corroboration with our findings.

Our results indicate that cropland microbes have low stability, indicated by significant changes in diversity due to seasonal changes compared to grassland microbes. This implies that conventional tillage practices in Kamishihoro cropland (frequent tillage, chemical fertilizer use) may have negative effects on soil microbial stability.

3.5.3 Effect of season on shared phyla and predicted functions in cropland

Of the shared microorganisms in cropland seasonal treatments, *Crenarchaeota* were significantly ($p < .05$) higher in cropland-spring while *Actinobacteria* and *Cyanobacteria* were significantly ($p < .05$) higher in cropland-summer (Figure S3.2B). Higher relative abundance of *Crenarchaeota* in cropland-spring compared to cropland-summer may have been caused by differences in chemical properties between the two seasons. In our experiment cropland-spring had lower pH (5) and higher chemical properties (Mg-30.8, K-50) than cropland-summer (pH-5.46, Mg-22.85, K-36.8) (Table S3.2), which may have enhanced the relative abundance of *Crenarchaeota*. Additionally, based on the correlation analysis, *Crenarchaeota* were negatively correlated to soil pH and positively correlated to Mg and K (Table S3.3). Lehtovirta et al. (2009) conducted research on how soil pH (4.5 to 7.5) regulates abundance and diversity of *Crenarchaeota* in soils. Their results showed that *Crenarchaeota* abundance declined as pH increased from 4.5 to 7.5, in agreement with our findings. Furthermore, Hoshino et al. (2011) and Furtak and Galazka (2019) reported that soil chemical properties such as Mg and K were significant in driving changes in abundance of *Crenarchaeota* in agricultural soils, with increasing values having higher abundance of the phyla.

In our study, predicted functional analysis showed that *amoA* and *nirK* gene was significantly ($p < .05$) higher in cropland-spring compared to cropland-summer (Figure 3.7 A). Nicol and Schleper (2006) reported that *Crenarchaeota* contained genes related to *amoA.* Additionally, Mardanov et al. (2012) found that cultured and uncultured strains of *Crenarchaeota* contained the *nirK* gene which is associated with denitrification process of the nitrogen cycle. We therefore suggest that

the significantly higher relative abundance of *Crenarchaeota* in cropland-spring compared to cropland-summer may have led to higher expression of *amoA* and *nirK* gene in cropland-spring.

The significantly ($p < .05$) higher relative abundance of *Actinobacteria* in cropland-summer may have been caused by higher pH observed in cropland-summer (5.46) compared to cropland-spring (5) (Table S3.2). This was further supported by the positive correlation between *Actinobacteria* and soil pH (Table S3.3). Wang et al. (2019) conducted research on the effect of soil pH (4.5 to 8.5) on the distribution and functions of microorganisms in farmlands in northeastern china. They reported that the relative abundance of *Actinobacteria* was positively correlated to soil pH with higher relative abundance observed as pH increased, in agreement with our study. Additionally, the significantly ($p < .05$) higher relative abundance of *Cyanobacteria* in cropland-summer may be attributed to higher rainfall experienced in cropland-summer compared to cropland-spring. Similar findings have been reported by Moreno-Espíndola et al. (2018) who found that *Cyanobacteria* are positively correlated to increased rainfall, which would be enhanced during summer in our study.

Predicted functional analysis of our study showed that *nifH* was significantly ($p < .05$) higher in cropland-summer compared to cropland-spring (Figure 3.7 A). Reports by Gtari et al. (2011) and Calderoli et al. (2017) identified that *Actinobacteria* and *Cyanobacteria* play functions related to nitrogen fixation through *nifH* gene expression. We therefore suggest that the significantly higher presence of members of *Actinobacteria* and *Cyanobacteria* in cropland-summer lead to higher expression of the *nifH* gene and subsequent functions related to nitrogen fixation in cropland-summer treatments.

Soil pH, Mg and K were significantly ($p < .05$) correlated to differences in soil microbial communities between seasons in cropland (Figure 3.7A). Our results corroborate those of Wessen et al. (2010) and Wang et al. (2019) who reported that pH was one of the primary factors determining soil nutrient availability and therefore the distribution of soil microorganisms in agricultural soils. Additionally, Nicolitch et al. (2019) reported that base cations such as Mg and K had a significant effect on soil microbial distribution. More specifically, Mg and K have been found to improve the soil base saturation and increase nutrient availability, affecting microbial abundance and diversity in agricultural soils (Kreutzer, 1995; Rosberg et al., 2006).

In our study the significant changes in the relative abundance of specific microbes in seasonal treatments was linked to predicted soil functions in cropland. This indicates the importance of cropland seasonal treatments and their associated changes in soil physical and chemical properties, in relation to driving changes in soil microbial diversity, relative abundance and related soil functions. A limitation of our study is it was conducted for only two seasons i.e summer and spring, although the influence of land use has been shown to change in all seasons in various long term research experiments (Li et al., 2019; Sengupta et al., 2020). Therefore, continuous studies to monitor and clarify the effect of land use on soil microbes in different seasons over time should be conducted in Kamishihoro.

3.5.4 Effect of season on shared phyla and predicted functions in grassland

Overall, the season had relatively minor impact on soil microbes in grasslands, when compared to those in croplands, but phylum *Bacteriodetes* was significantly ($p < .05$) higher in grassland-spring than grassland-summer (Figure S3.1C). This may have been due to lower BD during grassland-spring (0.66) compared to grassland-summer (0.77) (Table S3.2). Although zero tillage was conducted in grasslands, tractors were used to apply anaerobic digestate once during spring (April) and the application repeated during summer (August). The higher BD of soils in grassland-summer compared to grassland-spring can therefore be related to soil compaction due to the use of the tractor twice for summer samples compared to once for spring samples. Alaoui et al.(2018) reported that the increased use of farm machinery like tractors contributed to increased soil compaction and BD, in agreement with our findings. In addition, *Bacteriodetes* were negatively correlated to BD further supporting the findings (Table S3.3). Our results are in line with Guo et al. (2019) whose research on the linkage between soil nutrients and microbial characteristics found that the abundance of *Bacteriodetes* were negatively correlated to soil BD. *Bacteriodetes* contain the nitrogen fixing gene *nifH* and subsequently play important roles in agricultural nitrogen fixation (Inoue et al., 2015). In our study predicted functional analysis showed that *nifH* was significantly ($p < .05$) higher in grassland-spring compared to grassland-summer (Figure3.7). We therefore infer that the significantly higher abundance of *Bacteriodetes* in grassland-spring may have resulted in higher expression of *nifH* gene. In our study K and BD were significantly ($p < .05$) correlated to microbial distribution in grassland season treatments (Figure 3.8 B). Pan et al. (2014) similarly found that K was one of the nutrients that had strong correlations with bacterial

abundance in grassland soils. Additionally, Lee et al. (1996) and Li et al. (2002) reported soil BD to be one of the factors that influenced soil microbial populations, with increase in BD accompanied with a decline in bacterial abundance in agricultural soils. Our results suggest that both soil physical (BD) and chemical (K) properties are significant in causing changes in grassland microorganisms community structure in Kamishihoro grasslands.

3.6 Conclusion

Land use (grasslands and croplands) and season (summer and spring) significantly affected the soil microbial community in Kamishihoro region in Japan. Grassland soil microorganisms were resistant to seasonal changes compared to cropland. This was demonstrated by the significant changes in alpha and beta diversity observed in the cropland due to seasonal fluctuations. Conventional tillage practices in cropland may reduce microbial stability. Additionally, the effect of season on diversity varied based on land use with spring having higher values in grassland and summer in cropland. The relative abundance of both shared and unique OTUs in land use and seasonal treatments were dominated by similar microorganisms belonging to *Proteobacteria, Planctomycetes, Actinobacteria* and *Acidobacteria*. This suggests that these phyla play important roles in shaping regional core microbial community in the studied agricultural soils. Of the shared microorganisms, significantly higher soil microorganisms in cropland and grassland seasonal treatments seemed to influence relative abundance of predicted functions relating to the nitrogen cycle (*nifH, nirK, amoA*). These results show the importance of seasonal changes on soil microorganisms and the resultant provision of related soil functions within the agricultural environment. The study should provide more insight on the effect of combined effects of land use and seasonal treatments on specific microorganisms within the agricultural system at a regional level. Additionally, the study should contribute towards development of sustainable best management practices within the agricultural environment, that will ensure the continued provision of microbial ecosystem services.

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Supplementary

Table S3.1: The significant effects of land use on soil physical and chemical properties. Asterisks indicate a significant effect of each treatment (*** 0.001, ** 0.01, * 0.05) and “ns” shows “no significance” based on ANOVA.

	Land use		
	Cropland	Grassland	*p*
pH	5.23±0.43	5.90±0.30	***
N (%)	0.51±0.04	0.39±0.02	***
K	43.71±8.74	9.23±1.37	***
Mg	26.83±5.98	28.14±4.62	ns
CEC	36.78±3.25	30.50±2.11	***
BD (g/cm^2)	0.73±0.08	0.70±0.57	ns
Humus	12.94±1.76	9.42±0.58	***

Table S3.2: The significant effects of season on soil chemical properties in cropland and grassland. Asterisks indicate a significant effect of each treatment (*** 0.001, ** 0.01, * 0.05) and "ns" shows "no significance" based on ANOVA.

	Cropland			Grassland		
	Summer	Spring	*p*	Summer	Spring	*p*
pH	5.46±0.31	5.00±0.42	**	5.80±0.20	5.98±0.36	ns
N (%)	0.52±0.01	0.50±0.03	ns	0.40±0.02	0.39±0.02	ns
K	36.80±3.41	50.63±6.61	***	9.65±0.94	8.80±1.63	ns
Mg	22.83±3.38	30.83±5.34	***	28.35±1.16	27.98±4.58	ns
CEC	37.03±3.60	36.53±3.00	ns	31.70±2.41	29.30±0.61	*
BD (g/cm^2)	0.73±0.09	0.73±0.07	ns	0.75±0.03	0.66±0.03	***
Humus	13.00±1.59	12.87±1.99	ns	9.79±0.40	9.05±0.49	***

Table S3.3: Correlation analysis (spearman) among soil properties (pH, bulk density-BD, nitrogen-N, potassium-K, magnesium-Mg, cation exchange capacity-CEC, calcium-Ca and humus) and selected phyla based on land use (cropland and grassland) and season treatments. Selected microbes showed significant increase in each treatment. Red color shows significant ($p < .05$) interactions.

Cropland		pH	N	Mg	K	Hu	CEC	Ca	BD
Actinobacteria	rho	-0.330	0.363	-0.176	0.382	0.397	0.372	-0.305	0.085
	p	0.005	0.002	0.140	0.001	0.001	0.001	0.009	0.475
Firmicutes	rho	-0.159	0.107	-0.148	0.286	0.170	0.175	-0.194	0.096
	p	0.183	0.373	0.215	0.015	0.154	0.142	0.102	0.425
Gemmatimonadetes	rho	-0.391	0.376	-0.125	0.492	0.421	0.438	-0.297	0.142
	p	0.001	0.001	0.294	0.001	0.001	0.001	0.011	0.236
Cropland season		pH	N	Mg	K	Hu	CEC	Ca	BD
Actinobacteria	rho	0.118	0.118	0.118	0.118	0.118	0.118	0.118	-0.039
	p	0.641	0.641	0.641	0.641	0.641	0.641	0.641	0.877
Crenarchaeota	rho	-0.131	-0.066	0.131	0.131	-0.066	-0.066	0.066	-0.066
	p	0.604	0.796	0.604	0.604	0.796	0.796	0.796	0.796
Cyanobacteria	rho	-0.177	-0.177	-0.177	-0.177	-0.177	-0.177	-0.177	0.413
	p	0.482	0.482	0.482	0.482	0.482	0.482	0.482	0.088
Grassland		pH	N	Mg	K	Hu	CEC	Ca	BD
Nitrospirae	rho	0.505	-0.473	0.093	-0.598	-0.524	-0.465	0.305	-0.290
	p	0.001	0.001	0.437	0.001	0.001	0.001	0.009	0.014
WS3	rho	0.438	-0.372	0.184	-0.444	-0.438	-0.368	0.338	-0.287
	p	0.001	0.001	0.121	0.001	0.001	0.001	0.004	0.014
Euryarchaeota	rho	0.389	-0.341	0.199	-0.447	-0.396	-0.339	0.343	-0.195
	p	0.001	0.003	0.093	0.001	0.001	0.004	0.003	0.100
Bacteroidetes	rho	-0.098	0.199	-0.143	0.098	0.201	0.207	-0.131	-0.061
	p	0.412	0.094	0.229	0.413	0.091	0.081	0.273	0.613
Grassland-season		pH	N	Mg	K	Hu	CEC	Ca	BD
Bacteroidetes	rho	-0.178	0.210	-0.231	-0.122	0.182	0.173	-0.240	-0.235

	p	0.298	0.219	0.176	0.477	0.287	0.313	0.159	0.168

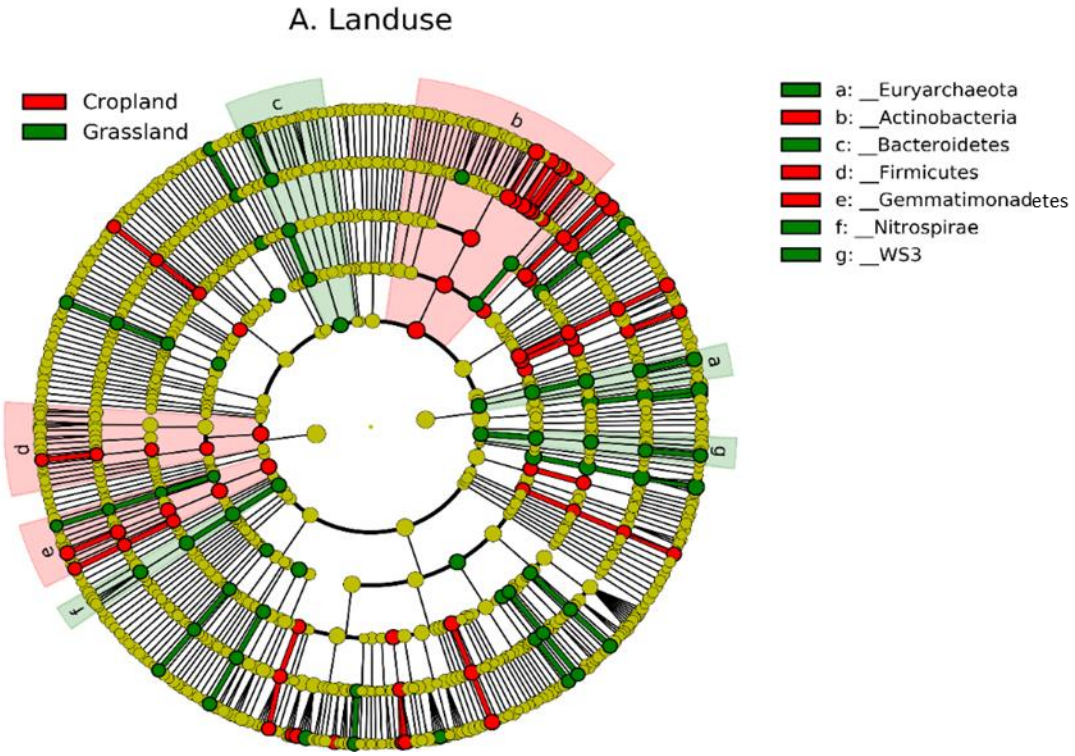


Figure S 3.0.1A: Cladogram showing significantly abundant taxa of shared OTUs. Taxa with significantly different abundance are represented by red and green dots. From the center outwards each ring represents kingdom, phylum, class, order, family and genus levels. The colored shadows represent trends of significant ($p < .05$) taxa based on Kruskal–Wallis test.

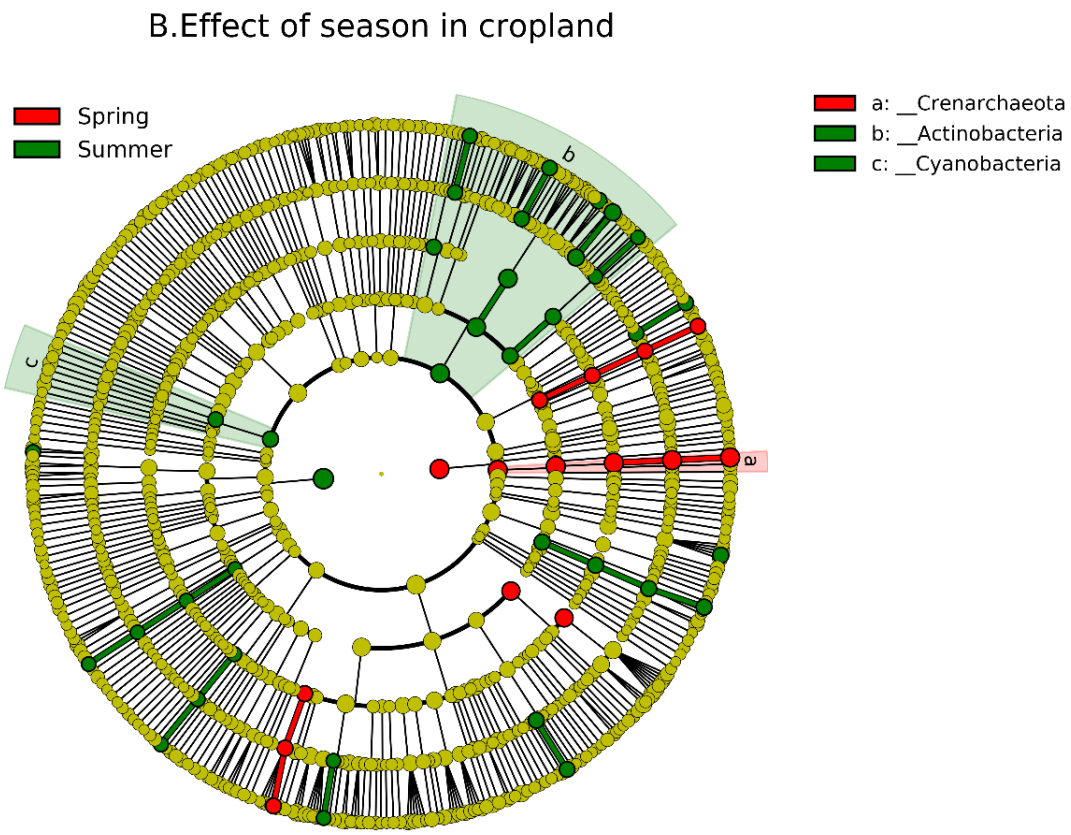


Figure S3.1B: Cladogram showing significantly abundant taxa of shared OTUs based on season treatment in cropland. Taxa with significantly different abundance are represented by red and green dots. From the center outwards each ring represents kingdom, phylum, class, order, family and genus levels. The colored shadows represent trends of significant ($p < .05$) taxa based on Kruskal–Wallis test.

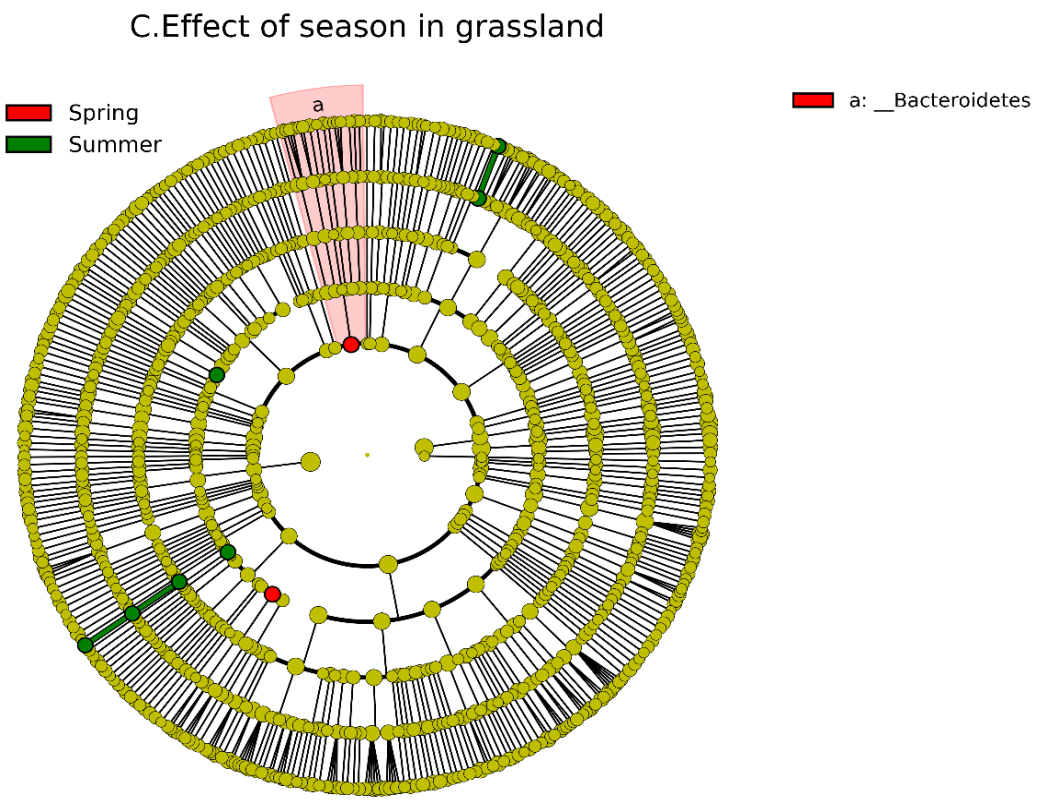


Figure S3.1C: Cladogram showing significantly abundant taxa of shared OTUs based on season treatment in cropland. Taxa with significantly different abundance are represented by red and green dots. From the center outwards each ring represents kingdom, phylum, class, order, family and genus levels. The colored shadows represent trends of significant ($p < .05$) taxa based on Kruskal–Wallis test.

Chapter 4 Liming improves the stability of soil microbial community structures against the application of anaerobic digestate made from dairy wastes

4.1 Abstract

Lime is used to reduce soil acidification in agricultural soils. However, its effects on the soil microbial community are not well understood. Additionally, the soil microbial community is known to be influenced by organic fertilizers. However, the question still remains whether liming influences the magnitude of organic fertilizers impact on soil microbial communities. Therefore, an incubation experiment was performed to understand the effect of lime application (pH = 6.5 and 5.5 for the soils with and without lime, respectively) and fertilizer (digestate, urea and control) on the soil microbial community structures, stability and gene functions. Soils were sampled weekly after the application of fertilizers for a month. For microbial community analysis, DNA was extracted and sequenced targeting 16S rRNA region. For gene abundances i.e 16S rRNA, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), nitrous oxide reductase (*nosZ*) and nitrite reductase (*nirS*) quantitative PCR was conducted. In result, the relative abundance of *Actinobacteria* was influenced more strongly by digestate in lime soils, while *Alphaproteobacteria* was influenced more strongly by digestate in the no lime soil. In no lime treatments, digestate had a significant effect on more operational taxonomic units (146) compared to lime (127), indicating that lime application increased soil microbial community's stability. Liming and fertilizer had a significant effect on 16S rRNA gene copy numbers with the highest values observed in lime plus digestate treatments. Soil pH had a significant on AOA, *nosZ* and *nirS* gene copy numbers with the highest values observed in lime treatments. In the lime treatments digestate application had a positive impact on AOB gene copy numbers but this was not the case for soils without liming treatments. These results indicate that soil pH and fertilizer type should be taken into consideration for the management of functional gene abundance in agricultural soils.

4.2 Introduction

About 50% of agricultural areas globally are affected by soil acidity (Von Uexküll and Mutert, 1995). Within the agricultural environment, soil acidification is a natural process that occurs constantly (Goulding, 2016). However, the process can be enhanced or reduced based on farmer management practices (Kunhikrishnan et al., 2016). Application of ammonium-based fertilizers such as urea is one of the management practices that significantly enhances soil acidification (Goulding, 2016; Yang et al., 2012). High levels of acidity negatively affect soil nutrient immobilization and mineralization and (Fageria and Baligar, 2008). Additionally, acidification directly and indirectly causes shifts in microbial community composition and related soil functions (Pang et al., 2019). To reduce the negative effects of soil acidification, there have been considerable efforts towards identifying effective soil management practices that can reduce acidity in agricultural soils.

Liming has been identified as one of the most effective agricultural management practices that can reduce the negative effects of soil acidity (Caires et al., 2010; Chatzistathis et al., 2015; Kunhikrishnan et al., 2016; Mkhonza et al., 2020). Liming directly reduces acidity by neutralizing excessive hydrogen ions in the soil (Bolan et al., 2003). Research shows that reduced soil acidification through liming increases the availability of essential soil nutrients such as nitrogen among others (Fageria and Baligar, 2008; Mkhonza et al., 2020; Daniel et al., 2013, Pang et al., 2019). Changes in soil pH and nutrient availability due to liming are usually accompanied by shifts in the soil microbial community structure (Dai et al., 2018; Zhalnina et al., 2014).

Through its influence on soil pH and nutrient availability, lime application directly or indirectly influences the soil microbial community structure (Xue et al., 2010). Studies researching the effect of lime application on soil microbes are vital because soil microorganisms are significant to the maintenance of soil health by providing ecosystem services such as nitrogen cycling (Singh et al., 2016). Research by Xun et al. (2010) and Narendrula-Kotha et al. (2017) on the response of soil microbial community to changes in soil pH determined that liming regulated the soil microbial community, by enhancing the growth of neutrophilic soil microbes. However, there are contrasting results on the effect of liming on specific soil microbes. For instance, Xun et al. (2010) reported that short term liming enhanced the growth of *Alphaproteobacteria* and *Bacteriodetes* while decreasing the growth of *Actinobacteria* in agricultural soils. However, Narendrula-Kotha et al.

(2017) reported that the relative abundance of *Actinobacteria* and *Proteobacteria* were enhanced with lime application, while *Chloroflexi* and *Firmicutes* were reduced. Nevertheless, there are few studies researching the effect of lime application on the soil microbial community. Most of the available research on lime is focused on the effects of its application on soil pH, nutrients and crop yields (Cifu et al., 2004; Hollad et al., 2019; Li et al., 2018). Therefore, research needs to be conducted to clarify the effect of lime application on the soil microbial community in agricultural soils.

Increased soil pH by liming directly and indirectly affects the soil nitrogen transformation processes (Guo et al., 2019; Zhang et al., 2016). Considering that nitrogen is one of the nutrients that limits agricultural crop production, the effect of pH on its transformation should be studied (Hofstra and Bouwman, 2005). Through its influence on the nitrifier (AOB and AOA) and denitrifier (*nosZ* and *nirS*) community, liming affects soil inorganic nitrogen content and atmospheric nitrogen losses through N_2O gas emissions (Jha et al., 2020; Liu et al., 2010). The first step of nitrification is catalyzed by AOA and AOB (Nunes-Alves, 2015). Studies on the ecological niches of AOA and AOB have discovered that soil pH is one of the major factors that influences niche separation of the two genes (Schleper, 2010; Xu et al., 2017). In addition, lime influences denitrification (*nirS* and *nosZ*) gene abundance within the agricultural environment. Jha et al. (2020) reported that lime application increased the abundance of *nirS* gene copy numbers in agricultural soils. Furthermore, lime application has the potential to decrease soil N_2O emissions, through its influence on the denitrification *nosZ* gene (Clough et al., 2003; Zaman et al., 2007). However, due to variable findings (Huang et al., 2011; Jia and Conrad, 2009; Sun et al., 2015) and experimental setup differences among various studies, our understanding of the effect of liming on nitrifier and denirifier gene abundance is still limited.

Potential changes induced by lime on soil microbes and related functional genes are dependent on the microbial community stability. One of the main aspects of microbial stability is resistance, which is described as the ability of soil microbes to withstand a disturbance over a specific period (Fan et al., 2013; Shade et al., 2012; Ziegler et al., 2017). The soil microbial community composition is one of the factors that may affect stability within the agricultural environment. Microbial communities dominated by oligotrophs, which are characterized by low growth rates and high resource use activity, increase microbial community stability compared to copiotroph

dominated communities (Fierer et al., 2006). Another factor that may affect microbial stability is diversity. Specifically, high microbial diversity has been associated with increased ecosystems stability compared to low diversity (McCann, 2000). Additionally, the soil nutrient content effects microbial stability. Management practices that increase soil nutrient content have been found to enhance microbial stability (De Vries and Shade, 2013). Previous research shows that lime application causes changes in soil microbial community structure, diversity and soil nutrient content (Pang et al., 2019; Wang et al., 2019). Therefore, soil lime additions are expected to influence soil microbial stability. Despite this, there is limited research investigating the specific effects of lime application on soil microbial stability. More information is therefore required to understand the effect of liming on soil microbial stability and how that relates to soil microbe's response to disturbances such as fertilizer additions.

Thus, this study aimed to investigate 1) the interaction between the effects of liming and fertilizer (digestate or urea) application on soil microbial community structures, and 2) the impacts of the interaction on some of the microbial functions, including nitrification and denitrification in soils. Also, we hypothesized that adding lime to soils before digestate application would reduce shifts of the soil microbial community after digestate application.

4.3 Materials and methods

4.3.1 Soil sampling and treatments

Soils used in this research were sampled from fields located in Obihiro, Hokkaido, Japan (42° 45'01.9"N, 143° 08'20.4"E). Soils in the region are classified as Andosols (FAO/UNESCO). The sampled soils were sieved using a 4 mm mesh before incubation. Characterization of the initial soil chemical properties; pH, Phosphorous oxide (P_2O_5), Potassium oxide (K_2O), Nitrogen (N) and Carbon (C) (Table S1) was conducted by Tokachi Federation of Agricultural Cooperatives.

The experimental design was a completely randomized design with two levels of soil pH 5.5 without lime (NL) and 6.5 with lime (L) and three types of fertilizer inputs (digestate, urea and control), with three replicates. For lime treated soils, calcium carbonate ($CaCO_3$) was applied at the rate of 2 g $CaCO_3$ kg^{-1} dry soil. Two fertilizer inputs were applied as urea and digestate at the rate of 50 kg NH_4^+ ha^{-1} and no fertilizers as the control. Digestate was provided by Hokkaido

biogas plant (Tokachi, Japan). The digestate was produced from dairy cow manure and their bedding materials (wood straw, wood chip). Characterization of digestate chemical properties; pH, P_2O_5, K_2O, N, ammonium (NH_4^+) and nitrates (NO_3^-) (Table S1) was conducted by Tokachi Federation of Agricultural Cooperatives.

4.3.2 Incubation setup

For the incubation, 50 g of air-dried soil was placed in each plastic cup (6 cm of diameter and 5 cm of depth). The water filled pore space was maintained at 60 % using milli Q and bulk density established at 1 g cm^{-3}. The plastic cups were covered with lids which had 6 holes to prevent contamination and excess evaporation. For lime treatments, $CaCO_3$ was added, and soils incubated for three months to stabilize the pH at 6.5. Fertilizers were applied to the soils after stabilization. Each treatment was replicated 3 times, totalling 72 soil cores.

4 3.3 Soil sampling during the incubation experiments, chemical and microbial analysis

Soils were destructively sampled per week for soil chemical (pH and inorganic nitrogen) and DNA analysis. To determine soil pH, 5 g of fresh soils were mixed with milli Q in 1:5 ration (5:10) and shaken for 30 minutes. The soil pH was then determined using a pH meter (AS800, ASONE Co., Osaka, Japan).

To measure concentration of soil inorganic nitrogen (NH_4^+ and NO_3^-), 5 g of fresh soils were mixed with 25 ml of 2 M KCl, solution and shaken for 30 minutes. The solution was filtered with 1 µl filter paper (Toyo Roshi Kaisha No. 5C filter paper, Tokyo Roshi Kaisha, Ltd., Tokyo, Japan). Colorimetric measurements for inorganic nitrogen were determined using a flow injection analyzer using the filtrate (AQLA-700, Aqualab Co., Ltd., Tokyo, Japan).

4.3.4 Soil DNA extraction

Soil DNA was extracted from 0.5 g soil using the NucleoSpin ® Soil (Takara Bio Inc, Shiga, Japan) according to the manufacturer's instructions. Skim milk (0.2 g) was used to reduce DNA attachment to Andosol (Hoshino and Matsumoto, 2005). The extracted DNA was purified using Agencourt AM Pure XP Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufactures protocol. The purified DNA was stored at −30°C until further analysis.

4.3.5 16S rRNA gene amplicon library preparation and sequencing

Extracted soil DNA samples were diluted 10 times before the Polymerase Chain Reaction (PCR) targeting 16S rRNA gene using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5' GGACTACHVGGGTWTCTAAT-3'). Each PCR sample contained 10 µl of Amplitaq Gold® 360 Master Mix (Applied Biosystems™, Carlsbad, USA) DNA polymerase, 0.4 of forward primer-F515, 0.4 µl of reverse primer-R806, 8.2 µl of nuclease free water and 1 µl of DNA template, making up a final volume of 20 µl. PCR was conducted under the following conditions: 95°C for 600 sec and thereafter 30 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec with a final extension of 72°C for 60 sec. Amplicon purification was conducted using Agencourt AM Pure XP Kit in line with manufacturers protocol. Second PCR was thereafter conducted to attach Ion P1 adaptor (Ion Torrent, Life Technologies), specific to each sample. Second PCR samples each contained 10 µl of Amplitaq Gold® 360 Master Mix DNA polymerase, 7.2 µl of nuclease free water, 0.4 µl of reverse primer (P1-R806), 0.4 µl of forward primer (Ion A-Barcode) and 2 µl of DNA template, making up a final volume of 20 µl. The PCR was conducted under the same conditions as above but with 5 cycles. Bioanalyzer high sensitivity DNA Kit (Agilent Technologies, Palo Alto, CA, USA) was used to determine final library concentration and length, based on manufacturer's protocol. Libraries were then diluted to 50 pM using Low TE (Tris-EDTA, 19 mM Tris base, 0.1 mM EDTA, Ion Torrent, Life Technologies). Sequencing was done with Next Generation Sequencer (Ion PGM, Life Technologies). Ion Chef was used to load libraries to Ion 318 chip (Ion Torrent, Life Technologies) and thereafter sequencing performed with the Ion PGM™ sequencer (Thermo Fisher Scientific K.K., Japan) using Ion PGM™ Hi-Q™ view sequencing kit.

4.3.6 Sequence processing and data analysis

Raw sequenced data was processed using QIIME 2 software package version 2019.7 (Caporaso et al., 2010). DADA2 was used to cut primer sequences, denoise and select representative sequences. Classify-sklearn classifier, which uses naïve brayes classifier to set taxonomy, was used to set representative sequences. Clustering of operational taxonomic units (OTUs) at 99% identity was conducted based on Greengenes 13_8 database.

4 3.7 Quantitative Polymerase Chain Reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, Hercules, USA).

For 16S rRNA, *nosZ* and *nirS* each sample contained 10.4 µl of KAPA SYBR Fast qPCR kit (Agilent Technologies, Palo Alto, CA, USA), 0.8 µl of forward and reverse primers, 7.0 µl of nuclease free water and 1 µl of DNA template, making up a final volume of 20 µl. For AOA and AOB each sample contained 12.5 µl of KAPA SYBR Fast qPCR kit (Agilent Technologies, Palo Alto, CA, USA), 0.5 µl of forward and reverse primers, 9.5 µl of nuclease free water and 2 µl of DNA template, making up a final volume of 25 µl.

The qPCR was conducted with primers and cycling conditions as described in Table 4.1.

Table 4.1: Primer sequences and qPCR conditions used for the study

Target	Primer names and sequences	qPCR condition
16S rRNA (Caporaso et al., 2011)	515F (5'GTGCCAGCMGCCGCGGTAA3') 806R (5'GGACTACHVGGGTWTCTAAT-3')	95°C (600 sec), [30 cycles 95°C (30 sec), 57°C (30 sec),72°C (60 secs)]
nosZ (Kloos et al., 2001)	nosZ2F (CGCRACGGCAASAAGGTSMSSGT) nosZ2R (CAKRTGCAKSGCRTGGCAGAA)	95°C (600 sec), [40 cycles 95°C (15 sec), 60°C (30 sec), 72°C (60 secs)]
nirS (Braker et al., 1988)	nirS1F 5'-CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T NirS3R GCCGCCGTC(A/G)TG(A/C/G)AGGAA	95°C (600 sec), [40 cycles 95°C (15 sec), 60°C (30 sec), 72°C (60 secs)]
AOA (Meinhardt et al., 2015)	nosZ2R (CAKRTGCAKSGCRTGGCAGAA) GenAOAR (CCAAGCGGCCATCCAGCTGTATGTCC)	96°C (180 sec), [40 cycles 95°C (30 sec), 65°C (60 sec, 72°C (60 sec)]
AOB (Meinhardt et al., 2015)	1Fmod (CTGGGGTTTCTACTGGTGGTC) GenAOBR (GCAGTGATCATCCAGTTGC)	95°C (600 sec), [40 cycles 95°C (30 sec), 58°C (60 sec), 72°C (60 secs)]

4.3.8 Analysis of bacterial community composition

Permutational multivariate analysis of variance (PERMANOVA), alpha (Pielou evenness, OTU counts, Shannon) and beta (unweighted unifrac) diversity analysis was conducted in QIIME2. The "DESeq" function in R software (R Foundation for Statistical Computing, Vienna, Austria) was used to determine significant changes in OTUs based on different fertilizer treatments. Enhanced volcanoe package was used to visualize the changes in OTUs using volcanoe plots (Blighe et al., 2020). To determine the effect of fertilizers on microbial relative occurrence, ternary plot analysis was conducted using ggtern package (Hamilton and Ferry, 2018).

4.3.9 Statistical analysis

All statistical analyses were performed using R software. In all analysis, $p < 0.05$ were considered significant. For soil pH, inorganic nitrogen and gene abundance analysis, two-way and three-way analysis of variance (ANOVA) was performed to determine the effect of pH, fertilizers and sampling day. For significant interactions, Tukey's HSD test was performed.

4.4 Results

4.4.1 Beta Diversity

Principal Coordinate Analysis (PCoA) based on unweighted UniFrac distances showed that samples from the same pH and fertilizer treatments had significant ($p < 0.001$) distinct clustering between treatments (Figure 4.1). For fertilizer, digestate had its own cluster while urea and control treatments clustered together in both L and NL treatments. Therefore, samples from the same pH and fertilizer (digestate) treatments shared a high degree of similarity. Variability between fertilizer treatments was higher in NL compared to L treatments.

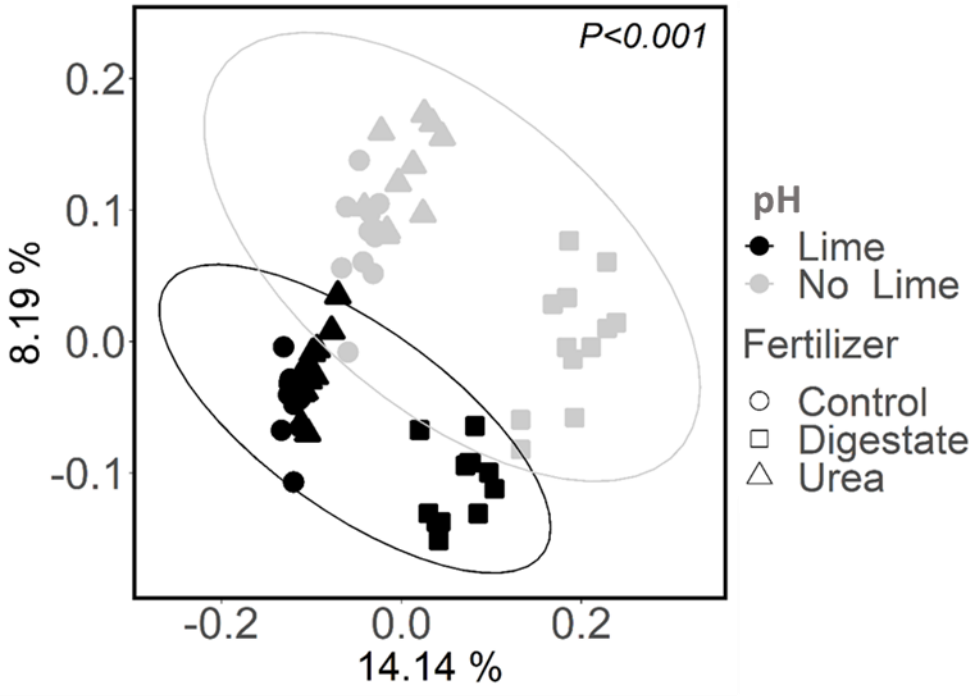


Figure 4.1: Principal coordinate analysis (PCoA) by unweighted unifrac based on soil pH and fertilizer treatments. Axis represent the two dimensions explaining greatest proportion of variances in microbial communities. *p* values calculated based on permutational multivariate analysis of variance.

4.4.2 Changes in OTU distribution and stability

Because our PCoA (Figure 4.1) analyses showed clear separation between the digestate treatment and other two treatments (control and urea referred to as “mix” treatment), volcano plots were

made to identify the OTUs significantly influenced by the digestate treatment, when compared to mix treatments.

Digestate significantly increased more OTUs (146 OTUs) in NL compared to L treatments (127 OTUs) (Figure 4.2). Considering the 127 OTUs that significantly increased due to digestate application in L treatments, most OTUs belonged to families *Sphingomonadaceae* (17 OTUs), *Xanthomonadaceae* (8 OTUs) and *Chthoniobacteraceae* (6 OTUs) (Figure 4.2 A). At phylum level most OTUs that were significantly increased with digestate application in L treatments belonged to *Proteobacteria* (52 OTUs), *Actinobacteria* (19 OTUs) and *Bacteroidetes* (10 OTUs) (Figure S4.1A). Furthermore, majority of OTUs that were significantly decreased in L treatments due to digestate application belonged to phyla *Acidobacteria* (9 OTUs), *Actinobacteria* (3 OTUs) *Euryarchaeota* (3 OTUs) and *Proteobacteria* (3 OTUs).

Considering the 146 OTUs that significantly increased due to digestate application in NL treatments, most OTUs belonged to families *Sphingomonadaceae* (17 OTUs), *Caulobacteraceae* (9 OTUs) and *Chitinophagaceae* (9 OTUs) (Figure 4.2 B). At phylum level majority of the OTUs that were significantly increased with digestate application in NL treatments belonged to *Proteobacteria* (65 OTUs), *Bacteroidetes* (19 OTUs) and *Actinobacteria* (15 OTUs) (Figure S4.1B). Additionally, majority of OTUs that were significantly decreased in NL digestate treatments belonged to phyla *Acidobacteria* (14 OTUs), *Actinobacteria* (4 OTUs) and *Planctomycetes* (4 OTUs).

Furthermore, we considered the overall effect of soil pH on OTU stability during the experimental period. In NL treatments more (121) OTUs significantly increased compared to L (96) treatments (Figure S4.2). The 121 OTUs that significantly increased in NL treatments, most belonged to families *Gaiellaceae* (15 OTUs) and *Sphingomonadaceae* (12 OTUs). At phylum level most of these OTUs belonged to *Actinobacteria* (40 OTUs), *Proteobacteria* (36 OTUs) and *Gemmatimonadetes* (10 OTUs). The 96 OTUs that significantly increased in L treatments, most belonged to families *0319-6A21* (6 OTUs), *Pirellulaceae* (3 OTUs) and *Gemmataceae* (3 OTUs). At phylum level most of these OTUs belonged to *Acidobacteria* (32 OTUs), *Actinobacteria* (13 OTUs) and *Chloroflexi* (11 OTUs).

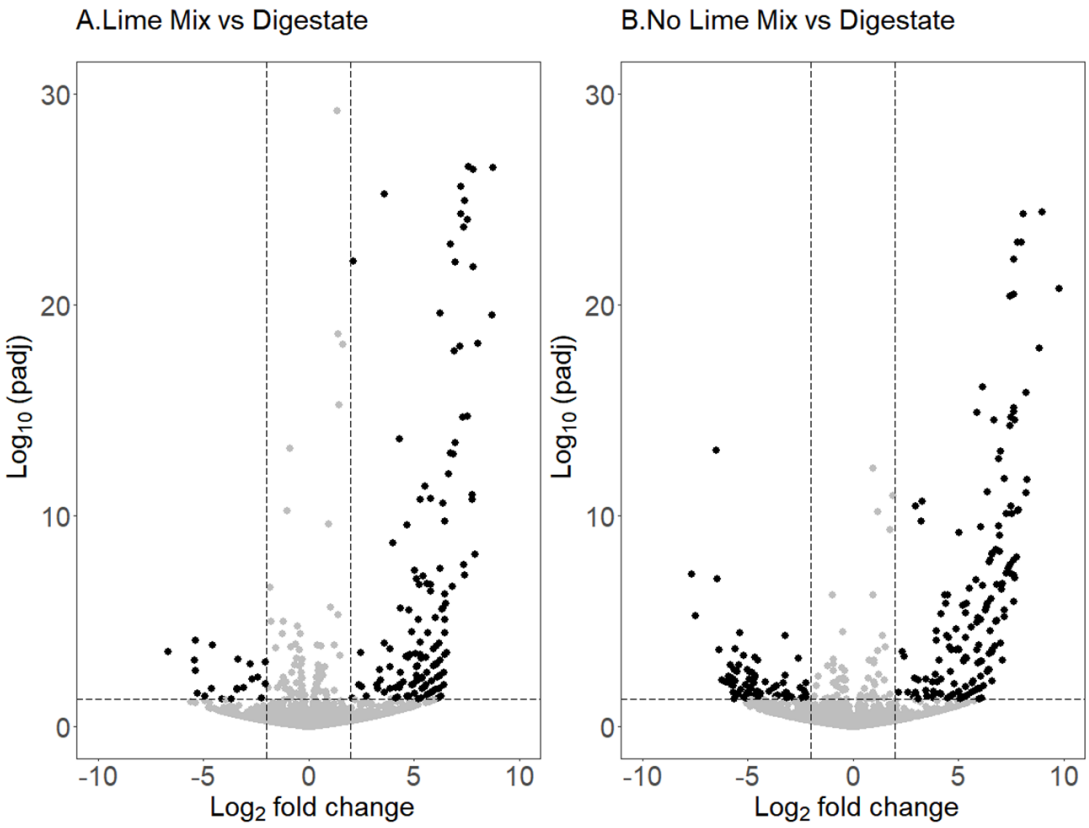


Figure 4.2: Volcano plots showing the distribution of OTUs abundance according to the adjusted *p* value (−log10 scale) and the log twofold change comparing mix versus digestate. Black nodes in the right side represent OTUs significantly more abundant in the digestate treatments while black one in the left side represent to OTUs more abundant in mix treatments.

4.4.3 Class level microbes that were influenced by fertilizer application based on their abundance

To identify shifts in microbial community abundance due to fertilizer application, ternary plots were prepared (Figure 4.3). Considering OTUs in the top 10 abundant taxa at class level, *Alphaproteobacteria* and *Actinobacteria* were the more abundant in digestate in both L and NL treatments. *Alphaproteobacteria* were more influenced by digestate in NL treatments while *Actinobacteria* were more influenced in L treatments. In NL treatments, the OTUs that were more abundant in urea belonged to *Planctomycetia* and *Gemmatimonadetes*.

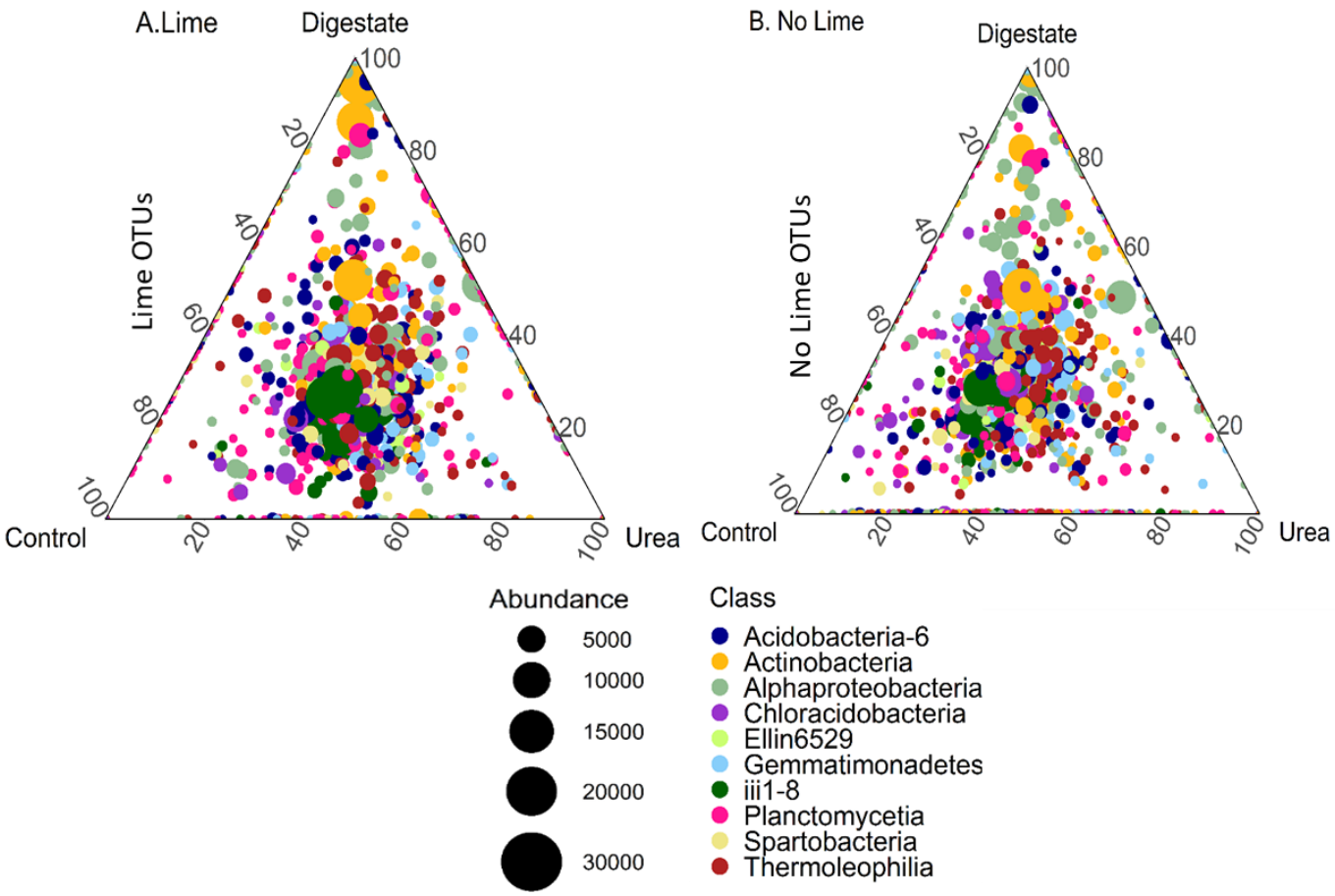


Figure 4.3:Ternary plots showing the relative occurrence of OTUs belonging to the top 10 class level microbes. Axes represent fertilizers (digestate, urea, control) treatments in lime and no lime treatments. Points closer to corners indicate that a great proportion of the class total relative abundance found in the fertilizer treatment.

4.4.4 Alpha Diversity

Diversity indices for the bacterial community structures were also influenced by treatments. Regarding Pielou evenness, overall soil pH and sampling day (D) had a significant ($p < 0.05$) effect with the highest values observed in L and D14, respectively (Figure 4.4 A and B). Considering digestate treatments, the interaction of pH and sampling day had a significant ($p < 0.05$) effect with the highest values observed in L treatments on D28. For urea treatments, sampling day had a significant ($p < 0.05$) effect with the highest values observed on D21. On D7, fertilizer had a significant ($p < 0.01$) effect with the highest values observed in L treatments. On D28 the

interaction of soil pH and fertilizer was significant ($p < 0.05$) with the highest values observed in L digestate treatments.

Considering OTU numbers, soil pH, fertilizer and sampling day did not have a significant ($p < 0.05$) effect in both L and NL treatments (Figure 4.4 C and D). However, there were more OTUs observed in NL compared to L treatments. Additionally, digestate and urea fertilizer had the most OTUs in L and NL treatments, respectively.

Overall, the interaction of soil pH**, f**ertilizer and sampling day had a significant ($p < 0.05$) effect on Shannon diversity, with the highest values observed in NL treatments with digestate fertilizer on D21 (Figure 4.4 E and F).

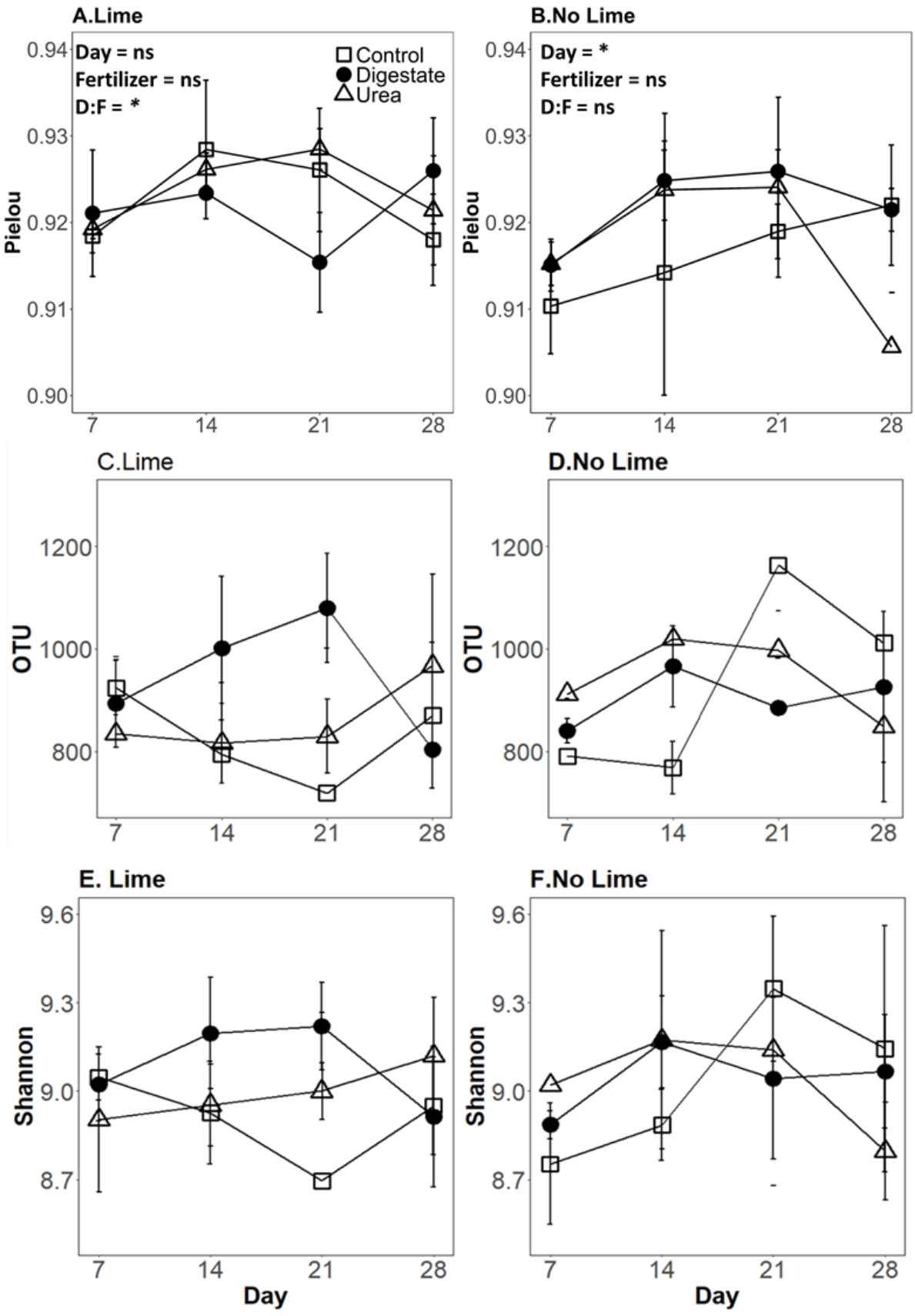

A.Lime
Day = ns
Fertilizer = ns
D:F = *
Control
Digestate
Urea
Pielou
B.No Lime
Day = *
Fertilizer = ns
D:F = ns
C.Lime
D.No Lime
OTU
E. Lime
F.No Lime
Shannon
Day

Figure 4.4: Line graphs illustrating alpha diversity based on Pielou evenness, observed OTU numbers and Shannon diversity based on soil pH, fertilizer (F) and sampling day (D). Error bars show mean standard deviation mean ± SD (n=3). Significant levels are displayed as * $p < 0.05$.

4.4.5 Gene Abundances

Overall, the effect of soil pH, fertilizer and sampling day had a significant ($p < 0.05$) effect on 16S rRNA gene copy numbers with the highest values observed in L, digestate fertilizer on D21. Considering the effect of pH on stability of 16S rRNA gene copy numbers, fertilizer had a significant ($p < 0.05$) effect in L with the highest values observed in digestate (Figure 4.5 A). In NL, the effect of sampling day was significant ($p < 0.05$) on 16S rRNA gene copy numbers with the highest values observed on D21 (Figure 4.5 B).

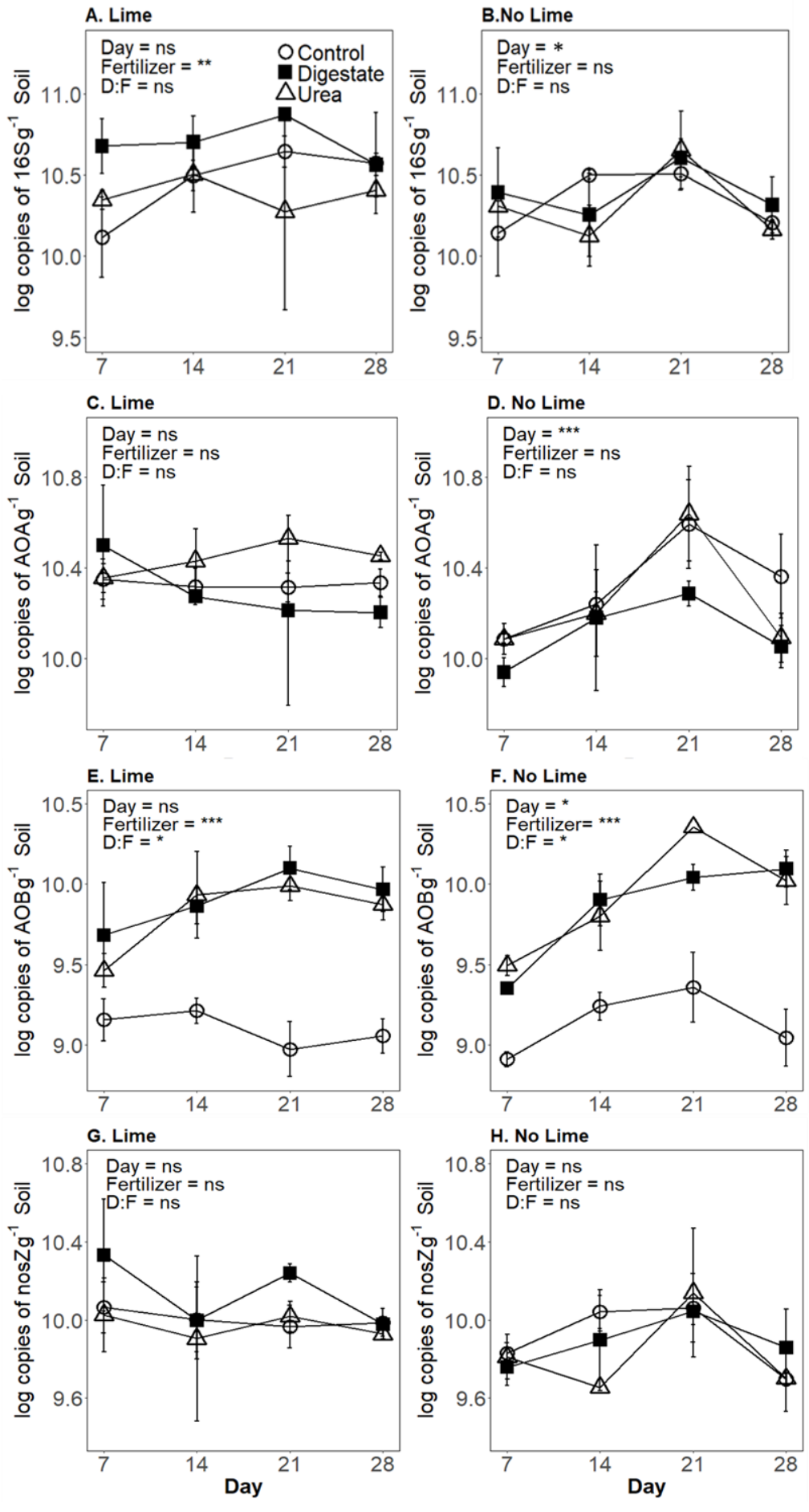
Overall, the interaction of pH and sampling day had a significant ($p < 0.001$) effect on AOA gene copy numbers with the highest values observed in NL on D21. Although the effect of fertilizer was not significant, digestate application had a negative effect on AOA gene copy numbers in both L and NL treatments (Figure 4.5 C and D). In control and urea treatments the interaction of pH and sampling day had a significant ($p < 0.05$) effect on AOA gene copy numbers with the highest values observed in NL on D21. On D7 the AOA gene copy numbers were significantly ($p < 0.001$) affected by soil pH with the highest values observed in L. On D21 the AOA gene copy numbers were significantly ($p < 0.05$) affected by fertilizer with the highest values recorded in urea treatments. Additionally, on D28 the interaction of soil pH and fertilizer had a significant ($p < 0.05$) effect on AOA gene copy numbers with the highest values observed in L urea treatments. Considering the effect of pH on stability of AOA gene copy numbers, sampling day had a significant ($p < 0.001$) effect in NL with highest copy numbers recorded on D21 (Figure 4.5 D).

Overall, the interaction of fertilizer and sampling day had a significant ($p < 0.001$) effect on AOB gene copy numbers with the highest values observed in urea on D21. The interaction of pH and sampling day was significant effect on AOB gene copy numbers ($p < 0.01$) with the highest values observed in NL treatments on D21. The effect of fertilizer had a significant effect on all sampling days with the highest values observed in digestate except on D21 where urea was highest. Soil pH had a significant effect on D7 with the highest values observed in L treatments. The effect of fertilizers on AOB gene copy numbers varied based on soil pH. Highest AOB gene copy

numbers in L and NL treatments were observed in digestate and urea, respectively (Figure 4.5 E and F). In both L and NL treatments, fertilizer and sampling day had a significant ($p < 0.05$) effect on AOB gene copy numbers. In L highest AOB gene copy numbers were observed in digestate on D21 and in NL the highest values were in urea on D21 (Figure 5E and F).

Although pH was not significant to *nosZ* gene copy numbers, higher values were observed in L compared to NL (Figure 4.5 G and H). Day had a significant ($p < 0.01$) effect on *nosZ* gene copy numbers with higher values observed in L compared to NL treatments. In control treatments the interaction of soil pH and sampling day had a significant ($p < 0.05$) effect on *nosZ* gene copy numbers with the highest values observed in L treatments on D7. In NL treatments, sampling day had a significant ($p < 0.001$) effect on *nosZ* gene copy numbers with the highest values observed on D21.

Soil pH had a significant ($p < 0.05$) effect on *nirS* gene copy numbers with higher values observed in L treatments. Considering the effect of fertilizers, in digestate treatments, pH and sampling day had a significant ($p < 0.05$) effect on *nirS* gene copy numbers with the highest values observed in L treatments and D21. In Urea, pH had a significant ($p < 0.05$) effect on *nirS* gene copy number numbers with the highest values observed in L treatments. Considering the effect sampling day, on D7 the interaction of soil pH and fertilizer had a significant ($p < 0.05$) effect on *nirS* gene copy numbers with highest numbers observed in L treatments with urea fertilizers. On D14, pH had a significant effect on *nirS* gene copy numbers with the highest values observed in L treatments (Figure 4.5 I and J).


A. Lime
Day = ns
Fertilizer = **
D:F = ns
Control
Digestate
Urea
log copies of 16Sg-1 Soil
B.No Lime
Day = *
Fertilizer = ns
D:F = ns
C. Lime
Day = ns
Fertilizer = ns
D:F = ns
log copies of AOAg-1 Soil
D. No Lime
Day = ***
Fertilizer = ns
D:F = ns
E. Lime
Day = ns
Fertilizer = ***
D:F = *
log copies of AOBg-1 Soil
F. No Lime
Day = *
Fertilizer= ***
D:F = *
G. Lime
Day = ns
Fertilizer = ns
D:F = ns
log copies of nosZg-1 Soil
H. No Lime
Day = ns
Fertilizer = ns
D:F = ns
Day

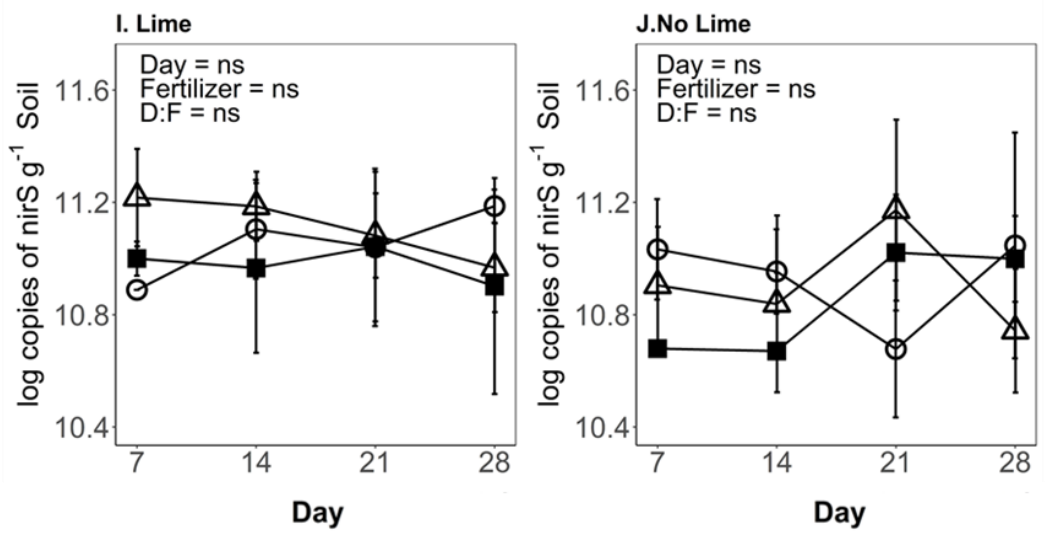


Figure 4.5:The temporal change of 16S rRNA, AOA, AOB , *nosZ* and *nirS* gene copy numbers in soil pH and fertilizer treatments. Error bars show mean standard deviation mean ± SD (n=3). Significant levels are displayed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.5 Discussion

4.5.1 Influence of soil pH on microbial response to digestate application

The effect of digestate on the relative abundance of soil bacterial community compositions was different depending on the liming treatment (Figure 4.3). For example, the abundance of *Alphaproteobacteria* and *Actinobacteria* increased in both L and NL treatments due to digestate application. However, *Alphaproteobacteria* was influenced more strongly by the digestate in NL compared to L treatments. To support our results, Shen et al. (2013) conducted research on the effect of soil pH (3.5 to 6.5) on the spatial distribution of bacterial communities and showed that the relative abundance of *Alphaproteobacteria* increased with decrease in soil pH in line with our results. Contrastingly, the abundance of *Actinobacteria* was influenced more by digestate in L compared to NL treatments. Similarly, Wang et al. (2019) conducted research on the effect of pH range from 4.5 to 9.0 on different microbes within the agricultural environments and reported that the abundance of *Actinobacteria* increased in higher pH levels. Thus, the impact of digestate on soil microbiome could be partially manipulated by liming the soil prior to application of digestate, but further studies are needed because our study was not fully focusing on the functional aspects of the microbiome.

4.5.2 Influence of soil pH on the effect of digestate on OTU stability

Lime application increased the stability of soil microbiome (i.e. relatively smaller shifts in soil microbial community structure after the application of digestate in the L treatments compared to NL, Figure 4.1 and 4.2). Our findings can be attributed to the increased abundance of oligotrophic microbes in L compared to NL treatments (Figure S4.3). In our study, 7 out of 10 of the most abundant top 10 microbes at phylum level had higher relative abundance in L compared to NL treatments (Figure S4.3). More specifically *Acidobacteria*, *Chloroflexi*, *Planctomycetes*, *Verrumicrobia*, *Nitrospirae*, *AD3* and *Crenarchaeota*, all characterized as oloigotrophic (Janssen et al., 2002; Kabore et al., 2020; Kits et al., 2017; Moreno-Espíndola et al., 2018; Ramirez-Villanueva et al., 2015; Ward et al., 2009) were higher in L compared to NL treatments (Figure S4.2), and the increase of these microbes may have enhanced the stability of soil microbial community structure in L. De Vries and Shade (2013) reported that there was a direct relationship between soil microbial community structure and their response to external disturbances. Microbial communities dominated by oligotrophs (such as in our study) which have low growth rates and high resource use efficiency (Fierer et al., 2006) are more stable to external disturbance compared to communities dominated by copiotrophs (Bapiri et al., 2010; Lennon et al., 2012) in agreement with our results.

4.5.3 Influence of soil pH on OTU response to digestate application

The current study showed that, in the L treatment, digestate increased the abundance of OTUs belonging to *Xanthomonadaceae* and *Chthoniobacteraceae*. *Xanthomonadaceae* is involved in the decomposition of carbon, nitrogen and phosphorous (Lueders et al., 2006; Folman et al., 2008). *Chthoniobacteraceaeare* play a role in methane oxidation, nitrogen fixation and polysaccharide degradation (Dunfield et al., 2007; Khadem et al., 2010). Contrastingly, in NL treatments, digestate increased the abundance of OTUs belonging to *Caulobacteraceae* and *Chitinophagaceae. Caulobacteraceae* is involved in the decomposition of lignin and cellulose (Pepe-Ranney et al., 2016; Wilhelm et al., 2019). *Chitinophagaceae* has been associated with cellulose decomposition and increased activity of β-glucosidase (Bailey et al., 2013; De Vries et al., 2015). Thus, the impact of digestate application on some soil microbes and related functions can be different depending on soil lime addition prior to the application of digestate.

Some of the families that increased due to digestate application i.e *Xanthomonadaceae*, and *Caulobacteraceae* are categorized as *Proteobacteria* at phylum level (De Vries et al., 2019; Kersters et al., 2006). Their increase in digestate treatments can be related to the copiotrophic nature of *Proteobacteria*. Copiotrophs rapidly increase in nutrient rich conditions (Fierer et al., 2011), which were observed in digestate (Table S4.1) in our study. In addition, *Chitinophagaceae* which belong to phylum *Bacteroidetes* increased in digestate NL but not in L treatments. In our study, *Bacteroidetes* was one of the families that was dominant in digestate fertilizer (Figure S4.4). We therefore infer that the high *Bacteroidetes* content of digestate fertilizer may have increased the phylum abundance in digestate NL treatments. Our results are in agreement with those of Jiang et al. (2019) and Walter et al. (2018) who reported that *Bacteroidetes* was one of the major phyla in digestate fertilizer samples.

Further phylum level analysis showed that *Synergistetes* and *Armatimonadetes* significantly increased exclusively in digestate NL treatments. Because they are not part of the soil major phyla, these species are poorly characterized and their soil functions not well understood (Bandhari and Gupta, 2012; Wang et al., 2019). Although the number of OTUs that were exclusively increased in NL treatments were few, we believe that the result provides more information on the phyla that may be stabilized with the addition of L in agricultural soils. Long term research should therefore be conducted to clarify the mechanisms and influence of soil pH on the stability of these phyla and related soil functions.

4.5.4 Influence of soil pH and fertilizers on microbial diversity

Soil microbes in the L treatment had relatively higher Pielou evenness across all treatments, when compared to the NL treatments (Figure 4.4 A and B). Our results contrast with those of Narendrula-Kotha and Nkongolo (2017) who conducted research on the effect of lime (as one of the treatments) on the soil microbial community structure. Their results showed that lime did not have a significant effect on microbial evenness. The contrasting results may have been due to differences in soil types. Narendrula-Kotha and Nkongolo (2017) conducted their research on sandy soils while our research was conducted on an acidic volcanic soil with high nutrient content. Surprisingly, for Shannon diversity, NL treatments had significantly higher values compared to L (Figure 4.4 E and F). Although we are not sure why the effect of L was limited to evenness, we believe that the

results show the significance of soil pH in influencing specific diversity indices within the agricultural environment. Additionally, different diversity indices should be considered to understand the effect of soil pH on soil microbial diversity in agricultural soils.

Regarding the effect fertilizers on beta diversity, digestate had its own separate microbes cluster while urea and control treatments clustered together in both L and NL treatments (Figure 4.1). This might have been caused by differences in soil chemical properties between digestate and urea treatments. Although we applied the same amount of NH_4^+ from digestate and urea (50 kg NH_4^+ ha^{-1}), digestate contained other nutrients such as P_2O_5 (0.03%), K_2O (0.27%), NO_3^- (1.92 mg NO_3^- -N kg^{-1} soil) and high pH (7.85) (Table S4.1) which may have enhanced shifts in the soil microbial community. To support this, the results by Tayyab et al. (2018; 2019) reported that increases in soil chemical properties such as pH, nitrogen, phosphorous and potassium caused changes in soil microbial properties, in agreement with our study. Our results demonstrate that in the short term, organic fertilizers such as digestate have more impact on the soil microbial community structure compared to inorganic fertilizers such as urea.

4.5.5 Effect of soil pH and fertilizer application on the abundances of bacteria and their functional genes

4.5.5.1 16S rRNA

An increase in soil pH enhanced the 16S rRNA gene copy numbers, averaging across the fertilizer treatments (Figure 4.5 A and B). Similar results were found by Rousk et al. (2010) who reported that increased soil pH due to liming had a positive correlation to 16S rRNA gene copy numbers. Shen et al. (2010) indicated that soil pH was a major factor affecting bacterial abundance in agricultural soils, with higher bacterial population size observed in less acidic soils, in agreement with our findings.

Digestate had the highest numbers of 16S rRNA gene copy numbers compared to urea and control (Figure 4.5 A and B). This may have been due to digestate fertilizer increasing the soil pH, P_2O_5, K_2O, and NO_3^- (Table S4.1) which enhanced abundance of the16S rRNA gene. A similar trend was observed by Nõlvak et al. (2016) and Truu et al. (2008) who reported that digestate enhanced 16S rRNA gene copy numbers compared to chemical fertilizers. Their result was attributed to

digestates' high nutrient content that increased 16S rRNA gene abundance, in agreement with our study.

Additionally, in L treatments, the effect of fertilizer was significant with the highest values observed in digestate, while in NL treatments, the effect of fertilizers was not significant (Figure 4.5 A and B). Jezille et al. (2009) conducted research on the effect of liming on microbial activity and mineralization in manure amended soils. In agreement with our study, their results showed that fertilizer addition (manure) increased microbial activity in lime treatments than in treatments without lime. They attributed this observation to lime creating favorable conditions for soil microbes to grow efficiently. In their study, lime treatments provided favorable pH, increased soil Ca^{2+} and nitrogen content, which enhanced efficient microbial growth with manure treatments, in agreement with our study. Our findings show that soil pH and fertilizer type are important factors to consider in the expression of 16S rRNA gene copy numbers. More specifically, near neutral to basic pH combined with organic fertilizer such as digestate should enhance 16S rRNA gene copy numbers in acidic agricultural soils.

4.5.5.2 Nitrification: AOA, AOB

Soil pH also had a significant effect on AOA gene copy numbers but was not significant for AOB (Figure 4.5). Regarding AOA gene copy numbers, unexpectedly, higher values were observed in L compared to NL treatments (Figure 4.5 C and D). These results contrast with previous reports (He et al., 2012, Zhang et al., 2016) which found that AOA gene copy numbers usually dominate in acidic (pH<5.5) soils. The discrepancy between the two studies may be attributed to differences in the range of soil pH. The acidic pH range for He et al. (2012) and Zhang et al. (2016) were below 5.5, which increased AOA gene abundance. In our research however, although L treatments had higher soil pH compared to NL, after addition of fertilizers, the NL treatments had a pH that was greater than 5.5 (Figure S4.5) which may have reduced AOA gene copy abundance in NL acidic soil. Alternatively, soil pH caused very little variation in AOB gene copy numbers (Figure 4.5 E and F). Nicol et al. (2008) and Wessen et al. (2010) similarly reported that changes in soil pH did not have a significant effect in AOB gene copy numbers in agreement with our study. Wessen et al. (2010) attributed this result to AOB microbial community having a high stability and resistance to changes in soil pH, which may have been the case in our study.

Fertilizers did not have a significant effect on AOA gene copy numbers but had a significant impact for AOB (Figure 4.5). Additionally, for AOA, digestate application had a negative effect on gene copy numbers in both L and NL treatments (Figure 4.5 C and D). Saunders et al. (2012) conducted research on bacterial responses to digestate application on incubated soils. Based on his results, digestate application reduced AOA gene copy numbers in corroboration with our study. A possible reason for the low gene copy numbers in digestate treatments may be due to AOA preference for oligotrophic environments which have low nutrient content (Erguder et al., 2009; Sterngen et al., 2015). We therefore suggest that the nutrient rich digestate fertilizer treatments would have decreased AOA gene abundance. The positive impact of fertilizer application on AOB gene copy numbers could be due to the high NH_4^+ content of digestate and urea treatments that stimulated the AOB gene abundance. Our results are in agreement with Wang et al. (2013) and Zhang et al. (2016) who found that increase in soil NH_4^+ content stimulated the growth of AOB gene copy numbers in agricultural soils. Based on our findings we concluded that the AOB abundance was enhanced by organic (urea) and inorganic (digestate) fertilizer, while organic fertilizers (digestate) reduced the abundance of AOA.

4.5.5.3 Denitrification: *nosZ, nirS*

Soil pH had a significant effect on *nosZ* and *nirS* gene copy numbers (Figure 4.5). For *nosZ*, the significant effect of pH was observed on D7 with the highest values recorded in lime (Figure 4.5 G and H). In agreement with our findings, Bergaust et al. (2010) and Čuhel et al. (2010) reported that increasing soil pH stimulated *nosZ* gene copy numbers in agricultural soils, subsequently increasing the conversion of N_2O to N_2 gas. Regarding *nirS*, our results are in agreement with those of Herold et al. (2018) who conducted research on the effect of pH (4.2 to 6.6) on *nirS* gene copy numbers. Their results showed that *nirS* abundance increased with soil pH in line with our study.

Fertilizers did not have a significant effect on *nosZ* gene copy numbers but had a significant effect on *nirS* (Figure 4.5). Additionally, for *nosZ*, higher gene copy numbers were observed in control treatments compared to digestate and urea (Figure 4.5 G and H). This may have been caused by fertilizers increased NO_3^- content that reduced the *nosZ* gene abundance. Our results agree with Huang et al. (2013) who conducted research on the effect of fertilizers on *nosZ* gene. Based on their findings, fertilizers did not enhance *nosZ* gene copy numbers as the gene's abundance was decreased by the presence of NO_3^-. Our findings show that fertilizers may increase agricultural

N_2O emissions by reducing the *nosZ* gene abundance, although more field level studies are needed to confirm this. For *nirS* the interaction of soil pH and fertilizer was significant on D14 with the highest values observed in urea treatments (Figure 4.5 I and J). Our results for *nirS* agree with Dong et al. (2015) who found that fertilizers had a significant effect *nirS* gene copy numbers due to their high NH_4^+ content that enhance the genes abundance.

Based on our findings the gene copy numbers of 16S rRNA and nitrification (AOA and AOB) were fluctuating lesser in L compared to NL treatments (Figure 4.5). Although more long term studies need to be conducted to verify the underlying mechanisms that may have increased 16S rRNA and nitrification gene stability in lime treatments, we believe that the results are important towards understanding the influence of soil pH on soil functions, especially on the nitrification process of the nitrogen cycle.

4.6 Conclusion

Liming increased the stability of soil microbial community structures, when digestate was applied. This was indicated by the lower number of operational taxonomic units that significantly changed due to digestate application in lime treatments compared to no lime. Digestate also had a larger impact on microbial community structures compared to urea. This indicates that in agricultural soils, organic fertilizers may have a larger influence on microbial diversity compared to inorganic fertilizers in the short term. Regarding the functionality of soil microbiomes, lime increased the relative abundance of 16S rRNA, AOA, *nosZ* and *nirS* gene copy numbers. Additionally, lime additions enhanced the positive effect of digestate on 16S rRNA gene copy numbers. The results show the importance of soil pH in modulating changes in 16S rRNA gene abundance and its response to inorganic fertilizer application. Although fertilizers (digestate and urea) increased the abundance of AOB, digestate decreased the abundance of AOA. This indicates that fertilizer sources are important to consider in the management of nitrification gene abundance in agricultural soils. The study should provide more information on the significance of soil pH as an important variable influencing changes in microbial community structure, diversity, stability and functional gene abundance. The study should contribute towards development of sustainable management practices within the agricultural environment that are focused on soil pH management.

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Supplementary

Table S4.1: Digestate and Kamishihoro soil chemical properties

Parameter	Soil	Digestate
Water content %	-	98.4
pH (H_2O)	5.5	7.85
P_2O_5 (mg kg^{-1})	86	0.03 (%)
K_2O (mg kg^{-1})	250	0.27 (%)
Total N %	0.35	0.22
NO_3^--N (mg N kg^{-1})	-	1.92
NH_4^+-N (mg N kg^{-1})	-	2125.49
Total C %	4	-

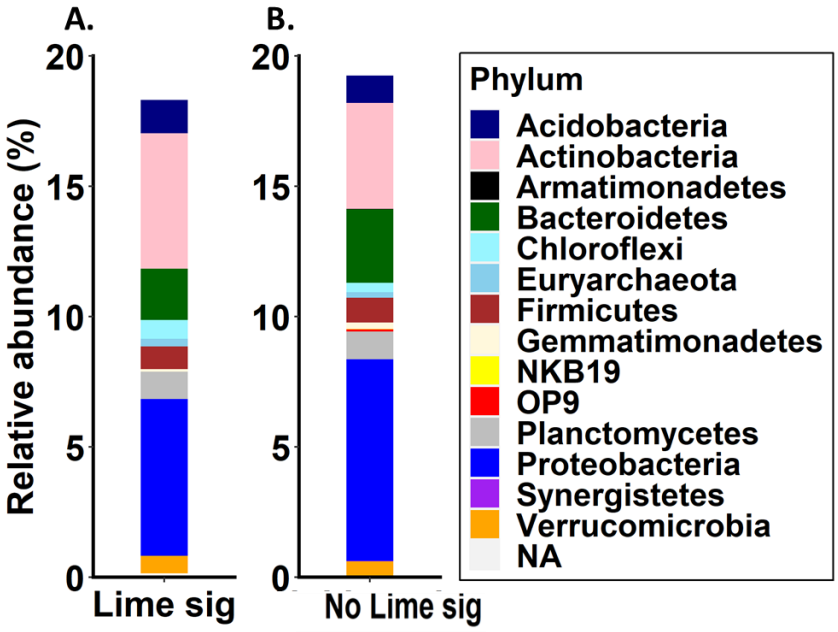

Figure S4.1: The relative abundance of phylum level OTUs that significantly increased in digestate treatments in L and NL treatments based on volcano plots.

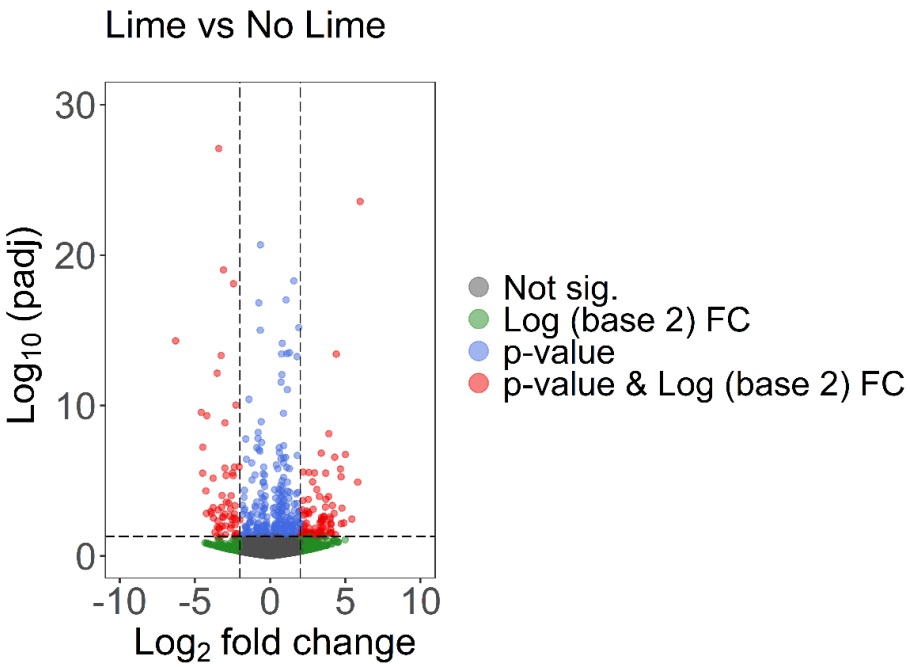

Figure S4.2: Volcano plots showing the distribution of OTUs abundance according to the adjusted p value (−log10 scale) and the log2 fold change between lime and no lime treatments. Each point represents an individual OTUs. Black points indicate OTUs that were not significant p > 0.05), blue dots indicate OTUs that were significant (p <0.05), green dots indicate OTUs that had a log_2old change that was greater or less than 2 and red dots indicate OTUs that were significant (p < 0.05) and had a log_2fold change that was greater or less than 2.

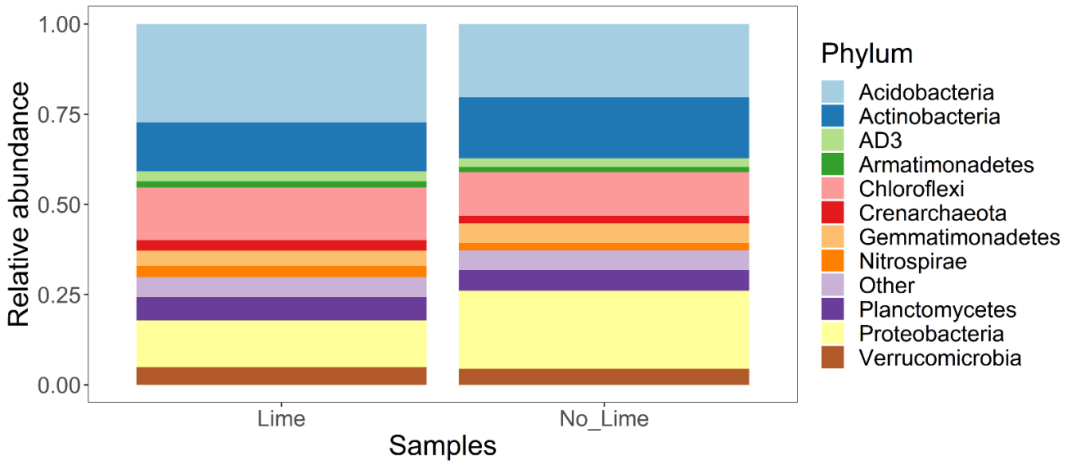

Figure S4.3: Relative abundance of the most abundant microbes at phylum level in lime and no lime treatments.

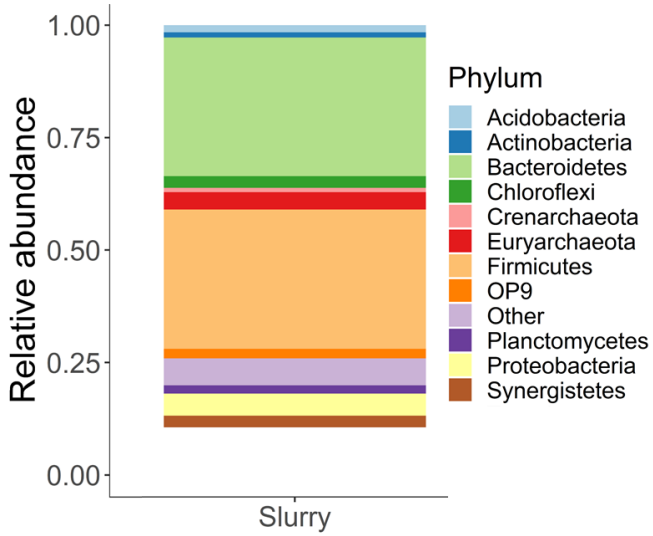

Figure S 4.4: Relative abundance of the most abundant microbes at phylum level in digestate.

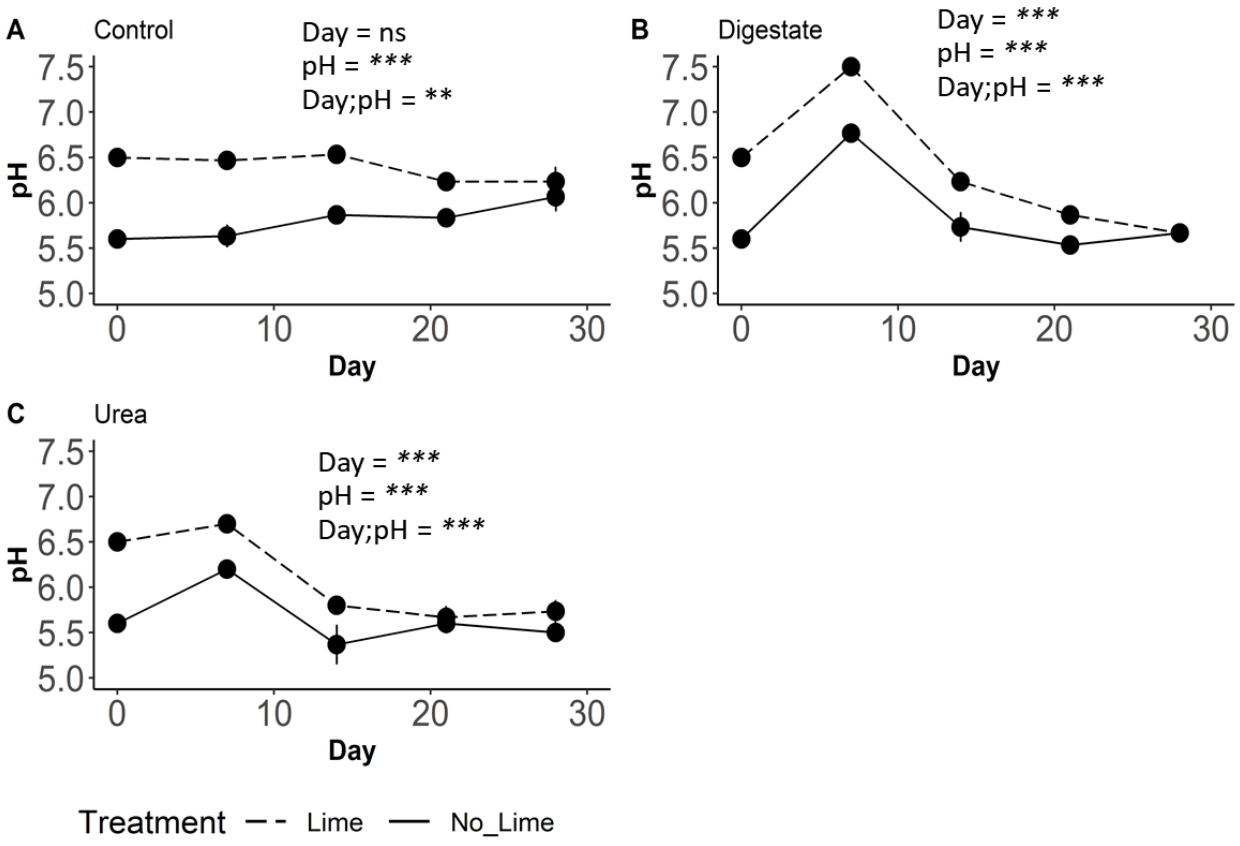

Figure S4.5:The temporal change of soil pH in control, digestate and urea treatments. Error bars show mean standard deviation mean ± SD (n=3). Significant levels are displayed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

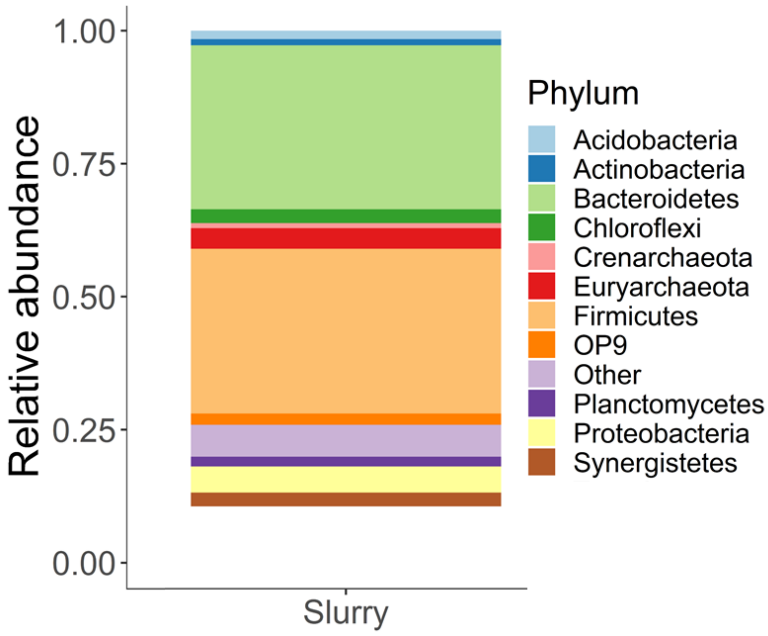

Figure S 4.6: Relative abundance of microbes at phylum level in digestate.

Chapter 5 The impact of separating anaerobic digestate on soil nutrients, yields and microbial functions in agricultural soils

5.1 Abstract

The use of digestate as an organic fertilizer has been gaining popularity in recent years. High transportation costs and soil flood risks are some of the main challenges that have been associated with digestate use as a fertilizer. Separation of digestate in solid and liquid fractions has been proposed as a potential solution to address these challenges. However, the question remains whether the use of separated digestate fractions influences soil nutrient dynamics and microbial properties in agricultural soils. Therefore, an incubation experiment was setup to analyze the effect of soil types (Kamishihoro and Arakida) and fertilizers (digestate, solid digestate, liquid digestate, chemical and control) on soil and plant (Japanese mustard spinach) nutrient dynamics, microbial abundance and crop yields. Based on the results, soil type significantly influenced the above ground plant biomass and Nitrogen (N) content with higher values observed in Kamishihoro compared to Arakida. Kamishihoro had significantly higher soil inorganic N (NO_3^- and NH_4^+) content compared to Arakida soil. Fertilizer had a significant effect on soil NO_3^- (solid-Kamishihoro, digestate-Arakida) and NH_4^+ (liquid-Kamishihoro, chemical-Arakida) content. The results indicate that digestate and its derived fertilizers (solid digestate, liquid digestate) can be used to influence soil inorganic N content in agricultural soils. Digestate derived fertilizers influenced soil pH with highest values observed in liquid in both Kamishihoro and Arakida soils. Regarding gene abundance, Arakida soil had significantly higher 16S rRNA gene copy numbers compared to Kamishihoro. Regarding soil N_2O emissions, Kamishihoro soil had significantly higher emissions compared to Arakida. Additionally, digestate and solid fertilizer had significantly higher N_2O emissions compared to liquid in both Kamishihoro and Arakida soils. The results indicate the importance of soil type and digestate derived fertilizers in modulating soil gene abundance and N_2O emissions in agricultural soils. Furthermore, the separation of digestate into solid and liquid fractions can be considered a reliable method to regulate various aspects of soil nutrient dynamics in agricultural soils.

5.2 Introduction

Digestate is the semi liquid byproduct of the biogas production process (Weiland, 2009). The use of digestate as an organic fertilizer within the agricultural environment has been increasing, attributed to its high nutrient content (Athurson, 2009). More specifically, digestate application increases the soil organic carbon (OC), inorganic nitrogen (N), phosphorous (P), potassium (K) among other nutrients (Šimon et al., 2016; Smith et al., 2014). Due to digestates' high ammonium (NH_4^+) content, various researchers consider the fertilizer as a practical substitute for reducing the use of inorganic N fertilizers in the agricultural environment (Risberg et al., 2017; Weiland, 2009). Additionally, with the increasing negative effects of inorganic chemical N fertilizers use on the environment such as eutrophication, soil acidification and increased nitrous oxide N_2O emissions, (Bouwman et al., 2005; Guo et al., 2010), the use of organic fertilizers such as digestate has been increasing globally (Weiland, 2009; Gielnik et al., 2019).

Although the use of digestate has been increasing in recent years, there have been observed challenges associated with its use. Some of the challenges include the high storage and transportation costs, increased soil flood risks and challenges in applying specific nutrient concentrations of digestate compared to chemical fertilizers. Digestate use as a fertilizer has associated high transportation and storage costs due to its low nutrient content and large volume compared to chemical fertilizers (Camilleri-Rumbau et al., 2021). The high soil moisture (97%) of digestate presents agricultural challenges to its use as a fertilizer (Greenberg et al., 2019). This problem is amplified in humid environments or during periods of high rainfall when the use of digestate has been found to increase temporary soil flooding risks (Vaneeckhaute et al., 2013). Additionally, the high soil moisture and varying nutrient contents complicate precision nutrient application of digestate based on soil nutrient requirements (Drosg et al., 2015).

The separation of digestate into solid and liquid fractions before soil application has been proposed as a potential solution to address the identified challenges accompanied with its use (Heviánková et al., 2015). Various methods have been developed to separate digestate into solid and liquid portions, with the most common being the combined use of press pretreatments and filters (Dosch, 1996). However, to increase efficiency of the process, the use of chemical treatments such as flocculants (water soluble metal salts) has been gaining popularity (Nowostawska et al., 2005; Meixner et al., 2015). Holm-Nielsen et al.(2009) reported that the separation of digestate to solid

and liquid fractions was a simple and efficient method to reduce high transport costs and simplify soil application of digestate. The separated solid and liquid fractions of digestate contain different soil nutrients, which helps farmers to apply the fertilizer based on soil requirements (Bauer et al., 2009). This is especially important for different soil types, with research showing that the impact of digestate application on soil nutrients varies based on soil types (Möller et al., 2015). The liquid fraction is considered a suitable substitute to the use if inorganic N fertilizers due to its high NH_4^+ -N content (Sigurnjak et al., 2017). On the other hand, the solid fraction usually has a high OC and P content (Egene et al., 2020). These observed differences in soil nutrient content of the solid and liquid fractions are expected to influence the effect of their application on agricultural soils. Despite this observation, there is limited research investigating the effect of separated fractions of digestate on soil nutrient dynamics and microbial abundance in agricultural soils. Most of the available research is focused on the effect of digestate on crop yields (Tampio et al., 2015; Šimon et al., 2015; Risberg et al., 2017). Research therefore needs to be conducted to clarify the effects of separated solid and liquid fractions of digestate on soil and plant nutrient dynamics and microbial abundance in agricultural soils.

Thus, this study aimed to investigate 1) the impact of solid liquid separation of digestate on soil and plant nutrient dynamics and Japanese spinach yields 2) the impacts of solid liquid separation of digestate on soil N_2O emissions 3) the impacts of solid liquid separation of digestate on microbial abundance. We hypothesized that the digestate liquid fraction would have the highest soil N content, crop yields, N_2O emissions and microbial abundance compared to the solid fraction.

5.3 Materials and methods

5.3.1 Study site and soil characteristics

Two types of soils (Andosols and Alluvial) were used for the study. The soils were sampled from Kamishihoro and Kawagoe, Japan. Kamishihoro soils, classified as Andosols (FAO/UNESCO), were sampled from farmers' fields (0-15 cm) located in Obihiro, Hokkaido, Japan (42° 45'01.9"N, 143° 08'20.4"E). Soils from Kawagoe (Arakida soils), classified as Alluvial (FAO/UNESCO), were commercially sourced and had been sampled from the bottom layer of a paddy field in Kawagoe city, Saitama, Japan. Both soils were sieved using a 4 mm diameter mesh before incubation. Characterization of initial soil chemical and physical properties; pH, Phosphorous

oxide (P_2O_5), Potassium oxide (K_2O), Magnesium oxide (MgO), Calcium oxide (CaO), cation exchange capacity (CEC) and bulk density (BD) are as described in Table S5.1 and were measured using the following methods. Soil pH was measured with a glass electrode pH meter. The P_2O_5 K_2O and MgO and CaO were analyzed using Flow Injection Analysis (FIA). CEC was measured according to the method by Scollenberger and Simon (1945), with an atomic absorption spectrophotometer. The soil BD was determined by comparing the change in soil moisture content before and oven drying to a constant weight .

5.3.2 Digestate separation to solid and liquid phase

Digestate was obtained from a biogas plant located in the Field Science Center for Northern Biosphere in Hokkaido University, Japan (N43.0781050, E141.334344). The digestate was produced from dairy cow excreta. A polymer flocculant was used to separate digestate into solid and liquid fractions. Analyzed chemical properties (pH, P_2O_5, nitrates- NO_3^-, ammonium- NH_4^+) of digestate and its derived fertilizers are as described in Table S5.2.

5.3.3 Incubation setup

The experimental setup was a randomized design with 2 soils (Kamishihoro and Arakida) and 5 fertilizer treatments; Digestate (106 kg inorganic nitrogen [N] ha^{-1} and 27 kg P_2O_5 ha^{-1}), ADS (200 g), ADL (200 g), chemical fertilizer-CF (120 kg inorganic-N ha^{-1} and 100 kg P_2O_5 ha^{-1}) and no fertilizer control, replicated 3 times. Fertilizer application rates were based on recommended N standards for Japanese mustard spinach (Department of Agriculture, Hokkaido Government, 2015).

For the incubation, 1500 g of air-dry soil were placed in Wagner pots. Water filled pore space was maintained at 70 % using milli Q and BD established at 0.7 g cm^{-3} for Kamishihoro soils and 1 g cm^{-3} for Arakida soils. Fertilizers were added to the pots (Day 0) and Japanese mustard spinach planted 10 days after fertilizer application. Each fertilizer treatment had 3 replicates making up a total of 30 soil cores.

5.3.4 Soil and plant sampling and analysis

For soil chemical and leachate analysis soils were sampled on day 1, 7, 16, 23, 30 and 37. To determine soil pH and inorganic N (NH_4^+, NO_3^-), 5 g of soil was extracted with 2 M potassium chloride and shaken for 1 hour. Immediately after extraction, soil pH was determined using a pH meter (AS800; AS ONE Co., Osaka, Japan). The solution was then filtered with 1 µl filter paper (Toyo Roshi Kaisha No. 5C filter paper; Tokyo) and colorimetric measurements for inorganic N determined using FIA (AQLA-700; Aqualab Co., Ltd., Tokyo, Japan).

To determine soil P_2O_5, 1 g of soil was extracted with 100 g mixture of ammonium sulfate and 0.5 M sulfuric acid. After shaking for 30 min, the solution was filtered with 1 µl filter paper (Toyo Roshi Kaisha No. 5C filter paper; Tokyo) and P_2O_5 colorimetric measurements determined using FIA (AQLA-700; Aqualab Co., Ltd., Tokyo, Japan).

Above ground biomass was collected on day 38. After oven drying at 65°C for 48 hours, carbon (C) and N measurements were analyzed using CN coder (Perkin Elmer 2400).

5.3.5 Nitrogen mass balance analysis

The N mass balance was determined by analyzing the distribution of inorganic N from the soil and chemical fertilizers during the incubation period. For plants, N uptake was determined by multiplying plant dry weight with analyzed N content. For leachates, inorganic N loss was determined by multiplying collected leachate (ml) with analyzed leachate NO_3^--N and NH_4^+-N content.

5.3.6 N_2O gas sampling and analysis

Gas samples were collected on day 7, 16, 23, 30 and 37. Each soil core were placed in a sealed plastic container (25 L) with a septum. At 0 and 60 min after sealing the container, 30 mL of headspace gas sample was collected through the septum using a gas-tight syringe. During the preliminary experiment, we confirmed that the N_2O gas concentration linearly increased in the plastic container during the first 60 min of incubation. Sampled gases were stored in pre vacuumed 20 mL glass vials. The N_2O was measured with a gas chromatograph (GC-2014, Shimadzu Co.,

Kyoto, Japan) and analyzed within 24 hours of sampling. The N_2O flux was calculated by dividing the increased N_2O concentration (from 0 to 60 min) by the incubation time (60 min).

5.3.7 Soil DNA extraction and microbial abundance

For microbial analysis, soils were sampled on day 1 and 37. Using 0.5 g soil, DNA was extracted with the NucleoSpin ® Soil (Takara Bio Inc, Shiga, Japan) according to the manufacturer's instructions. To reduce DNA attachment to the soils, 0.2 g of skim milk was added during extraction (Hoshino and Matsumoto, 2005). Extracted DNA was purified using Agencourt AM Pure XP Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufactures protocol and thereafter stored at −30°C until further analysis.

To determine microbial abundance, the qPCR (Quantitative Polymerase Chain Reaction) was performed using CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, Hercules, USA). DNA samples were 50-fold diluted before qPCR targeting 16S rRNA. Each sample contained 10.4 µl of KAPA SYBR Fast qPCR kit (Agilent Technologies, Palo Alto, CA, USA), 0.8 µl of forward (515F-5'-GTGCCAGCMGCCGCGGTAA-3') and reverse (806R-5' GGACTACHVGGGTWTCTAAT-3') primers, 7.0 µl of nuclease free water and 1 µl of DNA template, making up a final volume of 20 µl. The qPCR was conducted under the following conditions: 95°C for 30 sec and thereafter 30 cycles of 95°C for 30 sec, 57°C for 30 sec with a final extension of 72°C for 60 sec.

5.3.8 Statistical analysis

All statistical analysis was conducted in R software version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). To obtain a normal distribution, N_2O emissions data was log transformed (log 10 (flux+1)) before analysis. In all analysis, $P < 0.05$ were considered significant. Two-way analysis of variance (ANOVA) with multiple pairwise comparisons were conducted to determine the effect of soil type and fertilizer on soil and plant chemical properties, N_2O emissions and microbial abundance. For significant interactions, Tukey's HSD test was performed.

5.4 Results

5.4.1 Plant Analysis

Regarding above ground biomass, Kamishihoro soil had significantly ($P < 0.001$) higher values compared to Arakida soil (Figure 5.1). Fertilizer had a significant ($P < 0.001$) effect on above ground biomass with highest values observed in chemical fertilizer in both Kamishihoro and Arakida soil. Below ground biomass was not significantly affected by soil type and fertilizer (data not shown).

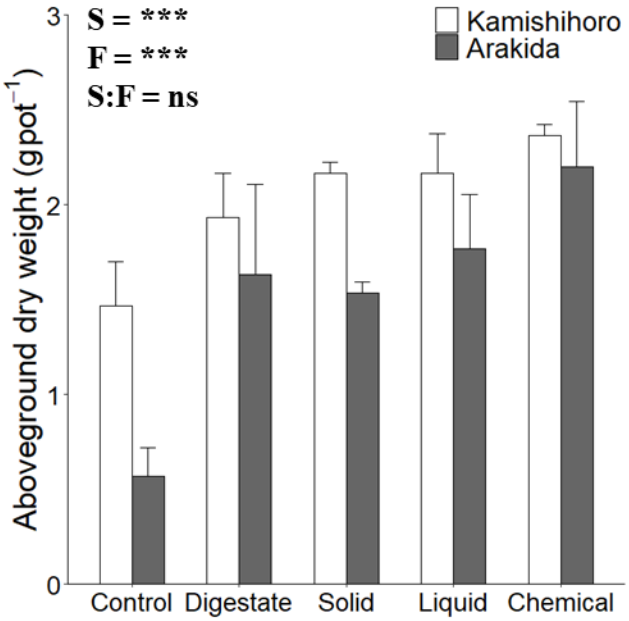

Figure 5.1:The effect of fertilizer on dry weight of aboveground plant biomass in Kamishihoro and Arakida. Vertical bars indicate standard deviation (n=3). Abbreviations; S-Soil, F-Fertilizer.

For above ground plant biomass C, there was an interaction between fertilizer and soil type ($P < 0.05$). The highest above ground plant biomass C content was recorded in Arakida control treatments (Figure S5.1). Below ground plant biomass C content was not significantly affected by soil type and fertilizer (data not shown).

For plant biomass N, the effect of soil type and fertilizer had a significant ($P < 0.001$) effect with highest values recorded in Kamishihoro soil and chemical fertilizer, respectively (Figure S5.2). Considering Kamishihoro and Arakida soil separately, fertilizer had a significant ($P < 0.01$) effect

on above ground N content with highest values recorded in chemical fertilizer in both soils. Soil type had a significant effect on below plant biomass N content with highest values observed in Kamishihoro soil (Data not shown).

5.4.2 Soil Analysis

Regarding soil NO_3^- content Kamishihoro soil had significantly ($P < 0.001$) higher values compared to Arakida soil (Figure 5.2). Considering Kamishihoro and Arakida soil separately, fertilizer had a significant ($P < 0.05$) effect on soil NO_3^- content with highest values recorded in solid (Kamishihoro) and digestate (Arakida). Soil NO_3^- content was more responsive to fertilizer in Kamishihoro soil compared to Arakida soil (Figure 5.2).

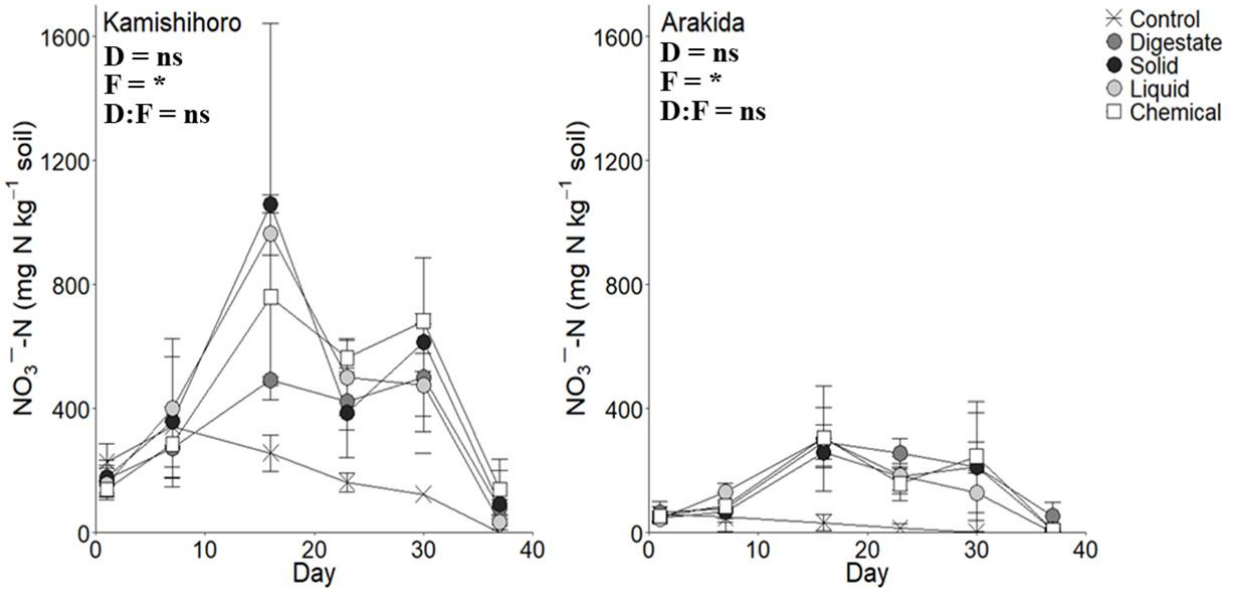

Figure 5.2: : Effect of fertilizer on temporal variations in NO_3^--N concentration in the Kamishihoro and Arakida soil. Vertical bars indicate standard deviation (n=3). Abbreviations; D-Day, F-Fertilizer.

Considering soil NH_4^+ content, there was an interaction between soil types and fertilizer (Figure 5.3). The highest soil NH_4^+ content was recorded in Kamishihoro soil in chemical fertilizer. Considering Kamishihoro and Arakida soil separately, fertilizer had a significant ($P < 0.01$) effect on soil NH_4^+ content. In Kamishihoro significantly higher values were observed on in liquid and chemical fertilizer while in Arakida significantly highest values were observed in chemical

fertilizer. Soil NH_4^+ content was more responsive to fertilizer in Kamishihoro soil compared to Arakida soil (Figure 5.3).

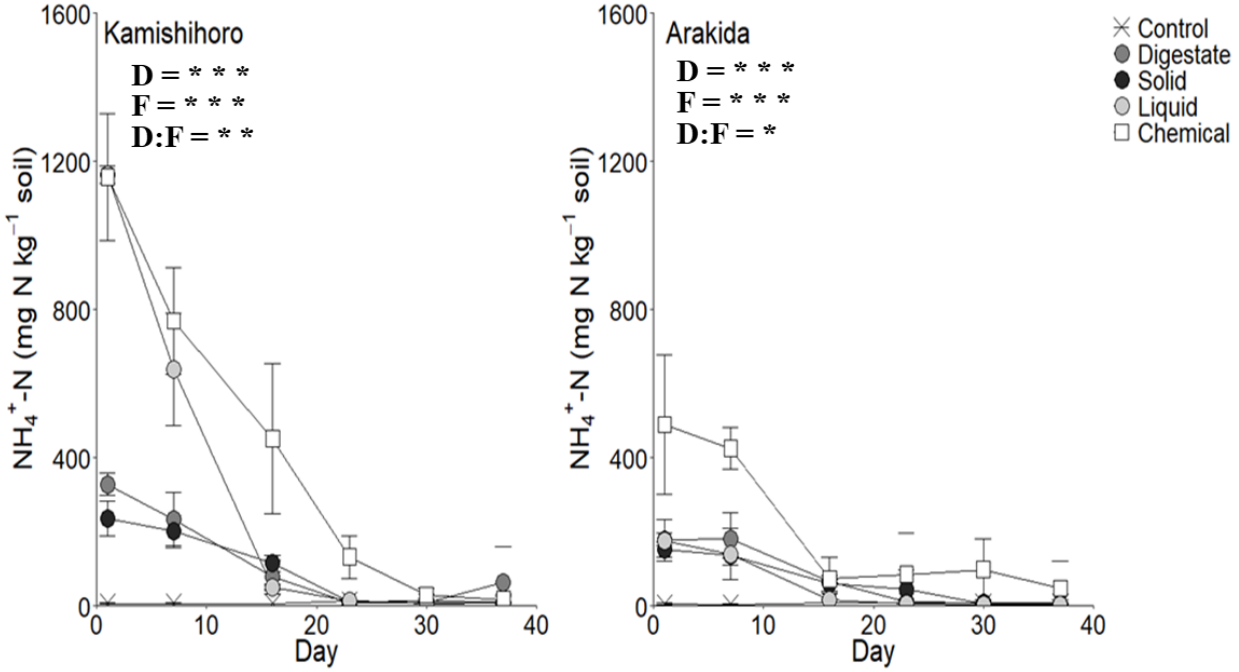

Figure 5.3: : Effect of fertilizer on temporal variations in NH_4^+-N concentration in the Kamishihoro and Arakida soil. Vertical bars indicate standard deviation (n=3). Abbreviations; D-Day, F-Fertilizer.

For soil P_2O_5 content, there was an interaction between soil types and fertilizer ($P < 0.05$). The highest soil P_2O_5 content was recorded in Arakida soil in chemical fertilizer (Figure 5.4). Arakida soil had significantly ($P < 0.05$) higher soil P_2O_5 content compared to Kamishihoro soil. Considering Kamishihoro and Arakida soil separately, fertilizer had a significant ($P < 0.05$) effect on soil P_2O_5 content in both soils, with highest values recorded in chemical fertilizer. Soil P_2O_5 content was more responsive to fertilizer in Arakida soil compared to Kamishihoro soil (Figure 5.4).

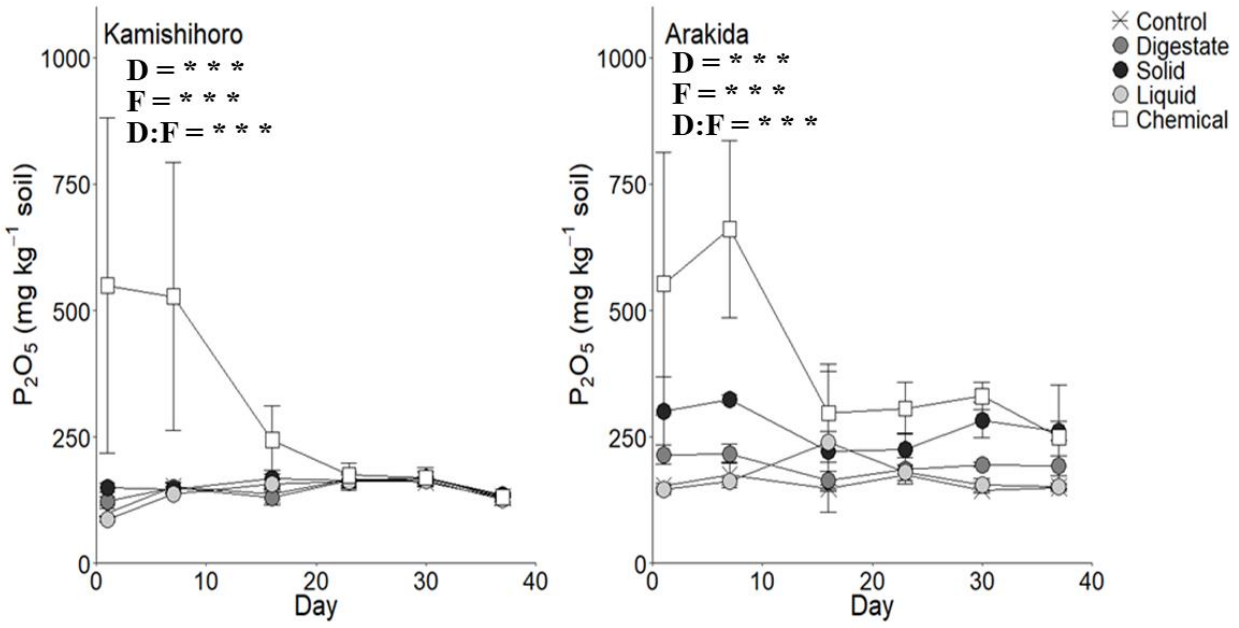

Figure 5.4: Effect of fertilizer on temporal variations in P_2O_5 concentration in the Kamishihoro and Arakida soil. Vertical bars indicate standard deviation (n=3). Abbreviations; D-Day, F-Fertilizer.

For soil pH, there was an interaction between soil types and fertilizer ($P < 0.05$). The highest soil pH content was recorded in Arakida soil in liquid fertilizer (Figure 5.5). Considering Kamishihoro and Arakida separately, liquid fertilizer recorded significantly ($P < 0.05$) higher soil pH values in both soils, while chemical fertilizer had lowest soil pH values. Soil pH was more responsive to fertilizer in Arakida soil compared to Kamishihoro soil (Figure 5.5).

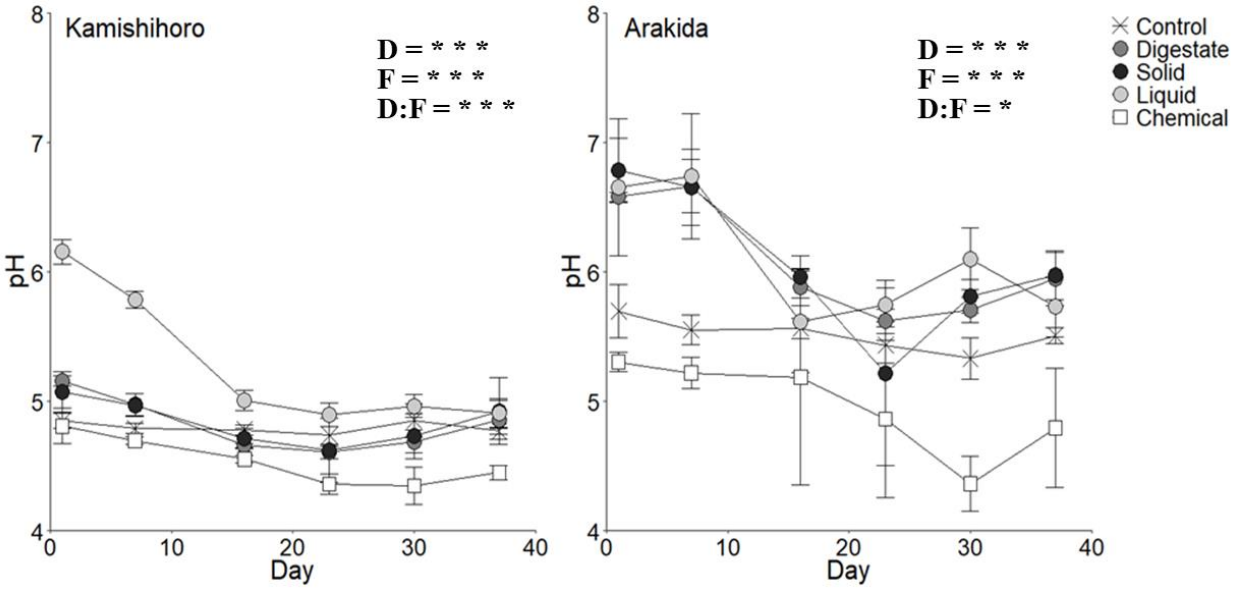

Figure 5.5: Effect of fertilizer on temporal variations of soil pH in Kamishihoro and Arakida soil. Vertical bars indicate standard deviation (n=3). Abbreviations; D-Day, F-Fertilizer.

5.4.3 Leachate Analysis

Regarding leachate NO_3^- content, Kamishihoro soil had significantly ($P < 0.05$) higher values compared to Arakida soil. Chemical fertilizer had significantly ($P < 0.05$) higher soil leachate NO_3^- content in both Kamishihoro and Arakida soil (Figure 5.6).

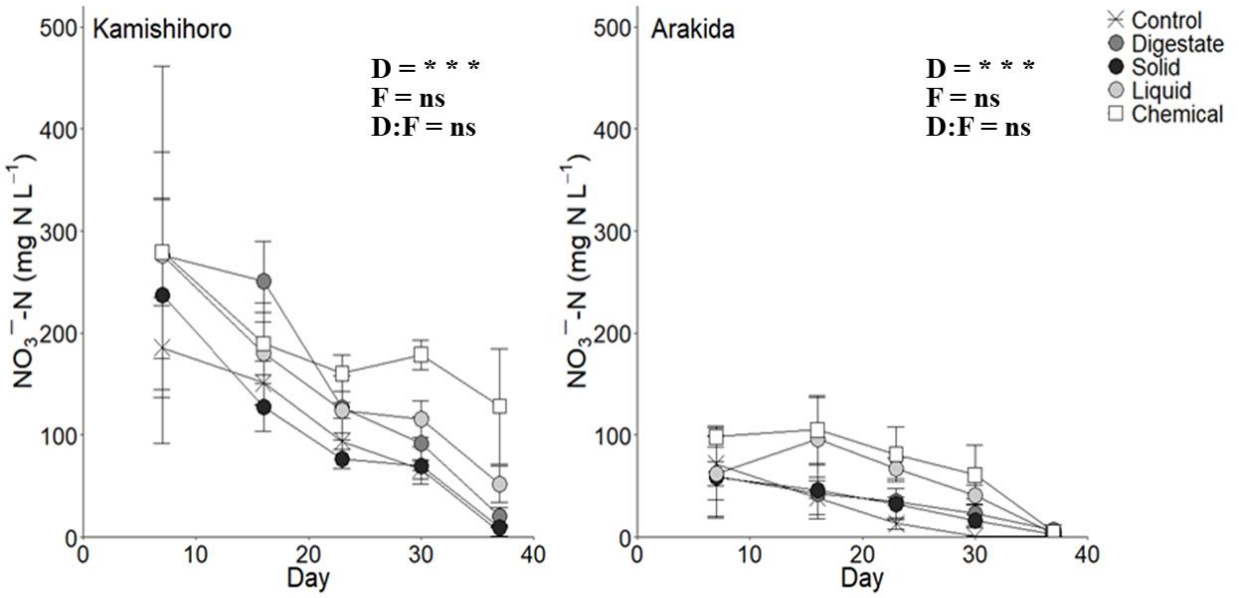

Figure 5.6: Effect of fertilizer on temporal variations of leachate NO_3^- content in Kamishihoro and Arakida soil. Vertical bars indicate standard deviation (n=3). Abbreviations; D-Day, F-Fertilizer.

For the leachate NH_4^+ content, there was an interaction between soil type**s** and fertilizer ($P < 0.05$). The highest leachate NH_4^+ content was recorded in Arakida soil in chemical fertilizer**s** (Figure 5.7). Considering Kamishihoro soil separately, fertilizer did not have a significant effect on leachate NH_4^+ content. However, in Arakida soil, chemical fertilizer had significantly higher leachate NH_4^+ content compared to other fertilizers.

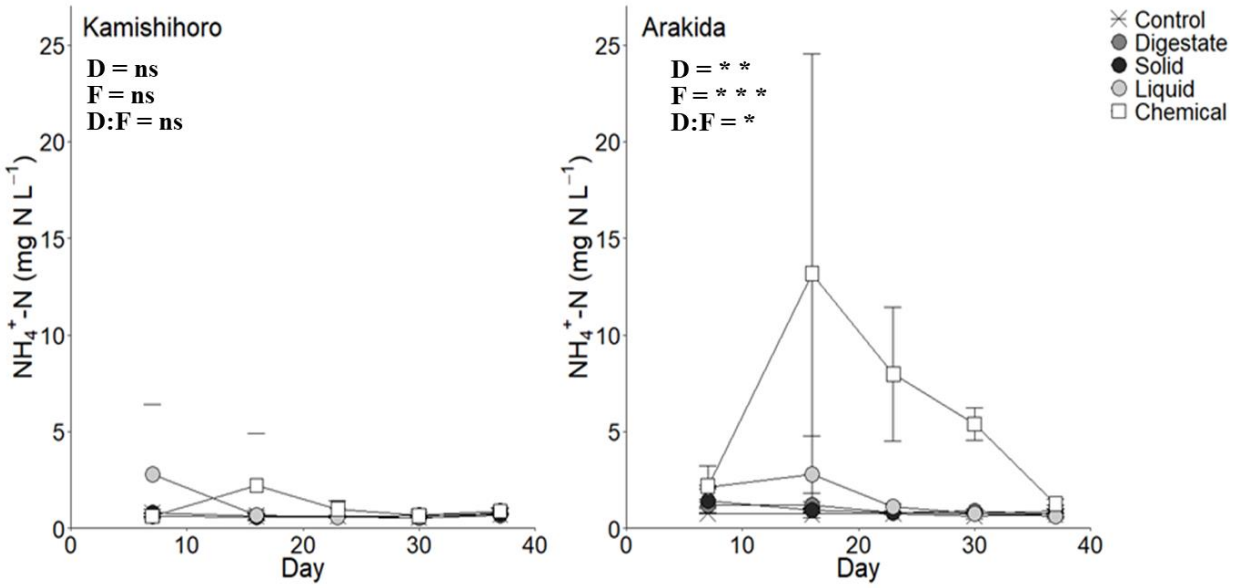

Figure 5.7:Effect of fertilizer on temporal variations of NH_4^+ leachate content in Kamishihoro and Arakida soil. Vertical bars indicate standard deviation (n=3). Abbreviations; D-Day, F-Fertilizer.

5.4.4 Nitrogen mass balance

Overall, Kamishihoro soil had higher (234.32 mg N $kg^{-1)}$ inorganic N content compared to (Arakida 63.72 mg N kg^{-1}). Considering fertilizer treatments, digestate had highest inorganic N content (164.50 mg N kg^{-1} Kamishihoro soil and 147.50 mg N kg^{-1} Arakida soil), followed by liquid (119.19 mg N kg^{-1} Kamishihoro soil and 106.87 mg N kg^{-1} Arakida soil) and solid fertilizer (91.20 mg N kg^{-1} Kamishihoro soil and 81.78 mg N kg^{-1} Arakida soil).

In Kamishihoro soil, solid and liquid fertilizer had higher above ground percentage distribution compared to digestate. In solid and digestate fertilizer, about 30 % of the added N remained in the soil while 25 % was lost in other ways other than leaching (Figure 5.8). In liquid fertilizer, 12 % of added N remained in the soil while 44 % was lost in other ways other than leaching. In Arakida soil, liquid fertilizer treatments had higher N accumulated in leachates and aboveground plant biomass compared to digestate and solid fertilizer (Figure 8). About 28 %, 10%, and 3% of N remained in the soil under the digestate, solid, and liquid fertilizer treatments, respectively. Additionally, more than 50 % of N applied to the solid and liquid treatments were lost in other forms other than leaching.

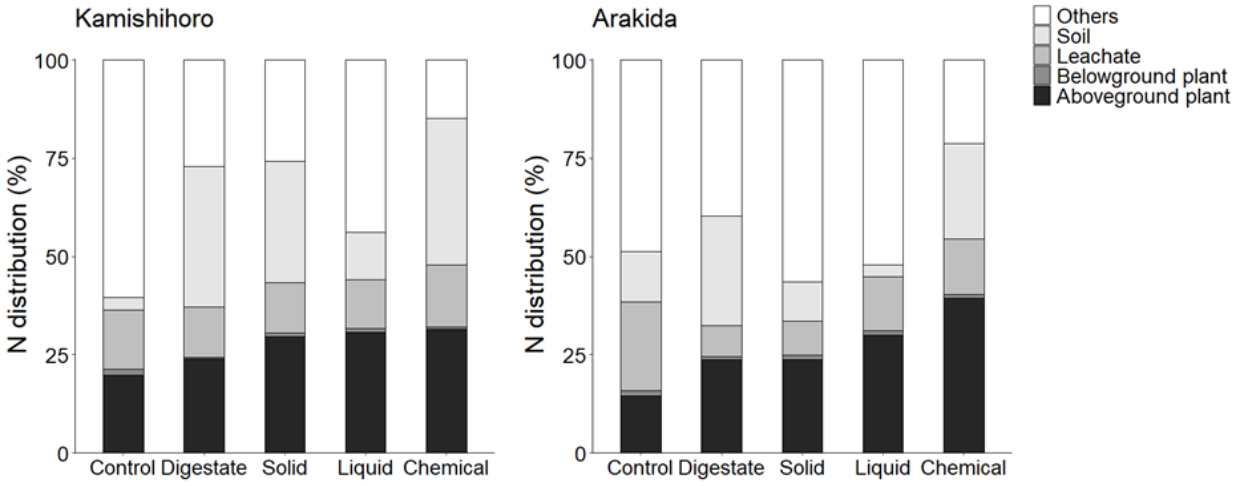

Figure 5.8: Effect of fertilizer on nitrogen mass balance in percentage in the Kamishihoro and Arakida soil.

5.4.5 Gene Abundances

Arakida soil had significantly ($P < 0.001$) higher 16S rRNA gene copy numbers compared to Kamishihoro soil. In Kamishihoro soil, fertilizer did not have a significant effect on 16S rRNA gene copy numbers. However, in Arakida soil, fertilizer had a significant ($P < 0.001$) effect on 16S rRNA gene copy numbers with the highest values observed in solid fertilizer.

5.4.6 Nitrous oxide emissions

Regarding soil N_2O emissions, there was an interaction between soil type and fertilizer ($P < 0.05$). The highest soil N_2O emissions were recorded in Kamishihoro soil in solid treatments (Figure 5.9). Fertilizer had a significant ($P < 0.05$) effect on soil N_2O emissions in Kamishihoro and Arakida soils. In both Kamishihoro and Arakida soils, digestate, solid and chemical fertilizer had significantly higher soil N_2O emissions compared to other fertilizers (Figure 5.9).

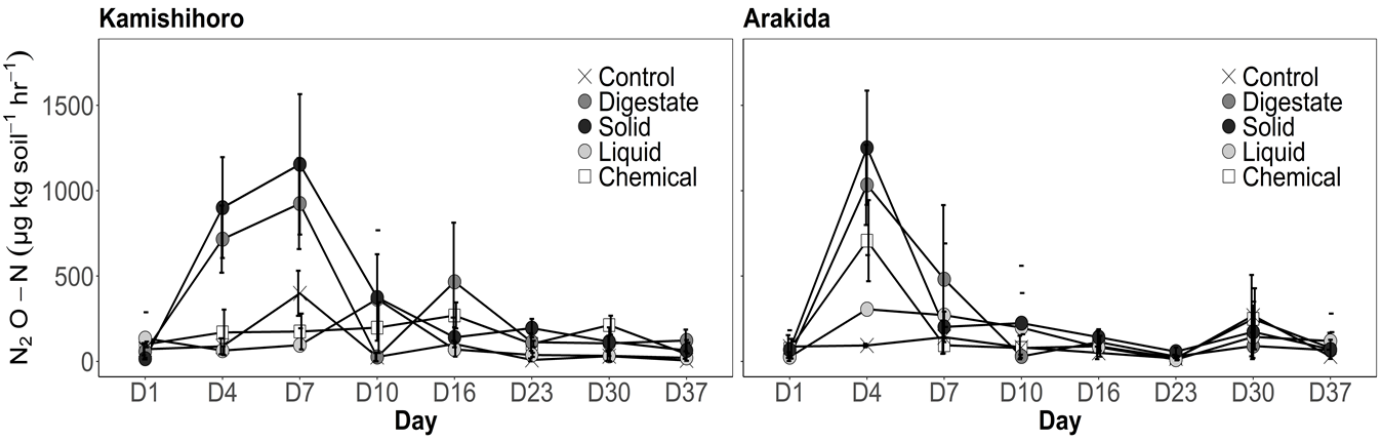

Figure 5.9: Temporal changes in soil N_2O emissions in Kamishihoro and Arakida fertilizer treatments. Error bars show mean standard deviation mean ± SD (n=3).

5.5 Discussion

5.5.1 The effect if separated digestate on soil inorganic nitrogen content

Digestate and its derived fertilizers influenced the soil inorganic nitrogen content (Figure 5.2 and 5.3). However, the effect of application varied based on soil types. For NO_3^- in Kamishihoro solid had the higher values while in Arakida digestate had the highest values. Solid having the highest values of NO_3^- in Kamishihoro is expected as results from the separated digestate chemical analysis showed that solid had higher NO_3^- content compared to digestate and liquid (Table S5.2). Möller and Müller (2012) similarly reported that the solid fraction of digestate contained a high amount of available nitrogen. These results contrast with those of various researchers (Drosg et al., 2015; Tambone et al., 2017) who have reported that after separation of digestate, the liquid portion contains high inorganic N content. The differences in results may be attributed to methods used to separate the digestate. Barampouti et al. (2020) reported that the different methods of digestate separation such as mechanical technologies, polymers and flocculants had different effects on the nutrient distribution in the solid and liquid fractions.

Considering soil NH_4^+ content, in Arakida chemical fertilizer had highest values (Figure 5.3). However, in Kamishihoro soils highest values were recorded in both liquid and chemical fertilizers (Figure 5.3). Chemical nitrogen fertilizers having high NH_4^+ content is expected as they directly increase the soil NH_4^+ content (Giday, 2019). Regarding liquid digestate NH_4^+ content, Sigurnjak

(2017) conducted research on nitrogen release and mineralization potential of various nitrogen fertilizers. Based on their results, liquid fraction of digestate had a high NH_4^+ content in agreement with our study. Subsequently it can be used as a substitute for inorganic nitrogen fertilizer (Sigurnjak, 2017). Tambone et al.(2017) and Möller and Müller (2012) similarly reported that due to the high NH_4^+ content (about 45–80% of N in the liquid fraction of digestate) the liquid part of digestate was a better fertilizer quality than digestate and could be used as a fast-acting nitrogen fertilizer.

Considering the soil P_2O_5 content, digestate derived fertilizers had an influence in Arakida soils (Figure 5.4). Although the values were not significantly different, solif had the highest values compared to digestate and liquid (Figure 5.4). Egene et al.(2020) and Möller and Müller (2012) reported that the solid fraction of digestate had a high phosphorous content and could subsequently be used as a phosphorous fertilizer. Bachman et al.(2015) conducted research on phosphorous distribution and availability in untreated and separated biogas digestate. Based on their results the solid fraction had higher phosphorous concentration than the liquid fraction in agreement with our study.

Digestate and derived fertilizers had a significant influence on soil pH values (Figure 5.5). In both Kamishihoro and Arakida liquid, solid and digestate had the highest soil pH. These results may have been due to the high soil pH of applied digestate and its derived fertilizers (Table S5.2). Previous research has found that application of digestate and its derived fertilizers increased soil pH content of agricultural soils in line with our findings (Galvez et al., 2012, Valentinuzzi et al., 2020). Chemical fertilizer had the lowest soil pH in both Kamishihoro and Arakida soils. Sun et al.(2020) and Gouling (2016) similarly reported that the use of chemical fertilizers reduced soil pH levels and was one of the main reasons for agricultural soil acidification. Chemical fertilizer use cause soil acidity in agricultural soils due to enhancing the oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) which produces hydrogen ions (H^+) (Garvin and Carver, 2003). Our results show that digestate and its derived fertilizers can be used to reduce soil acidity in agricultural soils.

5.5.2 Influence of soil types

Inorganic N content was responsive to fertilizer additions in Kamishihoro soils compared to Arakida soils (Figure 5.2 and 5.3). However, for soil P_2O_5 content and soil pH, Arakida soils were more responsive to fertilizer additions. Ohshiro et al.(2016) conducted research on the effects of soil types and fertilizers on growth, yield, and quality of edible Amaranthus tricolor lines in Japan. Based on their results, nutrient availability varied in different soil types based on differences in soil physical, chemical and biological properties, which may have been the case in our study. Lapwayi et al.(2012) similarly reported that the response of soil properties to fertilizers was dependent on soil types. These results indicate that soil type should be taken into consideration when determining agricultural fertilizer application rates. For example, the high clay minerals (allophane, imogolite) present in Kamishihoro soils have a high phosphorous retention capacity which may have reduced the increase in phosphorous content due to fertilizer application (Nyanzo, 2002). Additionally, the low difference in soil pH in Kamishihoro soils due to fertilizer additions may have been caused by the high soil pH buffering capacity of the soils. Shoji and Takahashi (2002) found that Andosols have high organic matter and cation exchange capacity which increase the soil pH buffering capacity in agreement with our study.

5.5.3 Leachate analysis

Interestingly digestate and its derived fertilizers did not influence the inorganic N content of leachates (Figure 5.6 and 5.7) . This may have been due to the soil types used in the experiment. The Kamishihoro soils had a high organic carbon content while Arakida soils had high clay content, which both reduce leaching losses. Kanthle et al. (2016) similarly reported that soils with high organic carbon content reduced leaching losses. Cameron and Moir (2013) found that soils with high clay content had reduced leaching losses in agreement with our study. Chemical fertilizers had the highest inorganic N leachate losses compared to digestate and its derived fertilizers in Kamishihoro and Arakida soils. Wang et al.(2019) conducted research on the effect of fertilizer types on soil N leaching losses. Based on their results, chemical fertilizer treatments had the highest N leaching losses compared to organic fertilizers in agreement with our study. These results show that on soils with high clay and organic matter content, digestate and its derived fertilizers can be used without affecting inorganic N leaching losses.

5.5.4 Nitrogen mass balance

Considering digestate and its derived fertilizers, liquid had the highest N loss compared to digestate and solid fertilizer treatments (Figure 5.8). This may have been due to increased N losses through NH_3 volatilization in liquid fertilizers. To prevent these losses the liquid part of digestate is better suited as a fast acting fertilizer (Tambone et al., 2017, Nakamura et al., 2007). Nakamura et al.(2012) also reported that incorporating digestate into the soil immediately after application could reduce NH_3 volatilization losses. The solid digestate fertilizer treatments stored more loaded nitrogen into the soil compared to liquid, showing that it would be appropriate as a slow acting fertilizer. Nakamura et al.(2007) similarly reported that solid digestate contained N in organic form and would be best suited as a slow release fertilizer.

5.5.5 16S rRNA gene abundance

The effect of digestate and its derived fertilizers on 16S rRNA gene copy numbers varied based on soil type. In Kamishihoro the effect of digestate and its derived fertilizers was not significant. However, in Arakida, solid had significantly higher 16S rRNA gene copy numbers compared to digestate and liquid. This may have been due to the differences in soil pH and chemical properties of the digestate fertilizers after separation. Specifically, solid digestate had the highest soil pH (8.72), NO_3^- (178.59 mg N kg^{-1}) and P_2O_5 (1462.47 mg kg^{-1}) that may have enhanced the abundance of the 16S rRNA gene. Zhou et al.(2015) conducted research on the influence of fertilizers on soil bacterial communities in agricultural soils. Their results showed that soil NO_3^- content was one of the most important factors in shaping soil bacterial communities with higher abundance observed in higher NO_3^- concentrations. Regarding P_2O_5 our results contrast with those of Wang et al.(2018) who reported that soil phosphorous content did not have a significant effect on 16S rRNA gene copy numbers in agricultural soils. The variations in results may have been due to differences in soil types and experimental time periods. Wang et al.(2018) conducted their research on Ultisols for 35 years while our experiment was conducted on Andosol for 37 days.

5.5.6 N_2O emissions

Kamishihoro soils had higher N_2O emissions compared to Arakida soils (Figure 5.9). This can be related to Kamishihoro soils having higher NH_4^+ content compared to Arakida soils, which

stimulated nitrification and N_2O emissions. N_2O emissions peaked during the first week of the experiment. This can be attributed to nitrification of the added NH_4^+ from fertilizer additions, which were highest during the first week of the experiment. Similar trends in N_2O emissions were reported by De La Fuente et al.(2013) and Alburquerque et al.(2012). Digestate and its derived fertilizers influenced soil N_2O emissions with digestate and solid having the highest soil emissions compared to liquid. Askri et al.(2016) conducted research on the influence of origin and post treatment in greenhouse gas emissions after digestate application to soils. Based on their results, digestate had the highest N_2O emissions while liquid had the lowest emissions in line with our results. They related this to increased availability of decomposable organic matter in digestate and solid which stimulated denitrification, which may have been the case in our study. These results show that solid liquid separation of digestate may he used to reduce greenhouse gas emissions from agricultural soils. Specifically, the liquid digestate can be used to decrease N_2O emissions from digestate in agricultural soils.

5.5.7 Plant Analysis

Digestate and its derived fertilizers did not influence the Japanese mustard spinach yields (Figure 5.1). This result shows that digestate and its derived fertilizers can be used interchangeably on Japanese mustard spinach without affecting crop yields. Chemical fertilizer had the highest yields in both Kamishihoro and Arakida soils. This can be attributed to chemical fertilizer N P K application being suited to Japanese mustard spinach demand based on the Hokkaido government recommendation for the crop. However, for digestate and its derived fertilizers, only N was taking into account for this incubation. Specifically, P for digestate and its derived fertilizers was below the recommended amounts, which may have reduced Japanese mustard spinach yields. Ehmann et al.(2018) conducted research on the fertilizing potential of separated digestate on annual and perennial biomass production systems. Based on their results, increased maize yields were recorded in chemical fertilizers compared to digestate and its derived fertilizers. They attributed this to chemical fertilizers providing nutrients based on crop requirements, which may have been the case in our study. These results indicate that plant yields can be enhanced in digestate and its derived fertilizers by supplementing with chemical fertilizers, based on plant requirements.

Unexpectedly control treatments had significantly higher above ground biomass C compared to other fertilizers in both Kamishihoro and Arakida soils. This may have been due to the inorganic N in control treatments being below plan requirements. Hermans et al.(2006) and Hirai et al. (2004) similarly reported that soil N deficiency caused the accumulation of carbohydrates such as sugars and starch in leaves, increasing above ground C content.

5.6 Conclusion

Digestate and its derived fertilizers influenced soil inorganic nitrogen content. However, the influence varied based on soil type. In highest NO_3^- was recorded in solid-Kamishihoro and digestate-Arakida. Considering NH_4^+ highest values were recorded in liquid-Kamishihoro and chemical-Arakida. This indicates that soil types should be taken into consideration for soil inorganic nitrogen content when applying digestate and its derived fertilizers. Additionally, digestate and its derived fertilizers increased soil pH in Kamishihoro and Arakida soils, while chemical fertilizers decreased soil pH. Digestate and its derived fertilizers can therefore be used to reduce soil acidification in agricultural soils. Solid digestate enhanced the 16S rRNA in Arakida soils but not in Kamishihoro. Solid digestate can therefore be used to increase microbial abundance in alluvial agricultural soils. Considering N_2O emissions, liquid had the lowest emissions compared to digestate and solid fertilizer in both Kamishihoro and Arakida soils. This indicates that digestate separation can be used to reduce agricultural greenhouse N_2O emissions. Digestate and its derived fertilizers did not influence Japanese mustard spinach above ground biomass. However, chemical fertilizers had the highest above ground biomass of Japanese mustard spinach. This indicates that chemical fertilizers can be used to supplement digestate and its derived fertilizers to increase Japanese mustard yields. Digestate separation can therefore be used to modulate soil inorganic nitrogen content and N_2O emissions in agricultural soils. The study should contribute towards the development of efficient digestate management practices that can enhance soil nutrient content while reducing greenhouse gas (N_2O) emissions.

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Supplementary

Table S5.1: Physical and chemical properties of Kamishihoro and Arakida soils (means ± standard deviation, n=3).

Parameter	Kamishihoro	Arakida soil
pH (H_2O)	5.5	6.8 ± 0.1
P_2O_5 (mg kg^{-1})	135	178 ± 2
K_2O (mg kg^{-1})	253	123 ± 1
MgO (mg kg^{-1})	511	824 ± 16
CaO (mg kg^{-1})	2523	2582 ± 31
Lime saturation (%)	33.7	59.3 ± 1.3
Base saturation (%)	46.3	87.3 ± 1.2
Phosphate absorption coefficient	1384	893 ± 10
Cation exchange capacity (me kg^{-1})	281	155 ± 2
Bulk density (g cm^{-3})	0.78	1.10 ± 0.01

Table S5.2:Chemical properties of anaerobic digestate and separated solid and liquid fractions.

Parameter	Digestate	Solid fraction	Liquid fraction
Water content (%)	95.08 ± 0.22	93.09 ± 1.92	-
pH	8.27	8.72	8.42
NO_3^--N (mg N kg^{-1})	1.92	178.59	2.31
NH_4^+-N (mg N kg^{-1})	2125.49	1002.08	1085.72
P_2O_5 (mg kg^{-1})	544.65	1462.47	103.51
Mass distribution (%)	-	39.57	60.43

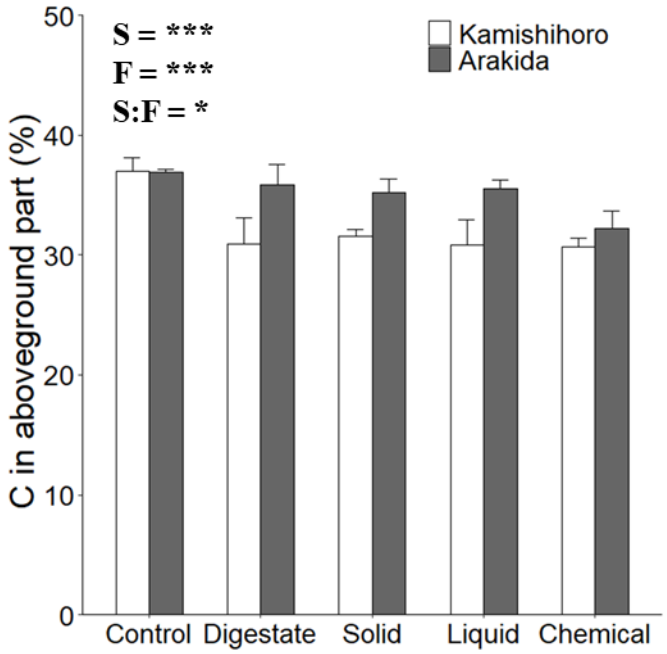

Figure S5.1:The effect of fertilizer on carbon content of aboveground plant biomass in Kamishihoro and Arakida. Vertical bars indicate standard deviation (n=3). Abbreviations; S-Soil, F-Fertilizer.

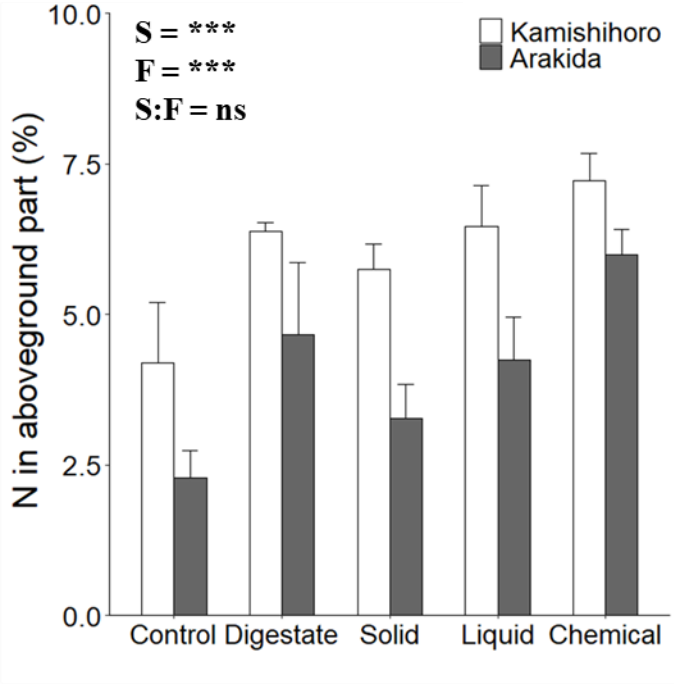

Figure S5..2:The effect of fertilizer on nitrogen content of aboveground plant biomass in Kamishihoro and Arakida. Vertical bars indicate standard deviation (n=3). Abbreviations; S-Soil, F-Fertilizer.

Chapter 6 Synthesis and Recommendations for further research

This chapter synthesizes the research and identifies emerging opportunities for advancement of further research

6.1 Overall summary and future research recommendations

6.1.1 Land use and season drive changes in soil microbial communities and related functions in agricultural soils

The study involved sampling of soils from farmers' fields in the Kamishihoro, Japan. The objective was to determine the effect of land use (cropland, grassland), season (spring, summer) and fertilizer (anaerobic digestate) on soil microorganisms and related functions. After sampling DNA extracted and sequenced targeting 16S rRNA region. Land use and seasons were identified as the main factors driving changes in soil microbial communities in the Kamishihoro region. Specifically, land use drove changes in alpha (evenness) and beta diversity (unifrac) indices with higher values observed in cropland compared to grassland. However, grasslands had a higher number of unique operational taxonomic units (OTUs) (10303) compared to cropland (5112). Based on these results, land use influenced different aspects of the soil microbial diversity indices. The influence of seasons on the soil microbial community varied based on land use. In cropland, season influenced microbial alpha (Shannon, evenness, OTU counts) and beta (unifrac) diversity. In grassland however season influenced beta (unifrac) diversity only. Thus, conventional tillage practices in cropland could have a negative effect on microbial stability in agricultural soils. Considering predicted soil functions, nitrogenase (*nifH*) had significantly higher values in cropland-summer while nitrite reductase (*nirK*) and ammonia monooxygenase (*amoA*) were significantly higher in cropland-spring. In grassland however, *nifH* was significantly higher in grassland-spring while *nirK* and *amoA* were significantly higher in grassland-summer. These results indicated that the impact of seasons on soil microorganisms' distribution and abundance in cropland and grassland may directly affected soil functions.

Our study was conducted on one crop (beet) although research shows that various crops influence the soil microbial community and related functions in different ways (Yang et al., 2020). Future research should therefore be conducted in Kamishihoro region involving different crops to clarify the influence of crop variations on the soil microbial community. Additionally, we conducted soil

sampling for 2 seasons (summer and spring), although research shows that all 4 seasons have an impact on the soil microbial community (Li et al., 2019; Sengupta et al., 2020). Therefore, future experiments should conduct this research on a continuous basis (with all seasons) for an extended time period to compare the long-term effects of seasonality on soil microbes in Kamishihoro region.

6.1.2 Liming improves the stability of soil microbial community structures against the application of digestate made from dairy wastes

The incubation experiment was conducted for a month to determine the effect of of lime application (pH = 6.5 and 5.5 for the soils with and without lime, respectively) and fertilizer (digestate, urea and control) on the soil microbial community structures, stability and gene functions. Soils were sampled for pH and microbial analysis every seven days after fertilizer application. Microbial analysis DNA was extracted and sequenced targeting the 16S rRNA region. To determine gene abundance for 16S rRNA, ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), nitrous oxide reductase (*nosZ*) and nitrite reductase (*nirS*), quantitative PCR was conducted. Based on the result, soil pH influenced the relative abundance of soil microbes. For example, *Actinobacteria* was influenced more strongly by digestate in lime soils, while *Alphaproteobacteria* was influenced more strongly by digestate in the no lime soil. Lime increased the stability of operational taxonomic units against the application of digestate. However, this was not the case for urea, where treatments without lime were more stable. The combination of lime and digestate fertilizer increased the 16S rRNA gene copy numbers in Kamishihoro soils. Considering nitrification and denitrification genes, higher values of AOA, *nosZ* and *nirS* gene copy numbers were observed in lime treatments compared to those without lime. Additionally, digestate increased AOB gene copy numbers but that was not the case for AOA. Based on the results soil pH and fertilizers play significant roles towards the management of microbial stability and functional gene abundance in agricultural soils.

Our study was conducted without plants for one month, although research shows that plants such as legumes increase soil acidity (Monaghan et al., 1998; Kiiya et al., 2010). Future research should include crops to clarify the effect of lime on stability of soil microbes in a cultivated agricultural environment. Additionally, we focused on the effect of soil pH on the nitrogen cycle. However,

soil pH may influence other nutrient cycles directly and indirectly. Malik et al.(2018) reported that soil pH influenced carbon accumulation in agricultural soils, decomposition rates among other processes. Therefore, future research should include analysis of different nutrient cycles and the potential effects of soil pH on their related functions.

6.1.3 The effect of solid-liquid separation of digestate on soil nutrient dynamics and Japanese mustard spinach (*Brassica rapa* var. *perviridis*) yields in different soils

The incubation experiment was conducted for 37 days to determine the effect of soil types (Kamishihoro and Arakida) and fertilizers (digestate, solid digestate, liquid digestate, chemical and control) on soil and plant (Japanese mustard spinach) nutrient dynamics, microbial abundance and crop yields. In the results, soil type influenced the above ground biomass and nitrogen content with higher values recorded in Kamishihoro compared to Arakida. A ssimilar trend was observed in soil inorganic nitrogen analysis with higher values observed in Kamishihoro. This indicates that soil types should be taken into consideration when using digestate and its derived fertilizers within the agricultural environment. The effect of separated digestate on soil inorganic nitrogen varied based on soil types as well. Specifically, fertilizer had a significant effect on soil NO_3^- (solid-Kamishihoro, digestate-Arakida) and NH_4^+ (liquid-Kamishihoro, chemical-Arakida) content. Therefore, digestate and its derived fertilizers can be used to regulate soil inorganic nitrogen content in agricultural soils. Soil pH was increased by digestate and its derived fertilizers showing their potential to reduce acidification in agricultural soils. Liquid had the lowest N_2O emissions compared to solid and digestate. This indicates that separating digestate can reduce agricultural emissions of the greenhouse N_2O gas. Therefore, the separation of digestate into solid and liquid fractions may be a reliable method to reduce agricultural soil acidification, regulate nutrient dynamics and reduce greenhouse (N_2O) gas emissions from agricultural soils.

In our study we used a flocculant to separate the digestate. However, there are different methods that are used to separate digestate into solid and liquid fractions. Some of these include mechanical technologies and polymers, among others. Barampouti et al. (2020) reported that the method used to separate digestate effected nutrient distribution in the solid and liquid fractions. Future research should therefore include different methods of separation to determine the potential effect this would have on the use of digestate derived fertilizers on soil and plant nutrient dynamics in

agricultural soils. Additionally, based on our experimental results, digestate and its derived fertilizers had low above ground biomass production compared to chemical fertilizers. A possible solution to this problem would be using chemical fertilizers to supplement nutrient deficiencies in digestate and its derived fertilizers. Therefore, future research should be conducted to clarify whether this is a viable method to improve the yield production of digestate and its derived fertilizers.

6.2 General Comments

- In chapter 3 we considered bulk soil samples for our analysis. Research has shown that the rhizosphere environment is very different from the bulk soil which leads to the establishment of distinct microbial communities. Including the rhizosphere soils could have provided a different perspective on the effect of imposed treatments on soil microbes.

- In chapter 4 stabilizing the soil pH at 6.5 took about 3 months. It is therefore important to take this adjustment time into consideration when conducting pH adjustment experiments in andosols as they have a high pH buffering capacity. We also considered one soil type (andosol) for our experiment. The pH adjustment period is expected to change based on different soils buffering capacity and should be taken into consideration for lime based experiments.

- In chapter 5 we considered only the plant required nitrogen recommendations for digestate and its derived fertilizers, due to the challenge associated with the use of organic fertilizers for precision nutrient application in agricultural soils. Resultantly, chemical fertilizer, where we considered plant required nitrogen, phosphorous and potassium requirements, had the highest plant yields compared to digestate and its derived fertilizers. Therefore, to improve yields it would be more practical to try and supplement digestate and its derived fertilizers with the appropriate chemical fertilizers.