Understanding the dynamics of soil microbial communities and gas emissions under different soil amendments

（異なる土壌改良資材施用が土壌微生物叢ダイナミクスとガス排出量に及ぼす影響評価）

北海道大学　大学院農学院

農学専攻　博士後期課程

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Abstract

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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Soil microbes play important roles in regulating the soil health. The addition of organic materials to the soils can improve the activities of the microbes. Specifically, soil microbes utilize carbon (C) from the applied organic materials to increase their abundance and activities. Contrastingly, soil microbes decompose the added materials and emit the C to the atmosphere as carbon dioxide (CO_2). Studies have shown that charred organic materials like biochar can store C in the soil and improve the microbial activities. However, research to verify the impact of biochar on microbial community under different biochar applications is needed. Also, compared to biochar, manure can support microbial activities but the factors regulating the variabilities in manure decomposition are underexplored. First study investigated the effects of different biochar materials on the bacterial community. Second study examined microbial community using network analysis. Third experiment examined the decomposition of cow dung and gas emissions.

A pot experiment was conducted to investigate the effects of different materials (chicken manure CM, rice straw RS, and rice husk RH) used to produce biochar on soil microbiome. The biochar was applied as single (CM, RS, and RH), combined form CM+RS, or CM+RH as mixed or surface under a dent corn. In results, surface applications increased the microbial diversity in the soil. This increase was attributed to the increased numbers of OTU such as *Actinobacteria* and *Proteobacteria* at the phylum level. Also, RS treatments impacted the microbial richness, and evenness under surface application. Thus, the effect on microbial diversities found in this study depends on the feedstock biochar, therefore biochar materials and application methods should be considered when interpreting its impact on the microbial community.

The second study was aimed at investigating the microbial community interactions among different biochar materials using network analysis. Statistical analysis investigating the co-occurrence of microbial taxa were evaluated. The analysis was performed in R software and the network visualizations and correlation statistics were carried out in Gephi software. The results showed dominate phyla to be *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* within the biochar materials. Further, rice husk biochar increased *Euryarchaeota*, while chicken manure biochar increased *Planctomycetes* in the soil. Therefore, variabilities of biochar feedstocks should be considered when choosing biochar type for soil amendment because different biochar materials have different impact on the microbial community structure.

The third experiment was carried out to investigate the soil and dung properties influencing the decomposition of cow dung and gas emissions. An incubation study was set-up with the dung and soil sampled from 15 different farms within Hokkaido, Japan. Gas emissions was also measured. During the incubation, samples were taken at three different timings (before, middle, and final incubation) for microbial analysis. Results showed that *Firmicutes* and *Bacteroidetes* were significantly decreased while *Proteobacteria* and *Actinobacteria* increased during dung decomposition. Also, in each location, there are differences in CO_2 emissions pattern which were categorized as high and low CO_2 emissions. Following this trend, higher numbers of OTUs were found in low CO_2 (3750) compared to the high CO_2 (3438). Further insight revealed that soil properties strongly influenced the emissions pattern based on the positive Pearson correlation coefficient between high CO_2 emissions and soil properties such as soil C, nitrogen, and CEC. These results indicate that soil properties were the strong determinant of dung decomposition and gas emissions.

Acknowledgement

I want to thank the almighty God for His protection, grace, and guidance in completing my studies and finishing up my thesis.

My special thanks to my supervisor Associate Professor Yoshitaka Uchida for his support and guidance throughout my study. I am grateful to Prof. Toshihiro Watanabe, Prof. Toma, and Prof. Ishiguro for their academic support.

I also wish to appreciate Uchida lab members (Chikae Tatsumi, Lin Jin-Feng, Yvonne Madegwa, Takamitsu Ohigashi, Akari Kimura, Akira Nagata, Gen Takahashi, Ruka Kiyama, Terumasa Yamamoto, Nao Ishige, Hiroki Nishimine, Kotaro, Nagisa Sugahara, Yu Hirata, and Anna Saito) for their support and assistance throughout my research.

I'm also thankful to my family and friends for their prayers and support.

My finally appreciation goes to the DX Hokkaido University Doctoral Followership (grant number JPMJSP2119) for their financial support during my doctoral program.

Table of content

Abstract i

Acknowledgement iii

Table of content iv

List of tables ix

List of figures x

Supplementary files xiii

Chapter 1 Introduction 1

1.1 Background 1

1.2 Research objectives 3

Chapter 2 Literature review 5

2.1 An overview of biochar 5

2.2 Biochar as a carbon-rich material 6

2.3 Effect of biochar on gas emissions 6

2.4 Biochar impact on soil microbial communities 8

2.4.1 Biochar properties that affect soil microbial communities 9

2.5 Biochar impact on microbial habitat 10

2.5.1 Water content 11

2.5.2 Soil pH 12

2.5.3 Aeration condition 14

2.6 Application rates and methods of biochar on soil microbes 15

2.7 Concept of organic manure application 16

2.7.1 Animal manure 17

2.8 Overview of manure decomposition 18

2.8.1 Factors regulating decomposition 18

2.8.2 Temperature 18

2.8.3 Moisture 19

2.9 Impact of dung on grassland 21

2.10 Changes in the soil physicochemical properties due to dung decomposition 22

2.11 Greenhouse gas emissions and dung decomposition 22

2.12 Microbial impact on dung decomposition 24

2.12.1 Community structure in dung and pasture 24

2.12.2 Microbial community structure in general 26

2.13 Microbial Network Analysis 27

2.13.1 Understanding network analysis 27

2.13.2 Purpose of microbial network analysis 27

2.13.3 Challenges of microbial network analysis 28

2.13.4 Different approaches to microbial network analysis 29

2.14 Research needs 31

Chapter 3 Response of bacterial diversity to different application methods of charred organic materials on sandy soil 33

3.1 Abstract 33

3.2 Introduction 34

3.3 Materials and method 36

3.3.1 Preparations of soil and biochar 36

3.3.2 Experimental set-up 37

3.3.3 DNA extraction, amplification, and sequencing 37

3.3.4 Measurement of bacterial abundance 39

3.3.5 Statistical analysis 40

3.4 Results 40

3.4.1 Soil bacterial diversity and abundance 40

3.4.2 Community structure analysis 44

3.5 Discussion 50

3.5.1 The impacts of biochar on soil bacterial abundance and diversity 50

3.5.2 Changes in soil bacterial community structure as mediated by application method 51

3.6 Conclusions 52

Supplementary 53

Chapter 4 Revealing the effects of different biochar feedstock on the microbial communities using network analysis 56

4.1 Abstract 56

4.2 Introduction 57

4.3 Materials and method 58

4.3.1 Statistical analysis 59

4.4 Results 60

4.4.1 Application method of biochar materials 60

4.4.2 Microbial interactions among all biochar materials 61

4.4.3 Microbial interactions among each biochar materials 65

4.5 Discussion 65

4.6 Conclusion 66

Chapter 5 Bacterial communities and soil properties influencing the variations in dung decomposition and gas emissions among Japanese dairy farms 67

5.1 Abstract 67

5.2 Introduction 68

5.3 Methods 70

5.3.1 Sampling sites and preparation 70

5.3.2 Gas sampling and measurement 71

5.3.3 Soil sampling for DNA analysis 72

5.3.4 Measurement of bacterial abundance 73

5.3.5 Statistical analysis 73

5.4 Results 74

5.4.1 Cumulative greenhouse gas emissions 74

5.4.2 Influence of selected soils and dung properties on gas emissions 74

5.4.3 Microbial community structure analysis 76

5.4.4 Changes in bacterial communities during dung decomposition 79

5.4.5 Relationship between evenness, richness, bacterial diversity, and abundance 81

5.5 Discussion 83

5.5.1 Differences in gas emissions 83

5.5.2 Bacterial community structure and gas emissions 84

5.5.3 Bacterial diversity and abundance impact on dung decomposition 85

5.6 Conclusions 86

Supplementary 88

Chapter 6 Summary and recommendations 91

6.1 Overall summary and future research recommendations 91

6.1.1 Response of bacterial diversity to different application methods of charred organic materials in sandy soil 91

6.1.2 Revealing the effects of different biochar feedstock on the microbial communities using network analysis 92

6.1.3 Bacterial communities and soil properties influencing the variations in dung decomposition and gas emissions among Japanese dairy farms 93

6.2 General comments 94

References.....95

List of tables

Table 3-1 Soil water pH mean ± standard deviation of the application rates and methods. CM – chicken manure, RH – rice husk, RS – rice straw, CM+RH – chicken manure + rice husk, CM+RS – chicken manure + rice straw and con – control..... 49

Table 5-1 Pearson correlation coefficient between CO_2 emissions, soil, and dung properties 76

Table 5-2. Pearson correlation coefficient between CO_2 emissions and relative bacterial abundance 81

Table 5-3. Pearson correlation coefficient between CO_2 emissions, evenness, richness, bacterial diversity, and abundance..... 82

List of figures

Figure 2-1 A conceptual model illustrating the direct micro-scale and indirect large-scale influence of biochar on microbial activities by altering soil properties and providing them with more habitat and extended niches. (Adopted from Gul et al. 2015)..... 10

Figure 2-2. Ecological function associated with dung decomposition adopted from Slade et al. 2017. 20

Figure 3-1 a) Bacterial richness and b) evenness among different materials. CM – chicken manure, RH – rice husk, RS – rice straw, CM+RH – chicken manure and rice husk, CM+RS – chicken manure and rice straw, and CON – control with two methods of application; mixed and surface. Two application rates 15 and 30 g kg−1 soil. The symbols *** (P < 0.001), ** (P < 0.01) and * (P < 0.05). ns = non-significant. Means ± SE of three replicates..... 41

Figure 3-2. Shannon diversity index with different treatments at 15 and 30 g kg^{-1} soil. For each treatment (except the control (con)), first two bars from the left represent the bacterial diversity at M15, mixed 15 g and M30, mixed 30 g while the last two bars to the right are S15, surface 15 g and S30, surface 30 g kg^{-1} soil. The materials are single applied chicken manure biochar (CM), rice husk (RH), rice straw (RS) and combined chicken manure + rice husk (CM+RH), chicken manure + rice straw (CM+RS), with two methods of application: surface and mixed; and a control without biochar. The symbols *** (P < 0.001), ** (P < 0.01) and ns = non-significant. The letters on the bar plot represent the interaction effect of methods within each material. Means ± SE of three replicates..... 42

Figure 3-3 Quantitative Polymerase Chain Reaction with different treatments at 15 and 30 g kg^{-1} soil. For each treatment (except the control (con)), first two bars from the left represent the bacterial abundance at M15, mixed 15 g and M30, mixed 30 g while the last two bars to the right are S15, surface 15 g and S30, surface 30 g kg−1 soil. The materials are single applied chicken manure biochar (CM), rice husk (RH), rice straw (RS) and combined chicken

manure + rice husk (CM+RH), chicken manure + rice straw (CM+RS), with two methods of application: surface and mixed; and a control without biochar. The symbols *** (P < 0.001), ** (P < 0.01) and ns = non-significant. The letters on the bar plots represents the interaction effect of rate within each material. Means ± SE of three replicates. 43

Figure 3-4 Principal Component Analysis (PCA) plot. Treatments: M15, mixed 15g; M30, mixed 30g; S15, surface 15g; S30, surface 30g kg−1 soil. CM, single applied chicken manure biochar; RH, rice husk; RS, rice straw; CM+RH, chicken manure + rice husk; CM+RS, chicken manure + rice straw; and CON, control without biochar. Results from PERMANOVA showed interaction effects of materials and method, materials, and rate (P < 0.001). 46

Figure 3-5 Volcano plots of differences in OTUs abundance i) application methods; mixed and surface, ii) rates; 15 and 30g kg−1 soil of each biochar materials. (a) CM, chicken manure, (b) RH, rice husk, (c) RS, rice straw, (d) CM+RH, chicken manure + rice husk, and (e) CM+RS chicken manure + rice straw. Volcano plots showing the distribution of OTUs abundance according to the adjusted P value (−log10 scale) and the log twofold change between mixed and surface, and rates. 48

Figure 4-1 Network analysis showing different biochar materials and their application methods..... 60

Figure 4-2 Network analysis visualization of the top abundant microbes with all the biochar treatments. The nodes were colored at phylum level and the connections represent Spearman correlations. The strength of correlation is defined by the color label with red indicating positive and green negative correlations respectively. 61

Figure 4-3 Network analysis visualization of the top abundant microbes with each biochar material a) chicken manure CM, b) rice husk RH, c) rice straw RS, d) chicken manure with rice husk CM+RH, e) chicken manure with rice straw CM+RS f) control. The nodes were colored at phylum level and the connections represent Spearman correlations. The strength

of correlation is defined by the color label with red indicating positive and green negative correlations respectively. 64

Figure 5-1 Cumulative (a) CO_2 and (b) N_2O emissions from soil and dung treatments among different farms. Means ± SD of four replicates. 75

Figure 5-2 Principal Component Analysis (PCA) a) dung and b) soil at OTU level based on the differences in emissions. Period: initial, middle, and final represents different sampling timing. Farms B, I, and O have low CO2 while C, H, and M have high CO2 emission..... 77

Figure 5-3 Venn diagram showing the number of shared and unique OTUs in a) dung and b) soil based on the high and low CO_2 emissions. Percentage values represent relative abundance of OTUs in each section. 78

Figure 5-4 Relative abundance of the dominate taxonomic groups at phylum level under high and low CO_2 and their changes during dung decomposition. 80

Supplementary files

Figure 3-S 1 Community structure with relative abundance of top 10 taxa representing the whole treatments. CM – chicken manure, RH – rice husk, RS – rice straw, CM+RH – chicken manure and rice husk, CM+RS – chicken manure and rice straw, and CON – control with two methods of application; mixed and surface. Two application rates 15 and 30 g kg−1 soil. Results from PERMANOVA showed interaction effects of materials and method, materials, and rate (P < 0.001) ……. 53

Figure 3-S 2i Different OTUs that were significantly impacted by the surface application. 54

Figure 3-S 2ii Different OTUs that were significantly impacted by the application rate ... 55

Figure 5-S1. Cumulative CO_2 emissions at 100 days of measurement from soil and dung treatments among different farms. Means ± SD of four replicates. Bars with * represents the farms selected for further analysis ……. 88

Figure 5-S2i. Unique OTUs that are specific to the a) high CO_2 and b) low CO_2 emissions under dung applied treatments …… 89

Figure 5-S2ii. Unique OTUs that are specific to the a) high CO_2 and b) low CO_2 emissions under control treatments ……. 90

Chapter 1 Introduction

1.1 Background

Improvement in soil carbon content is essential to strengthen the soil quality, increase food production and limits increases in atmospheric carbon dioxide emissions from the soil (Lal et al. 2004). Previous study by Minasny et al. (2017) emphasized the importance of sequestering organic carbon to the soil as a measure to mitigate climate change by taking the atmospheric carbon dioxide and convert it into soil carbon. Thus, increasing the soil carbon provides an additional benefit of improving the soil structure and conditions which influences many processes in the soil such as water retention and infiltration, root penetration, nutrient dynamics and soil organic matter (Rabot et al. 2018). Organic materials amendment has proven to increase the carbon content in the soil. Using biochar (a carbonated organic material) for instance, Blanco-Canqui et al. (2020) found an increase in carbon content from 7.25 to 14.07 Mg biochar carbon per hectare under corn plant after 6 years of application. However, biochar application can affect the soil microbial communities through changes in the soil properties. Specifically, biochar can increase the soil pH which is the key drivers of microbial community structure (Rousk et al. 2010). This shows that biochar applications can modify the soil microbial community structure (Santos et al. 2012), however, biochar impact in the soil can be influenced by the application method.

Soil microbiota play an important role in biogeochemical cycles and their capacity and interactions with soil factors regarding soil biogeochemical processes could provide an in-depth explanation into the soil functions (Ma et al. 2016). Therefore, network analysis based

approaches have been used to investigate co-occurrence patterns between microorganisms in different environments including soil (Matchado et al. 2021). Thus, there is a need to explore more inter taxa correlations to further understand the microbial community structures.

The dynamics of carbon storage to the soil also affects climate change and crop productivity (Li et al. 2007) and grassland ecosystem contains large amount of carbon. Conant et al. (2017) mentioned that increasing the areas covered by grassland improves soil carbon stocks. Specifically, they stated that improving grassland management can lead to carbon increase by 0.47 Mg C ha^{-1} yr^{-1}. However, animal grazing is the key factor controlling the storage of soil carbon in grasslands (McSherry & Ritchie, 2013). During grazing, the animals deposit large amounts of nutrients to the soil as dung or urine which are decomposed by microbes to release nutrients to the soil (Cai et al. 2017). The decomposition process of dung contributes to the release of greenhouse gases which solely depends on the microbial interactions. Moreover, the microorganisms consume the nutrients in dung, resulting in changes of the concentration of greenhouse gas emissions (Shakoor et al. 2020). In the end, due to the differences in animal upkeep and geographical site, there are variations in the microbial structures in dung released by the animals (Manyi-Loh et al. 2016). Therefore, investigating the dissimilarities associated with dung has not been properly documented.

Therefore, to clarify the above research needs, the following objectives were considered,

1.2 Research objectives

To determine the effect of different biochar materials and application methods on the microbial community structure; Chapter 3.

Explore changes in the microbial community structures using network analysis, Chapter 4.

To investigate the variations in bacterial community structures from cow dung during decomposition and greenhouse gas emissions within farms in Hokkaido; Chapter 5.

Thesis structure

The thesis is divided into 6 chapters.

Chapter 1. This chapter introduces the general background of the thesis relating to carbon sequestration and dung decomposition. The chapter also highlights the research needs that will be clarified in the present study.

Chapter 2. This chapter covers the literature review of the study by providing more detailed information regarding the effects of biochar applications and how it interacts with soil microbial communities through changes in soil physicochemical properties. The chapter also feature dung decomposition and the factors controlling microbial community interactions.

Chapter 3. This chapter reports a pot experiment that investigated the effect of different biochar materials on the microbial community structure. The study used three different materials (chicken manure, rice husk, and rice straw) to produced biochar and applied at 15 and 30 g per kg soil.

Chapter 4. This chapter explored the dynamic changes using network analysis and how the microbial community structure influenced different treatments.

Chapter 5. This chapter reports the variability of dung decomposition and the bacterial impacts under different farms in Hokkaido. In this study, dung and soil samples were taken from 15 different farms in Hokkaido and the decomposition process of the dung were monitored.

Chapter 6. This chapter summaries major findings from the researches and provides recommendations for future research.

Chapter 2 Literature review

2.1 An overview of biochar

Biochar is a carbon (C) rich material that is produced through thermochemical decomposition of a biomass (Cha et al. 2016). It also designates as a material that is applied to the soil to improve soil properties, restore soil functions and increase water filtration and C sequestration (Sarauer et al. 2019). A further insight by Oliveira et al. (2017) stated that biochar is a by-product of thermochemical conversion such as pyrolysis, gasification, torrefaction, and hydrothermal carbonization of carbonaceous biomass at a temperature of 300–900°C under low oxygen. Even though there are varying definitions among researchers, the general idea is that biochar is produced through heating of a feedstock and applied to the soil for the sole purpose of improving soil productivity. In addition, many agricultural waste materials can be used as feedstock to produce biochar (Wang & Wang 2019). The applications of biochar as soil amendment have raised a lot of concern due to its diverse activities in the soil. The physical properties of biochar can strongly affect their activities in the soil system and in addition, alter soil nutrient nutrient compositions. Moreover, the impact of biochar based on reportedly reviewed publications had seemingly improve soil structure and crop yield both in a laboratory (Gao & Deluca 2016; Sohi et al. 2010) and field study (Griffin et al. 2017). Thus, the stern advantages of biochar such as rich C content, high cation exchange capacity, increase surface area and stability structure contributes to its dominant functions in the soil.

2.2 Biochar as a carbon-rich material

The production process of biochar releases carbon dioxide (CO_2) through burning of a certain biomass, however, it also stabilizes a significant amount of C. Thus, the evaporation of water during heating of the biomass and the release of volatile components causes an increase in the relative fixed C content of the solid biochar produced (Weber & Quicker 2018). Therefore, using biochar as a soil amendment can sequester C in the soil for a long period of time. More specifically, Windeatt et al. (2014) found that biochar application pyrolyzed at 600°C sequestered 0.55 Pg CO_2 yr^{-1} in the soil. Such a C rich material could also lead to a withdrawal of CO_2 from the atmosphere. Moreover, due to the intense effect of global warming on the environment, much attention had been drawn on the use of biochar to reduce gas emission.

2.3 Effect of biochar on gas emissions

Burning of fossil fuels has recently increase the concentration of CO_2 which resulted to about forty percent of anthropogenic C emission in the atmosphere (Spigarelli & Kawatra 2013). On the other hand, different agricultural practices have also triggered an increase in gas emission thereby reducing the sustainability in agriculture. The use of synthetic fertilizers and pesticides by farmers to improve agronomic yield causes rise in gas emissions (Shakoor et al. 2021). Therefore, the need to transition to sustainable agricultural management which can boost crop productivity and at the same time reduce gas emission is very important. More efforts have been geared towards this approach by the recent involvement in the use of biochar.

The use of biochar as soil amendments has its own advantages such as rich C contents and high surface area which increases its chances of acceptance in the control of gas emission. Addition of biochar to the soil has been reported to reduce the concentration of atmospheric CO_2 (Paustian et al. 2016; Zhang et al. 2019). Particularly, due to the larger surface area and recalcitrant nature of biochar, it has been widely used in environmental application to sequester C to the soil which contributes to CO_2 gas reduction (Wang & Wang 2019). Gupta and Kua (2017) acknowledged the effect of larger surface area of biochar as a means to capture and store CO_2 through adsorption in its pores. They added that a surface area of 1 mm is capable of adsorbing CO_2 of 4.55 mmol g^{-1}. However, these properties are being influenced by the pyrolysis temperature. In general, biochar produced at a high temperature has higher surface area and C content compared to when it is produced at a lower temperature. This is basically due to the increase in micro pore volume that is caused by the removal of volatile organic compounds when pyrolyzed at a higher temperature (Wang & Wang 2019). Additionally, Yuan et al. (2014) found a significant increase in surface area of biochar from medicinal herb residues when pyrolysis temperature was increased from 300 to 700°C.

The C content in biochar also offers other additional benefits such as nutrient loss, increase soil fertility and agricultural productivity.

2.4 Biochar impact on soil microbial communities

Microbes are very important in the soil ecosystem because they enhance soil productivity and plant growth, and their activities and diversity are mostly enhanced in the presence of soil organic matter. For example, microbes are beneficial to plant growth through stimulation of nutrient supply that helps plants devise a certain mechanism to manage with the biotic and abiotic stresses in the plant soil interactions (Porter et al. 2020). Moreover, microbes that are related to plants can be used to overcome problems affiliated to soil salinity, fertility, degradation, and habitat loss (Mishra et al. 2016). Therefore, soil microbes are needed to achieve a sustainable agriculture and thus, environmental management.

Biochar had demonstrated different effects on soil microbial communities and functions. In fact, biochar has been recognized as a pioneer for beneficial microbes in the soil ecosystem. This is due to the reports by previous studies that biochar incorporation favors soil microbial growth through biochar's direct such as nutrient and indirect effect like immobilization of soil toxic ingredients to provide a desirable environment (Graber et al. 2014; Jeffery et al. 2011; Yang et al. 2019). Further stated, soil microbial communities respond to biochar application due to their increase in microbial abundance and activities through provision of an environment with ample aeration, water and nutrients (Ameloot et al. 2013; Gul et al. 2015; McCormack et al. 2013). However, the physicochemical properties of the applied biochar can alter the microbial activities in the soil. That is, the properties and biochar type play a significant role regarding how they interact with the soil microbes even though their properties change with its aging in the soil.

2.4.1 Biochar properties that affect soil microbial communities

Biochar improves soil physical qualities which is needed to enhance crop productivity. These changes in the physicochemical properties of soil contributes to the microbial activities in the soil. As illustrated in Figure. 2-1, biochar has both direct and indirect effects on the soil properties which are of importance to the soil microbial communities. Biochar provides some unique properties that are favorable to the soil microorganisms such as microbial habitat and increase in microbial abundance and activity.

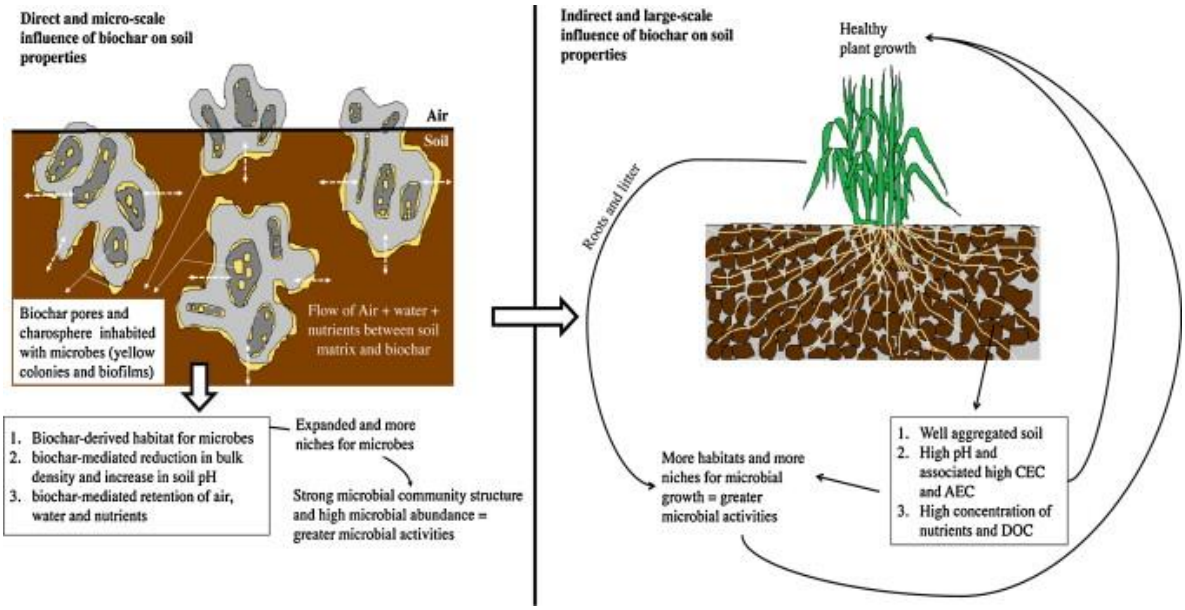


Figure 2-1 A conceptual model illustrating the direct micro-scale and indirect large-scale influence of biochar on microbial activities by altering soil properties and providing them with more habitat and extended niches. (Adopted from Gul et al. 2015)

2.5 Biochar impact on microbial habitat

The presence of pores structures in biochar provides a habitat for soil microorganisms and thus protect them from predatory soil microarthropods (Gul et al. 2015). Ameloot et al. (2013) added that the sorption of easily degradable organic compounds, dissolved organic matter, and chemisorption of ammonium at biochar surfaces within the presences of their functional groups considers biochar as the favorable habitat. Biochar contains both macropores, micropores, and mesopores respectively and each of them functions differently.

Basically, compared with other pores, biochar macropores which is >200 nm majorly protects the microbial habitat because they offer the right size to accommodate bacteria (Quilliam et al. 2013). Further, the presence of mesopores (2–50 nm) and micropores (<2 nm) contributes to storing of water and dissolved substances essential for microbial metabolism (Brewer et al. 2012). The pyrolysis temperature, aging, and biochar feedstocks also affects the pore sizes. For instance, Yang et el. (2021) revealed that the optimal temperature for producing porous biochar with bigger porosity was found at around 450°C when compared with lower temperatures. When Schnee et al. (2016) compared biochar made from different feedstock; mixed woods and miscanthus, they found that miscanthus biochar provided better habitat quality more than wood biochar. Also, Zhu et al. (2017) and Quilliam et al. (2013) noted that the colonization of microbes within the biochar pores improved around 3 years of biochar application. Therefore, biochar amendment has the capacity to alter the microbial habitat through changes in the soil properties that are beneficial to microbial growth. Such changes include water content, pH, and aeration condition.

2.5.1 Water content

Previous studies have recorded a positive impact of biochar on soil water content. For example, Abel et al. (2013) reported that biochar made from feedstock maize increased the water content at the permanent wilting point in sandy soil. Alternative to sandy soil, O'Toole et al. (2018) found a 5% increase in water content against control when miscanthus biochar was applied in a silty clay loam soil in a four-year field experiment. That being said, the capacity of biochar to store water is mostly attributed to its high porosity and surface area.

Therefore, the function of biochar surface charge and its porosity which enables the movement of water and nutrients within the biochar pores is needed to promote microbial growth and activity (Jaafar et al. 2014). The ability of the soil to retain water as a result of biochar amendment is necessary to maintain more stable microbial activities even when there's moisture change in the soil environment.

In contrast, there have been reports on the negative impact of biochar on soil water content. In this regard, Hardie et al. (2014) revealed that biochar made from acacia whole tree green waste had no significant effect on the soil moisture content or soil water availability when applied on a dark brown-black sandy loam. Additionally, Major et al. (2012) also reported no significant difference in soil water retention on clay soil. Also, Gaskin et al. (n.d.) found that at a lower application rate of 11 and 22 Mg ha^{-1} peanut hull pellets and pine chip pellets biochar, no significant effect was observed in water holding capacity. But on a higher rate of 88 Mg ha^{-1}, peanut hull pellets biochar increased water holding capacity in the soil. Therefore, it is necessary to acknowledge the diversities of biochar strength and activities in the soil.

2.5.2 Soil pH

Soil pH is the measure of acidity and basicity of a soil. Soil pH is very important because it gives information about the nutrient condition of the soil. It affects plant nutrient availability by controlling the chemical forms of different nutrient in the soil. Soil pH as described by Neina Dora (2019) is the "master soil variable" which influences the soil biological, chemical, and physical properties regulating plant growth and biomass yield. Soil pH strongly

influences the microbial activities in the soil and the required range of optimum productivity of microbes is 5.5–8.8 (Aciego Pietri & Brookes, 2008). Biochar has proven beyond measures to increase soil pH due to its alkaline nature. According to a meta-analysis conducted by Awad et al. (2018), they reported that biochar application instigated an overall increase of 11.8-16.0% in soil pH when compared to unamended treatment. The increase in soil pH as a result of biochar application is based on some reasons outlined by different studies; Gorovtsov et al. (2020), Gul et al. (2015), and Brewer and Brown (2012). First, biochar is alkaline and wood-based biochar tend to have higher pH than other sources of biochar. Although, the pyrolysis temperature plays an important role on the biochar pH due to biochar ash content. Secondly, due to the negative charged functional groups in biochar, it may attract the hydrogen ions, thus leading to a pH change in alkaline. Biochar feedstock and pyrolysis temperature works together to strongly influence pH in biochar. Consequently, this has been proven by Gaskin et al. (2008) when they compared three different biochar feedstocks namely, raw poultry litter, pelletized peanut hulls, and raw pine chips under two pyrolysis temperature 400°C and 500°C. they found that the biochar pH increased with temperature and the highest pH seen in pelletized peanut hulls compared to others. In addition, Wang et al. (2013) also reported an increase in pH with increasing temperature from 500°C to 700°C under wood and crop-based biochar.

Above all, previous studies have highlighted many significant changes in the microbial community because of pH change in soil. Specifically, Zhang et al. (2019) found that both in yellow-brown and fluvo-aquic soils, bacterial community was affected by the pH of the soil.

This was observed after peanut shell biochar pyrolyzed at 400°C with a pH background of 8.76 was applied. Further study by Rutigliano et al. (2014) reported a pH increase in acidic soil from 5.23 to 6.76 as a result of biochar application which supported microbial activity. Overall, these impacts of biochar on soil pH were basically recorded on acidic soil, showing that there can be a contrast to these findings. Following this argument, Zhang et al. (2019) found no significant effect of biochar on the bacterial community structure. This is because the soil they tested was an alkaline soil and biochar amendment rather showed a significant decrease in pH which led to their conclusion that biochar had almost no effect on the bacterial community structure under alkaline soil.

2.5.3 Aeration condition

Biochar can strongly impact the aeration condition of the soil which also affects the microbial activities in the soil. Gul et al. (2015) acknowledged in their review that biochar amendment can increase microbial abundance through providing an environment with ample aeration. Specifically, biochar enhances nitrification through improvement in aeration conditions which are favorable to nitrifying bacteria that converts ammonia to nitrate and thus minimizes gaseous nitrogen loss (Sanchez-Monedero et al. 2018). Sanchez-Monedero et al. (2018) also highlighted another importance of improved aeration conditions to favor methanotroph which is the bacteria that use methane as a source of C and energy.

Furthermore, biochar impact on soil aeration condition does not only favor microbial activities in the soil but also contributes to the reduction of gas emissions. This was reported

by Steiner et al. (2010) when they found a reduction in hydrogen sulfide of 58 and 71% under the application rates of 5 and 20% pine wood biochar. They attributed this change as the result of improved aeration in the soil due to biochar amendment. Even though biochar application has been successfully reported to enhance microbial abundance in the soil, therefore, there is need to understand the characteristics of the microbial community. In other to achieve this goal, network analysis has been used by researchers to gain more insight into the microbial community structure.

2.6 Application rates and methods of biochar on soil microbes

Generally, biochar applications to the soil influence microbial activities and community structure through changes in the pH, soil structure, and release of soluble C (Anderson et al. 2011; Lehmann et al. 2011). The efficiency of biochar in the soil can be influenced by its application rate. In this regard, Quilliam et al. (2012) did not found any significant differences in microbial growth between the biochar application rate of 25 and 50 t ha^{-1} residing on the soil for three years with the unamended soil, but a significant microbial growth was found when the application rates of biochar were doubled. This result could demonstrate that the increased application might have introduced more liable and diversified C sources consumed by the soil microbes (Xu et al. 2021). Also, Zhang et al. (2020) reported an increased abundance of actinomyces by 342.28% when 20 Mg ha^{-1} of corn stover biochar was applied to the soil. Furthermore, the biochar application method can pose different effect in the soil regarding their impacts on the soil microbes. Shen et al. (2016) mentioned that surface application of biochar may be an effective strategy to recover soil microorganism populations.

This was due to an increased basal respiration of 17.1 mg CO_2 kg^{-1} dry soil found in rice husk biochar compared to the unamended soil. Above all, biochar has been used as an amendment to improve soil quality and productivity because of its numerous benefits.

2.7 Concept of organic manure application

In general, manure application to agricultural soils serves as a source of nutrients and a method used to maintain soil organic C in the soil to mitigate climate change (Gross & Glaser, 2021). To efficiently control or manage gas emission from agricultural soils, it is necessary to pay rapid attention to the source of nutrient supplied to the soil. Compared to inorganic fertilizers which are manufactured artificially and contains minerals or synthetic chemicals, organic fertilizers referred to as plant or animal-based materials are more preferrable to enhance soil health. For instance, Rayne and Aula, (2020) stated that manures have beneficial effects on soil fertility and other soil properties that helps to improve soil health. On this note, manure is the collective term for different animal species, urine, plant materials and straw but also include livestock residues and human household waste (Gross & Glaser, 2021). A global data generated by Zhang et al. (2017) showed a steady increase in manure nitrogen production from 21.4 Tg N $year^{-1}$ in 1860 to 131.0 Tg N $year^{-1}$ in 2014 with an increasing annual trend of 0.7 Tg N $year^{-1}$. Among this increase, Zhang et al. (2017) also showed that on a global scale, cattle manure contributed most of the increase in manure nitrogen production, contributing about ~43.7% of the total manure nitrogen production in 2014, while one third of the global manure nitrogen was produced together by sheep and goats

respectively within the same year. Overall, manure is an important source of nutrients to agricultural soils.

There have been positive reports regarding the application of cattle manure as a soil amendment. In a long-term effect of manure application on cation exchange capacity conducted by Hao and Chang, (2002), they found out that cattle manure applied at the rate of 90 Mg ha^{-1} increased the cation exchange capacity by 5.6 cmol kg^{-1} under non-irrigated conditions while an increase from 19.6 to 33.5 cmol kg^{-1} was found under irrigated conditions at the same rate. Additionally, Nyamangara et al. (2001) found that cattle manure application to soil increased the water retention when compared to the control without manure. Above all, the soil microbial activities which contributes to the improvement of soil health can be enhanced through the addition of cattle manure according to Parham et al. (2003).

2.7.1 Animal manure

Animal manure as described by He et al. (2016) is the animal excreta comprising of urine and feces and bedding materials that are normally applied to the soils as fertilizer to enhance agricultural productivity. In addition to this and based on the particular management practices carried out in the farm, animal manure may also contain feed droppings, scurf, water, and soils basically from cattle and poultry manure, chicken feather, pig excrement, etc. (He et al. 2016; Q. Zhang et al. 2021). Generally, manure contains natural elements that promotes plant production and also, suitable for recycling in a natural environment (Abdellah & Li, 2020).

Particularly, manure is rich in C, nitrogen, oxygen, hydrogen, Sulphur, copper, zinc, and other nutritional resources according to Zeng et al. (2018). Even though manure application to agricultural soils could improve soil health and boost agricultural production, however, it also contributes significantly to greenhouse gas emissions. This occurs mostly during decomposition process of manure when microbes act on the available nutrient contents.

2.8 Overview of manure decomposition

2.8.1 Factors regulating decomposition

Decomposition is the process by which organic materials are broken down by microbial oxidation into simpler organic or inorganic matter. During decomposition of organic materials, most of the C present is lost as CO_2 into the atmosphere through microbial oxidation. Decomposition is driven by multiple factors that are being altered simultaneously due to the global environmental change and its sensitivity is dependent on the changes in temperature and moisture (Sierra et al. 2015).

2.8.2 Temperature

Temperature is an important factor regulating manure decomposition thereby affecting the microbial activities involved in decomposition. The change in temperature whether too high or too low influences the microbial growth and activities. Specifically, in a review by Mengqi et al. (2021) regarding the effect of temperature on decomposition, they reported that when temperature is less than 25°C, the microbial activity will decrease and in addition leads to decrease in decomposition rates. Consequently, Sierra et al. (2015) complied many

biogeochemical models targeted at evaluating temperature effects on decomposition rates and found that even at above 0°C, most functions diverge and predict a wide range of temperature effects on decomposition rates. In addition, they also added that the temperature range between 10 and 25°C were pronounced in enhancing decomposition rates. Overall, increase in temperature increases organic matter decomposition rates.

2.8.3 Moisture

Water content is an important factor influencing the existence and adaptability of microorganisms. For instance, when there is low moisture content (<40%), the microbial activity is affected and which results to decrease in decomposition and on the other hand, too high moisture content (>70%) also reduces organic matter decomposition. Thus, the recommended optimum moisture content is 55-65% (Guo et al. 2012; Mengqi et al. 2021). Nevertheless, Sierra et al. (2015) reported that the effect of moisture content on decomposition rates are not consistent. They further stated that decomposition can be affected both at low and high moisture content.

In a grazed dairy farm, the decomposition of livestock excreta such as cattle dung are being regulated by the two environmental conditions mentioned above. Moreover, dung decomposition is greatly needed to improve soil fertility and productivity. That being said, if appropriate decomposition of dung does not occur, there is high certainty that the farmers may incur a substantial number of economic losses arising from pasture recovery after grazing (Wall & Beynon, 2012). This is because a considerable amounts of nutrients in dung

can be transferred back to the soil in a form made available for plant uptake (Yoshitake et al. 2014). The decomposition of dung and its incorporation into the soil constitute a vital role in the ecosystem C and nutrient cycling and thus, stimulate microbial activity which results to the loss of dung C through microbial respiration (Menéndez et al. 2016). Slade et al. (2017) recorded the ecological functions associated with the decomposition of dung, recycling of nutrients between dung and plants, the fluxes of greenhouse gases from dung, and the microbial activity of dung and soil (Figure 2-2).

Thus, in other to utilize the use of cattle dung effectively, its contribution to greenhouse gas should be evaluated.

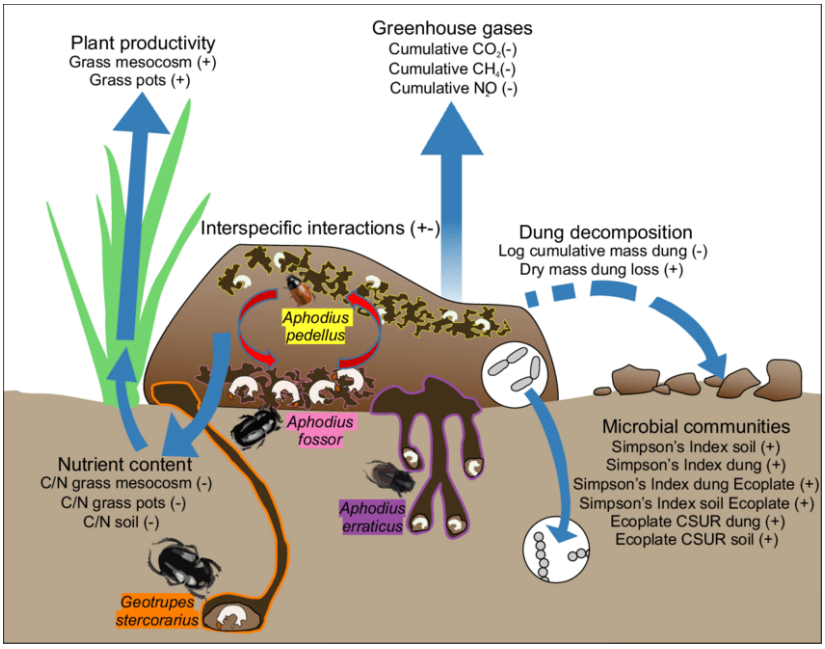


Figure 2-2. Ecological function associated with dung decomposition adopted from Slade et al. 2017.

2.9 Impact of dung on grassland

Grassland ecosystem cover about 40% of the earth terrestrial surface which have high inherent soil C and store 10-30% of the global soil organic C (Conant et al. 2001; Yoshitake et al. 2014). Grassland is reckoned on for food and forage production and in Japan just like all other temperate and humid regions use grassland for animal grazing (Yoshitake et al. 2014). In grassland, soil C can be determined by estimating changes in net C balance which is from C inputs and losses. For the C inputs, it can be through photosynthesis, decomposed dung, and fertilizer addition. While C can be lost through different processes including microbial respiration, biomass removal through grazing, and dissolved C through leaching and erosion (Mudge et al. 2011; Whitehead et al. 2018). In grazed grassland ecosystem, some of the nutrients ingested by the livestock are not utilized, about 60-99% of the nutrients are deposited to the soil as dung or urine (Cai et al. 2017). But the nutrients contained in the deposited dung varies because of the changes in the dietary composition and water composition (Cai & Akiyama, 2016).

Dung decomposition strongly influence grassland nutrient cycling. The process of dung decomposition provides a direct pathway for C and nutrients to enter the soils in a grazed grassland ecosystem due to the high concentration of C, nitrogen, and phosphorus contents in dung (Aarons et al. 2009). It has been reported previously that nutrient amendment as a result of dung decomposition impacted the composition of pasture species and enhanced plant growth (Bang et al. 2005; Yamada et al. 2007).

2.10 Changes in the soil physicochemical properties due to dung decomposition

Dairy cow excreta in the form of dung deposited during grazing and its application contributes to changes in the soil properties. The release of nutrients from dung during decomposition process greatly depends on its solubility and mobility (Aarons et al. 2004). Previous studies have extensively reported multiple impact of cow dung addition to soil properties. In this regard, Roy and Kashem (2014) found a significant increase in extractable nitrogen and electrical conductivity in a 60-day incubation study when cow dung was applied. Also, Ewulo (2005) recorded an increase in soil pH, organic C, nitrogen, phosphorus, calcium, magnesium, sodium, and cation exchange capacity under cattle manure in both clay and sandy clay soil. Above all, the chemical composition of dung and its C, nitrogen and phosphorus ratio strongly determines its rates of decomposition and nutrient release (Sitters et al. 2014). Nonetheless, in a grazed grassland, although dung decomposition contributes to the nutrient supply to the soil, it also represents a significant source of greenhouse gas emission.

2.11 Greenhouse gas emissions and dung decomposition

Globally, there has been an increasing attention to dairy farms regarding their impact on greenhouse gas emissions. The increasing rate of greenhouse emission is detrimental and threatens people's lives, food insecurity related to food availability, accessibility, utilization, and stability (FAO 2008). Thus, the potential impacts of the three main greenhouse gas emissions in the atmosphere CO_2, nitrous oxide (N_2O), and methane (CH_4) should be viewed on a larger scale of changing the environment and agricultural sector. In Japan, livestock

manure management produce an estimate of 2328 Gg-CO_2 eq. for CH_4 and 4768 Gg-CO_2 eq. for N_2O in 2008, while dairy manure contributed to 1878 Gg-CO_2 eq. (80.7%) for CH_4 and 619 Gg-CO_2 eq. (13%) for N_2O (Maeda et al. 2013). Therefore, understanding the related sources of gas emissions associated with livestock will provide an effective option for mitigation.

A couple of studies have reported the impact of dung on gas emission. Maljanen et al. (2007) and Flessa and Beese (2000) have found dung to be directly involved in greenhouse gas emission through increasing the availability of inorganic, organic nitrogen, and soluble C as a source of substrate for microbial metabolism. Further study to show dung impact on gas emission was presented by Lombardi et al. (2022). They reported that cattle dung contributed to the direct sources of greenhouse gas emission and according to them, the emission took place within a short period of one month after the dung was deposited. The decomposition of dung occurs in different ways to influence gas emission. Thus, dung can increase CO_2 production through microbial mineralization of organic matter that is present in fresh dung (Y. Zhu et al. 2020). While the organic nitrogen in the dung is mineralized into ammonium which will eventually change to nitrate with the aid of nitrifiers. Thus, the nitrate is transformed to dinitrogen through denitrifying bacteria and nitrous oxide will be finally produced as a byproduct of nitrification and denitrification process (Butterbach-Bahl et al. 2013; Cai et al. 2017; Lombardi et al. 2022). Moreover, these conversion processes are to a great extent influenced by the nutrient intake by the cattle.

2.12 Microbial impact on dung decomposition

In general, dung decomposition is controlled by the microbial characterizations both from the dung and soil. Moreover, the presence of dung provides different biological and physicochemical environment to the microorganisms (L. Wang et al. 2004) and thus, makes available nutrient supply to the soil. Decomposition of dung takes a longer period of time and literally requires a series of biological processes with interactions of microorganisms (Wu et al. 2011). Previous studies have shown that incorporating an additional material enhances dung decomposition. They reported that dung beetle plays an important role in dung decomposition by breaking down and transporting organic matter from the dung to the soil (Evans et al. 2019; Maldonado et al. 2019; Menéndez et al. 2016). Also, dung beetle can facilitate dung decomposition and alter soil properties through increasing nitrogen mineralization, ammonia volatilization and trigger dung microorganisms (Cheng et al. 2022; Iwasa et al. 2015). Microbial community play a significant role in the decomposition process and the relationship between similarities in microbial community composition and dung substrate usage relates to its decomposition (Slade et al. 2016). Thus, microbial interactions with dung can influence the amount of C inflow into the soil. This is important because dung represents the direct pathway by which C and nutrients enter the soil in the pasture ecosystem due to the nutrient content in dung (Aarons et al. 2009).

2.12.1 Community structure in dung and pasture

The productivity of pasture is dependent on some factors such as soil environment, water and nutrient availability, temperature, plant genetic, pasture management, and the soil

microbiome (Attwood et al. 2019; Harrison et al. 2016). Temperate grass and legume species makes up the mixture of pasture and the component of grass-legume microbiome consists of the symbiotic associated of *Epichloë* endophytes in grasses and *Rhizobium* in legume roots (Attwood et al. 2019). The effect of *Epichloë* on pasture has been reported by previous studies. Particularly, a pasture collapse was found when a ryegrass populated pasture contains strains of *Epichloë festucae* var. *lolii*. This was due to the minimal effect of *Epichloë festucae* on the insect pest black beetle which is a root feeding insect in grass (Popay & Hume, 2011). On the other hand, Thom et al. (2012) reported that ryegrass population was maintained when they contain *Epichloë* endophyte strain which is effective against black beetle.

In the dairy grazing farms, dungs are deposited on the pasture which are broken down by the combination of microbial, insect, and earthwork activity and the nutrient incorporated into the soil (Attwood et al. 2019). Generally, cattle manure is dominated by *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, and *Proteobacteria* (Callaway et al. 2010; J. Liu et al. 2016). Moreover, the type of diet consumed by the animal strongly influences their fecal microbiome (Shanks et al. 2011). These phyla can be affected during dung decomposition. In this regard, Ren et al. (2016) stated that during compositing of cattle manure, the relative abundance of *Proteobacteria* and *Firmicutes* which is 38.11% and 1.55% at the initial stage increased by 19.10% and 1.79% at the mesophilic phase of composting. They also found that during the early thermophilic phase, *Proteobacteria*, *Bacteroidetes*, and *Planctomycetes* were the dominate phyla and they concluded that temperature was a strong factor regulating the changes in the bacteria phyla.

2.12.2 Microbial community structure in general

Microorganisms contributes to many soil functions such as biogeochemical cycling, plant productivity, or climate regulations (Griffiths & Philippot, 2013). Soil microbial communities nurture thousands of species of bacteria and fungi per gram of soil and these have been proven through deoxyribonucleic acid (DNA) sequencing where millions of sequence reads are generated (Myrold et al. 2014). The application of this DNA sequencing can be used to explore the taxonomic diversity and composition of soil microbial communities using polymerase chain reaction (PCR) based approach which is focused on phylogenetically informative ribosomal genes (Buée et al. 2009; Roesch et al. 2007). And with the inclusion of barcoded primers or tag, provides more detailed information for describing the taxonomic composition of soil microbial communities (Hamady et al. 2008; Myrold et al. 2014). The extracted DNA yield and quality is very important due to its influence on the community structure (Thakuria et al. 2008). Several studies have shown that variations in the DNA extraction technique significantly influenced the microbial community structure (Carrigg et al. 2007; Luna et al. 2006).

Microbial communities are complex which makes it very challenging to study. Some molecular biological approaches are being used to gain better understanding into the microbial communities (Nakatsu, 2007). Co-occurrence patterns which can help define species identity, can be used to show how a particular organism in a system occur together

and differ with environmental parameters. Thus, this pattern provides details of a particular community structure which can be represented by a network (Fuhrman, 2009).

2.13 Microbial Network Analysis

2.13.1 Understanding network analysis

Network analysis is used to gain more understanding into the microbial complex interactions and thus helps to visualize and characterize microbial community structures. Poudel et al. (2016) added that network analysis provides a pathway to evaluate both direct and indirect interactions regarding community members and thus, offers a new perspective for analysis based on soil microbial communities.

Due to an interest in technology related to the microbial sequencing, microbial ecologists had explored several options to better understand the distribution and diversity of microbial taxa. These have proofed that microorganism from a vast diversity of taxa unknown could display distinct biogeographical pattern (Ma et al. 2016).

2.13.2 Purpose of microbial network analysis

In general, microbial network construction is an analytical tool used to analyze a massive data set. Specifically, it can recognize taxa sharing which is a common role in an ecosystem like cyanobacteria, an oxygen producing bacteria in a lake (Bush et al. 2017), can also link taxa to its function for instance C flux in the oceans (Guidi et al. 2016), and thus used to predict interactions (Durán et al. 2018). Also, Schmid et al. (2018) studied the impact of

manure and straw amendment on the co-occurrence of soil bacterial community using network analysis. Additionally, previous study suggested that network analysis can investigate the changes between soil microbial groups and their relationship with the soil parameters (Nielsen et al. 2014). Accordingly, studying the microbial community structure is quite burdensome due to the complexity of the interrelationships between the diverse microorganisms in the natural environment and thus can be explained through microbial network (Lu et al. 2021). Thus, the interactions between different species could be recognized to influence the key microbial communities and stability. Although microbial communities contain thousands of species coexisting and interacting with each other, an algorithm is needed to capture and describe the microbial ecological trends and consequently describe the structure and dynamics of microbial communities. Therefore, a computational expression of the microbial communities would initiate a better understanding of the factors responsible for community functions, stability, and resilience within the microbial communities (Cardona et al. 2016).

2.13.3 Challenges of microbial network analysis

Previous finding by Matchado et al. (2021) highlighted three main challenges that could affect the microbial relationships. First, microbial counts represent proportions instead of absolute abundances. Secondly, insufficient dataset can result to false association of microbes and thus, the presence of zero (0) could either mean absence of microorganisms or inadequate sequencing depth. And lastly, it is difficult to separate direct and indirect associations especially when they are based on environmental factors. Further, Karoline

Faust (2021) also highlighted other challenges in the use of microbial network analysis. In relation to the area of interest in this study, Faust (2021) mentioned that the challenges include invention and assessing a test for interaction driven community dynamics and relating it within the context of microbial network inference, comparing the performance of multiple preprocessing approaches over network inference tools and then evaluate which of the combinations works best. Additionally, investigating different methods to handle environmental factors and more understanding relating to the link between network and ecosystem properties. Also, microbes display a wide range of associations including linear, exponential, or periodic which makes the analytical approaches inefficient to detect them all. However, the ones that can are most likely to detect different functions with the same efficiency (Reshef et al. 2011).

2.13.4 Different approaches to microbial network analysis

There are proposed techniques in the study of microbial interactions. Barberán et al. (2012) investigated microbial interactions in soil microbiomes using correlation-based techniques such as the non-parametric Spearman correlation analysis. They further added that this approach was beneficial to explore an in-depth inter-taxa correlation which provide a better understanding of microbial community structure and the ecological rules guiding the community assemblage. Additionally, Lupatini et al. (2014) used Pearson correlation analysis to evaluate the associations between the microbial communities and describe how soil microbial community taxa interact with each other by detecting their positive and negative interactions. Furthermore, Brisson et al. (2019) developed an algorithm focused on

correcting habitat filtering effects on microbial correlation network analysis and found that their algorithm significantly improved correlation detection accuracy when they compared it with Spearman and Pearson correlations. Contrastingly, there have been complications regarding the correlation techniques. For instance, Weiss et al. (2016) evaluated eight different correlations analysis based on their strength and weaknesses and identify sparsity as the major significant unaddressed challenge amongst these approaches. Thus, they recommended filtering out extremely rare operational taxonomic units before network construction. Measuring correlation networks are computationally challenging due to the complexity of microbial communities because microbial data mostly have larger number of features (often more than 5000 features) which increases higher chances of two-feature correlations (Weiss et al. 2016).

An additional tools and software have also been recognized to evaluate the microbial correlation networks. For instance, CoNet was used by Faust and Raes (2016) to carry out microbial network inference from sequenced data. This app offers great features which considers the taxonomic levels and the environmental metadata necessary to interpret the microbial relationships. Local Similarity Analysis is used to detect non-linear, time sensitive relationships needed for correlation networks from time series data (Xia et al. 2013). Local similarity analysis was also used by Steele et al. (2011) to examine the relationship between the microbial populations and their environment by looking at the correlation among the operational taxonomic units over time. Quinn et al. (2017) have used a package implemented in R software called propr for network construction. They demonstrated that by default, propr

replaces all zero values in the data with one (1) during analysis which can be used to solve the issue when proportionality analysis failed after log-ratio transformation due to zero values. However, they noted that this should be properly considered to know the extent to which the zero values are replaced. SparCC (Sparse correlations for compositional data) is another tool designed to evaluate the correlation values from compositional data and the correlation estimated by SparCC measure the linear relationship between log transformed abundances (Friedman & Alm, 2012).

2.14 Research needs

The application of biochar to the soil has drawn rapid attention because of its alternative method in increasing long term soil C which also improves the soil quality and crop productivity. Also, previous studies have recorded a positive impact of biochar on soil microbial communities through changes in the soil physicochemical properties. Specifically, biochar can increase the soil pH as well as provide a habitat for microbial growth. The biochar impact on soil is dependent on the material used for pyrolysis. Studies that incorporated different biochar materials and application methods are limited. Therefore, research is needed to understand how biochar materials can be influenced by method of application. Additionally, the efficacy of biochar regarding their interactions with soil microbial community could offer more in-depth understanding on the choice of biochar as a soil amendment. This is because the soil microbial community is involved in the cycling of nutrients and C storage provided by biochar. Therefore, using network analysis, more insight will be provided on the microbial community structure interactions.

In addition to biochar, C from animal deposits contributes to soil C sequestration. In pasture ecosystems, livestock affects C balance through deposition of dung. Several studies have been conducted in dairy farms regarding dung decomposition and greenhouse gas emissions including the role of dung beetles in the decomposition process. Also, cow dung is known to harbor microbes which can be affected by its source and the animal management processes. However, information relating to the variations in dung and how microbes contribute to the dung decomposition is still scarce. Therefore, research is needed to fill in this gap by investigating the microbial communities influencing different sources of dung during decomposition.

Chapter 3 Response of bacterial diversity to different application methods of charred organic materials on sandy soil

3.1 Abstract

The use of charred organic materials (biochar) as soil amendment can alter soil nutrient, microbial abundance, and their diversities. These alterations can be influenced by the biochar source, application method and amount, but the details are still unknown. Thus, this study examined the effects of two methods of biochar application (surface or mixed) on microbial community under C-depleted sandy soil. Chicken manure (CM), rice husk (RH) and rice straw (RS), pyrolyzed at 350°C to produce biochar, were tested in a pot trial. The biochar was applied singly, and in combined forms (CM+RH and CM+RS) under dent corn, as mixed (incorporated) or surface application, at different rates of 0, 15, and 30 g kg^{-1} soil (equivalent to 0, 7.5 and 15.0 Mg ha^{-1}). Samples were taken at the end of the experiment to analyze bacterial relative abundance and community structure. Surface application of biochar increased microbial diversity on soil surface. The increase in diversity was characterized by an increase in OTU numbers within the phylum *Actinobacteria* and *Proteobacteria*. This study found that surface application of biochar increased the microbial diversities in the soil but was dependent on the biochar feedstock. Therefore, different biochar materials should be considered when interpreting biochar impact on the microbial community.

3.2 Introduction

Soil microbes contributes majorly to the recycling of nutrient content such as C and nitrogen in the soil. Their diversity is beneficial to maintain the variable processes within the nutrient cycles. Soil microbes depends on soil C for growth and functioning thus C must be supplied to the soils. The supply of C to soils can be achieved by plants and by organic amendments, including charred organic materials made from agricultural wastes (biochar). Soil microbes utilize the added C and release part of added C to the atmosphere through soil respiration but can also retain soil C in a form that is not easily decomposed (Liang et al. 2017).

Charred organic materials can also modify microbial diversity when applied to the soil (Abujabhah et al. 2018; Whitman et al. 2016). This is because charred organic materials can alter the soil properties such as soil pH, bulk density and moisture that would affect the bacterial communities (Wong et al. 2019). An increase in soil pH and water holding capacity favors the growth of microbes. Thus, these impacts of biochar on soil microorganisms are related to its material source and application rates, and soil type because specific biochars affects soil properties differently which in turn commensurate with the microbial communities (Abujabhah et al. 2018). However, this approach needs to be tested on a C depleted soil to observe their changes and efficiency after charred organic materials were applied.

The concept of the “efficiency” in the use of charred organic materials to modify C and nitrogen cycles needs to be studied because of the lack of organic resources to produce

biochar in certain regions. The application method of charred organic materials can also affect the relationship between biochar, CO_2 emissions and microbial biomass C. Thus, when the amount of available biomass for biochar production is not enough, the surface application of biochar might have better impacts to soils, compared to the application method aiming to uniformly mix biochar into the soils. The soil surface zone referred to as "pedoderm" as noted by Fey et al. (2006) is critically important to maintain the productivity of the soils as well as other soil health parameters, because of its direct interaction to sunlight and rainfall (Mills & Fey, 2004). Concentrating the charred organic materials on the surface zone might create an area with altered microbial biomass and diversities. Thus, the surface application of biochar may maintain (or improve) the positive impact of biochar with reduced application rates in the soil compared to the mixed application method.

The question remains whether we can improve the ability of charred organic wastes to increase microbial abundance and diversity, and to decrease nitrogen loss by optimizing the organic waste type and application method. Previous studies suggested that these positive characteristics of charred organic wastes positively depend on their application rates (Xu et al. 2016; Zhang et al. 2014). Thus, we speculated that the positive characteristics of charred organic wastes can be obtained even at relatively lower application rates by concentrating them on soil surfaces. To answer this question, we examined two different methods (mixed and surface) of biochar application to a C depleted sandy soil, regarding microbial biomass C with their corresponding impact on the bacterial community structure and abundance. We hypothesized that: 1) surface applied biochar can increase the abundance of microbes in soil

surface zone more efficiently when compared to the mixed application method at relatively lower biochar rate, 2) surface application of biochar can increase microbial diversity than when mixed in the soil.

3.3 Materials and method

3.3.1 Preparations of soil and biochar

A sandy soil was sampled at Ishikari, Japan (43.171°N, 141.316°E) in order to represent a C depleted soil. Topsoil (0–20 cm) was sampled using a shovel. Small portion of the sampled soils were air dried, analyzed and the following characteristics were determined; soil pH (6.6 ± 0.10), total C (0.53 ± 0.32%), total nitrogen (0.37 ± 0.02%), CEC (5.93 ± 3.26 me 100 g^{-1}), K (9.03 ± 0.47 mg 100 g^{-1}), P_2O_5 (3.80 ± 0.10 mg 100 g^{-1}), coarse sand (86.6 ± 1.07%), fine sand (3.22 ± 0.97%), silt (0.39 ± 0.18%), and clay (9.75 ± 0.14%), respectively (n = 3, errors were standard deviations). The soil was classified as sand based on the USDA system.

To produce biochar, three biomass materials; chicken manure (CM), rice husk (RH) and rice straw (RS) were used under a slow pyrolysis process. They were pyrolyzed using a Hi Cera Kiln (Nitto Kagaku Co. Ltd) under limited oxygen. The pyrolysis temperature was increased gradually starting from 100°C at the rate of 50°C 10 min^{-1} until the final temperature at 350°C. The final temperature was maintained for 2 h, before the furnace was turned off and the biochar allowed to cool at room temperature. The biochar samples were analyzed to determine the total C and nitrogen using a CN coder (Perkin Elmer 2400). The values were

22.3 ± 0.62% C and 2.63 ± 0.10% N, 45.3 ± 0.61% C and 0.68 ± 0.01% N and 56.2 ± 0.06% C and 1.31 ± 0.09% N for CM, RH and RS respectively.

3.3.2 Experimental set-up

A pot trial was prepared in the greenhouse in Hokkaido University, Japan (43.070°N, 141.340°E). Each pot (Wagner pot φ159mm × 190 mm) contained one kg of the sampled soil. The biochar was applied in two different methods; surface applied or mixed (incorporated) into the soil. Two different biochar application rates were used (15 and 30 g kg^{-1} soil, equivalent to 7.5 and 15.0 Mg ha^{-1}). Five biochar types were used, CM, RH, RS, "chicken manure biochar with rice straw biochar (CM+RS)" and "chicken manure biochar with rice husk biochar (CM+RH)". For the combined treatments, an equal amount of the two biochar types each was applied (e.g., 7.5 g each for 15 g kg^{-1} rates and 15 g each for 30 g kg^{-1} rates). The control pots (without biochar) were also prepared. All the treatments were replicated three times. This resulted in a total number of 63 pots (two application methods × two application rates × five biochar types (CM, RS, RH, CM+RS, and CM+RH) × three replicates = 60 plus three control pots). Dent corn was planted on each pot after one week of biochar application.

3.3.3 DNA extraction, amplification, and sequencing

Soil was sampled from the plant roots after the experiment and soil DNA extracted with PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturers protocols. The extracted DNA was purified with Agencourt AMpure XP

(Beckman Coulter) and the concentration measured using Promega Quantus™ Fluorometer (TM396J, USA). The DNA extracts were amplified through polymerase chain reaction (PCR) using primers (forward primer = 515F: 5´–GTGCCAGCMGCCGCGGTAA–3´, and reverse primer = 806R: 5´–GGACTACHVGGGTWTCTAAT–3´). For the PCR procedure, the samples were prepared with 10 µl of AmpliTaq Gold® 360 Master Mix (Applied Bio-systemsTM, Foster City, USA), 0.4 µl for both forward and reverse primers and 2 µl of DNA extract. The final volume was adjusted to 20 µl with nuclease-free water. The protocol used for the first PCR was 95°C for 10 min, then 30 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min. The final step was 75°C for 1 min. The first PCR products were then purified following the previous procedure.

The amplicon from the first PCR was used to perform second PCR and here the forward primer of 515F with a sequence Ion Xpress Barcode Adapters 1–63 kit (Life technologies) was attached to a specific sample to make it Ion Torrent for sequencing. The second PCR sample contained 10 µl of AmpliTaq Gold® 360 Master Mix (Applied Bio-systemsTM, Foster City, USA), 0.4 µl of specific forward primer, 0.4 µl of reverse primer (806F attached with the sequence of Ion P1 adaptor, Ion Torrent; Life Technologies) and 2 µl of purified first PCR product. Nuclease-free water was used to make up the final volume of 20 µl. The second PCR cycle was 95°C for 10 min, then 7 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min. The final step was 75°C for 1 min. The second PCR products were purified, and the DNA concentration measured using the same method as above. The length and concentration of the amplicons were determined through Bioanalyzer High Sensitivity DNA

Kit (Agilent Technologies, Palo Alto, CA, USA). The library was diluted to 50 pM using nuclease-free water. The library was loaded to the Ion PGM Hi-Q Chef Kit using Ion 318 chip (Ion Torrent Life Technologies, USA). The obtained sequence was further analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) software package version 2019.7 (Caporaso et al. 2010).

3.3.4 Measurement of bacterial abundance

The extracted DNA was purified and diluted 10 times with nuclease-free water for qPCR analysis. A standard curve was prepared from the first PCR product, a control treatment with 0 g biochar rate to determine the relative bacterial abundance among biochar treatments. For the measurement, samples were prepared with 10.4 µl of SYBR ROX, 0.8 µl of forward primer, 0.8 µl of reverse primer and 2 µl of DNA extract. Nuclease-free water was added to make up the final volume to 20 µl. The amplification temperature protocol was set-up as 95°C as the initial temperature for 30 s, annealing temperature 95°C for 35 cycles and the extension conducted for 30 s at 58°C. The final temperature was 72°C for 1 min with melting curve at 95°C for 1 min (Oka & Uchida, 2018).

3.3.5 Statistical analysis

For the relative amounts of soil bacterial DNA, first, the data was analyzed to determine the effect of biochar treatments by one-way ANOVA (biochar treatments vs. control). Then, for the biochar treatments, we performed three-way ANOVA, using the biochar application methods, biochar types and rates as factors, followed by Tukey's multiple comparison test at significant levels of $p < 0.05$. For soil bacterial community data at Operational Taxonomic Unit (OTU) level generated from QIIME 2, permutation multivariate analysis of variance (PERMANOVA) was used to determine the effect of biochar materials, application rates and method. Unique and shared OTUs related to mixed and surface biochar application respectively, were also identified. All statistical analysis was performed using R software version 3.6.2.

3.4 Results

3.4.1 Soil bacterial diversity and abundance

Regarding the diversity of soil bacterial communities, the effects of biochar application rates and methods were different depending on the materials used to produce biochar. RS exhibited increase in richness and evenness when applied at the surface compared to the mixed application but had no interaction effect with the application rates (Figure 3-1). Contrastingly, RH had no effect of application methods on richness and evenness, but the richness value increased with decreasing application rate. For Shannon-Wiener diversity under surface application, CM, RS and CM+RH were found with an increased bacterial diversity compared

to the mixed applied (Figure 3-2). The interaction effect of biochar material and application method (two-way ANOVA) was found in RS when averaged across the biochar types.

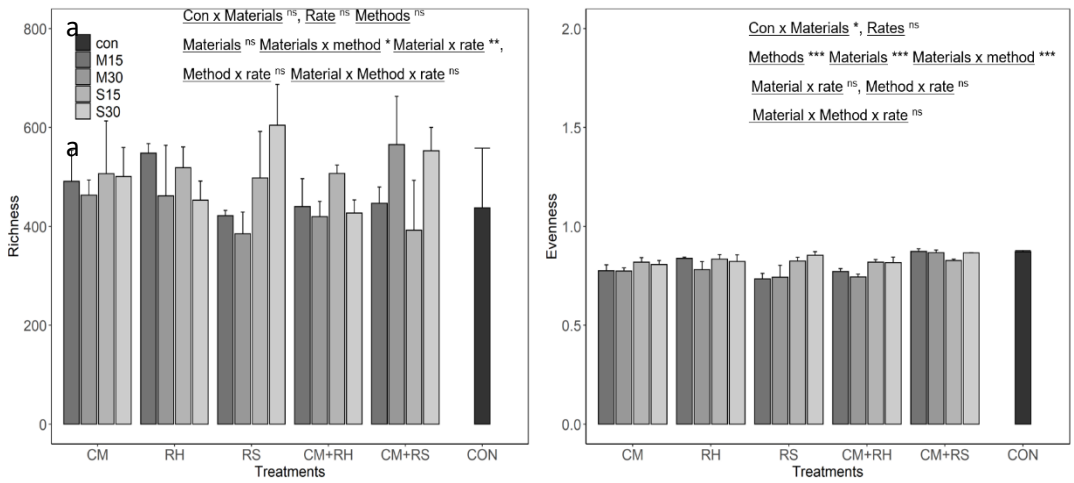


Figure 3-1 a) Bacterial richness and b) evenness among different materials. CM – chicken manure, RH – rice husk, RS – rice straw, CM+RH – chicken manure and rice husk, CM+RS – chicken manure and rice straw, and CON – control with two methods of application; mixed and surface. Two application rates 15 and 30 g kg−1 soil. The symbols *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$). ns = non-significant. Means ± SE of three replicates

The abundance of soil bacteria significantly increased with increasing application rate in RS treatment regardless of the application methods. For CM and RH, there was no impact of the application methods and rates. For CM+RH and CM+RS, generally bacterial abundance was

decreased in the surface applied when compared to the mixed applied, and the increasing application rates showed similar trend (decrease in bacterial abundance) (Figure 3-3).

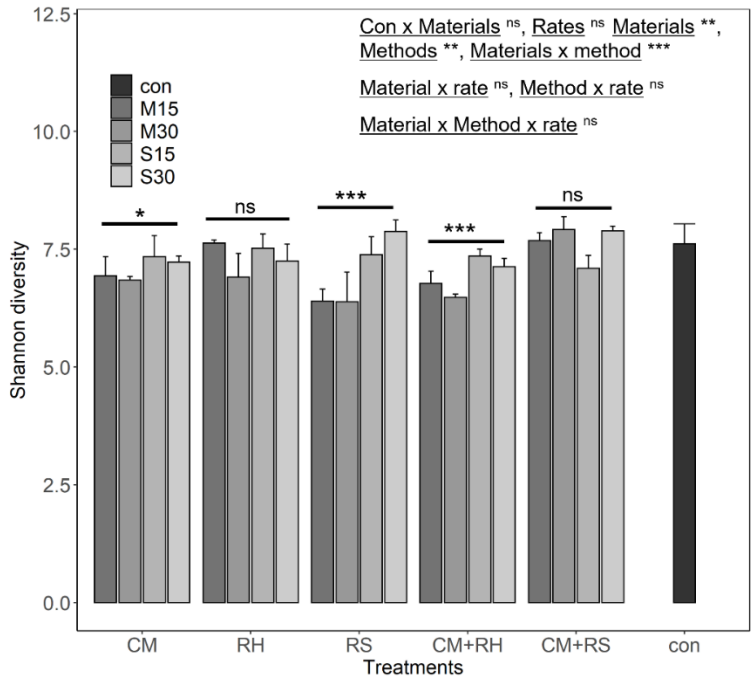


Figure 3-2. Shannon diversity index with different treatments at 15 and 30 g kg^{-1} soil. For each treatment (except the control (con)), first two bars from the left represent the bacterial diversity at M15, mixed 15 g and M30, mixed 30 g while the last two bars to the right are S15, surface 15 g and S30, surface 30 g kg^{-1} soil. The materials are single applied chicken manure biochar (CM), rice husk (RH), rice straw (RS) and combined chicken manure + rice husk (CM+RH), chicken manure + rice straw (CM+RS), with two methods of application: surface and mixed; and a control without biochar. The symbols *** ($P < 0.001$), ** ($P < 0.01$) and ns = non-significant. The letters on the bar plot represent the interaction effect of methods within each material. Means ± SE of three replicates.

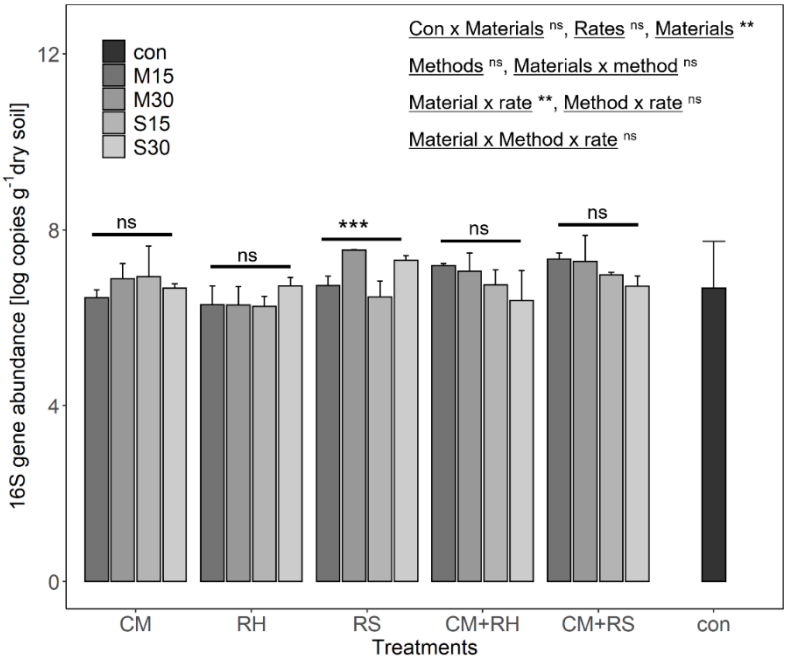


Figure 3-3 Quantitative Polymerase Chain Reaction with different treatments at 15 and 30 g kg^{-1} soil. For each treatment (except the control (con)), first two bars from the left represent the bacterial abundance at M15, mixed 15 g and M30, mixed 30 g while the last two bars to the right are S15, surface 15 g and S30, surface 30 g kg−1 soil. The materials are single applied chicken manure biochar (CM), rice husk (RH), rice straw (RS) and combined chicken manure + rice husk (CM+RH), chicken manure + rice straw (CM+RS), with two methods of application: surface and mixed; and a control without biochar. The symbols *** ($P < 0.001$), ** ($P < 0.01$) and ns = non-significant. The letters on the bar plots represents the interaction effect of rate within each material. Means ± SE of three replicates.

3.4.2 Community structure analysis

Regarding the impacts of the application methods and rates for the bacterial community structures of each material, a significant interaction effect ($P < 0.05$) was observed except in CM and RH (Figure 3-4). For RS, the bacterial communities between S30 and M30 were similar, whereas there was a clear difference between S15 and M15. Similar phenomenon was observed for CM+RS. Contrastingly, for CM+RH, the communities were similar between S15 and M15 but there was a clear difference in communities between S30 and M30 (Fig. 4).

Also, overall, the bacterial communities in RH and RS showed relatively higher proportions of *Proteobacteria* (> 30%) but decreased to less than 25% when combined with CM (Figure 3-S1). The control treatment also had a greater percentage of *Proteobacteria* than CM+RH and CM+RS with relatively smaller amount of *Euryarchaeota*.

The details of the OTUs in each biochar materials were shown in the volcano plots comparing mixed and surface, and application rates (Figure 3-5). Among the materials, differences in OTU numbers were found in RS treatment and surface applied displayed larger OTU counts compared to the mixed applied. In RS, 50 OTUs showed significant difference at surface application (twofold change > 2; $P < 0.05$, Figure 3-5ic), while CM+RS increased 37 OTUs at the surface compared to the mixed application (twofold change > 2; $P < 0.05$, Figure 3-5ie). Also, in CM+RH 8 OTUs was decreased at the surface application (Figure 3-4id), and

no significant changes were observed in CM treatment (Figure 3-5ia). RS increased the abundance of *Chloroflexi* and *Gemmatimonadetes* at the surface application (Figure 3-S2i). Further, 33 OTUs increased at 30 g kg^{-1} rate under CM+RH (twofold change > 2; $P < 0.05$, Figure 3-4iid). Increasing the application rates increased the relative abundance of *Proteobacteria* in RS and *Euryarchaeota* in RH and CM+RH, respectively (Figure 3-S2ii).

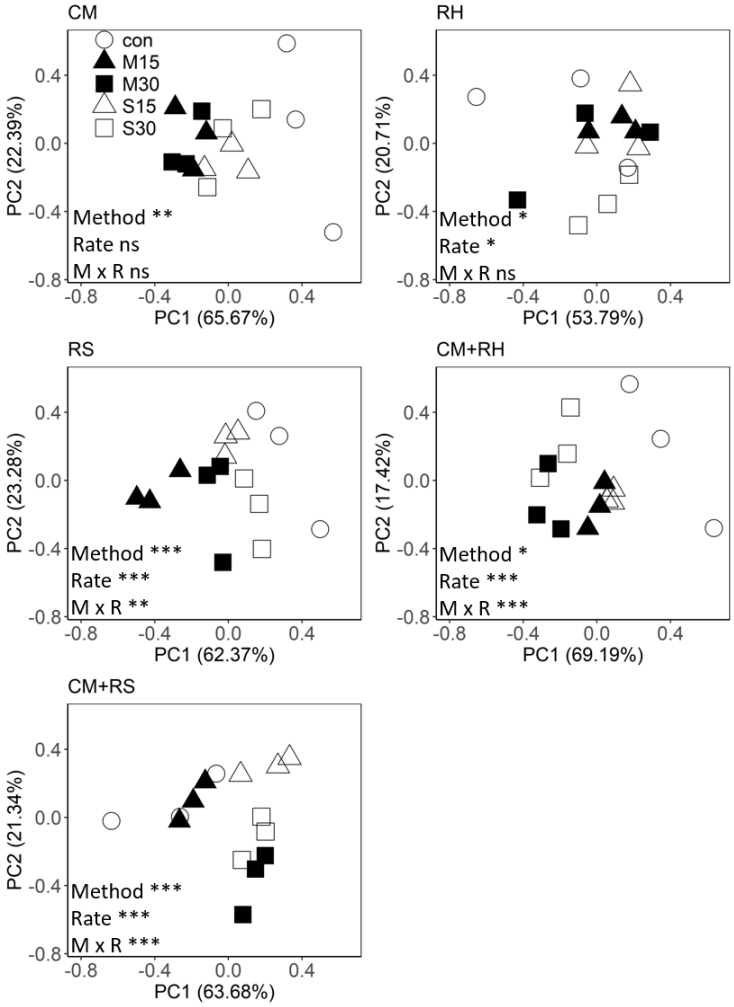


Figure 3-4 Principal Component Analysis (PCA) plot. Treatments: M15, mixed 15g; M30, mixed 30g; S15, surface 15g; S30, surface 30g kg−1 soil. CM, single applied chicken manure biochar; RH, rice husk; RS, rice straw; CM+RH, chicken manure + rice husk; CM+RS, chicken manure + rice straw; and CON, control without biochar. Results from PERMANOVA showed interaction effects of materials and method, materials, and rate ($P < 0.001$).

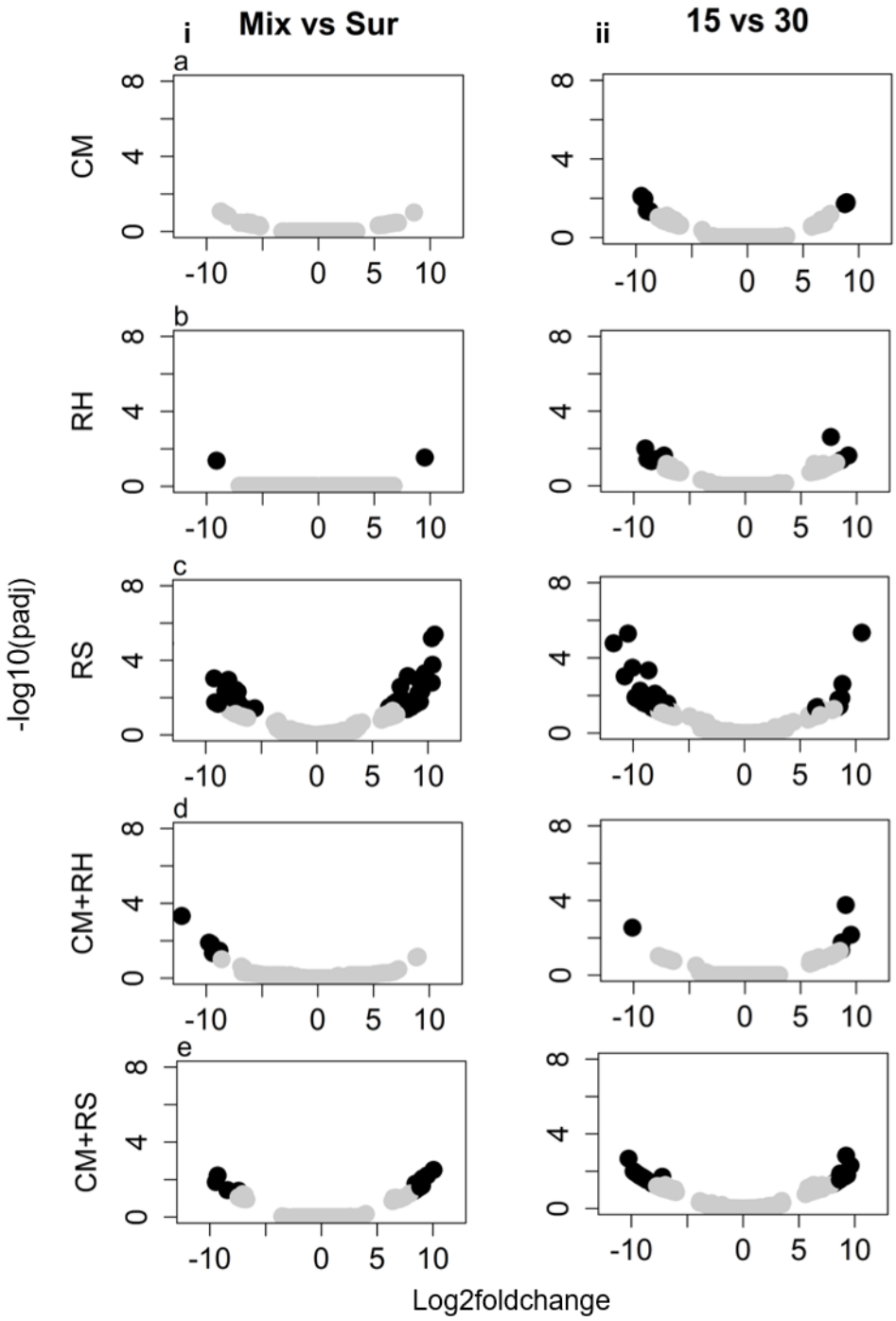

i
Mix vs Sur
ii
15 vs 30
a
b
c
d
e
CM
RH
RS
CM+RH
CM+RS
-log10(padj)
Log2foldchange
0
4
8
-10
0
5
10

Figure 3-5 Volcano plots of differences in OTUs abundance i) application methods; mixed and surface, ii) rates; 15 and 30g kg−1 soil of each biochar materials. (a) CM, chicken manure, (b) RH, rice husk, (c) RS, rice straw, (d) CM+RH, chicken manure + rice husk, and (e) CM+RS chicken manure + rice straw. Volcano plots showing the distribution of OTUs abundance according to the adjusted P value (−log10 scale) and the log twofold change between mixed and surface, and rates.

Table 3-1 Soil water pH mean ± standard deviation of the application rates and methods. CM – chicken manure, RH – rice husk, RS – rice straw, CM+RH – chicken manure + rice husk, CM+RS – chicken manure + rice straw and con – control.

Materials	Rate (g kg^{-1})	Mixed	Surface
CM	15	8.46 ± 0.16	8.05 ± 0.13
	30	8.80 ± 0.07	8.18 ± 0.11
RH	15	6.93 ± 0.14	7.16 ± 0.11
	30	6.77 ± 0.12	7.04 ± 0.11
RS	15	7.51 ± 0.05	7.12 ± 0.03
	30	7.73 ± 0.10	7.75 ± 0.36
CM+RH	15	8.07 ± 0.05	7.46 ± 0.06
	30	8.26 ± 0.14	8.02 ± 0.02
CM+RS	15	8.13 ± 0.12	7.70 ± 0.17
	30	8.46 ± 0.06	8.14 ± 0.18
CON	0	7.01 ± 0.07	

3.5 Discussion

3.5.1 The impacts of biochar on soil bacterial abundance and diversity

Biochar application did not increase the abundance of bacteria in the soil than the unamended soil, based on our qPCR approaches (Figure 3-3). It is difficult to fathom why qPCR-bacterial quantification showed no impacts of biochar while soil microbial biomass C showed a clear increase with the application of biochar. As a reason, we note that the DNA based approaches to evaluate microbial biomass in soils with and without biochar can have certain disadvantages because Hale and Crowley (2015) noted that biochar cation exchange properties can lead to DNA adsorption through cation bridging and the biochar can also contaminate extracted DNA with inhibitors of PCR. Further studies are needed to evaluate the relationship between soil microbial biomass C and qPCR-based bacterial abundance (i.e., copy number of 16S rRNA per gram soil). Contrast to our results, Yao et al. (2017) reported an increase in bacterial abundance resulting from increase in soil pH after biochar addition, but an increase in soil pH as observed in CM and CM+RH did not reciprocate in the bacterial abundance in our study. Therefore, we suggest that the relationship between soil pH and bacterial abundance should be monitored depending on biochar types. Further, we found an increase in the relative abundance of soil bacteria with increasing application rate only for the RS treatment. Thus, some biochar materials might increase soil microbial biomass even when they are applied at relatively lower rates, although this needs to be further studied.

Our study showed that different biochar materials had different effects on bacterial richness and evenness (Figure 3-1). Based on the application method, RS under surface application

had an impact on the bacterial richness and evenness while CM+RS only affected the richness at higher application rate. The different indices among the biochar material could be explained with the high C content availability in RS. Also, regarding the biochar properties, a study by Li et al. (2020) demonstrated that high volatile content in biochar produced at a temperature under ≤700°C can contribute to increased bacterial diversity which is probably associated with the differences in biochar material. In Shannon diversity, CM, RS and CM+RH contributed to increase in diversity. This could be related with the significant increase in soil pH (Figure 3-2; Table 3-1). As reported by Chen et al. (2015) who observed a greater increase in bacterial diversity under biochar soil amendment as a result of increased soil pH in the range of 5.99–6.29 when compared with unamended soil. Also, a previous study by Lauber et al. (2009) found that the overall phylogenetic diversity of bacterial communities correlated with soil pH and the greatest diversity was found in the soils with near-neutral pHs. The soil pH from the current study ranged from 6.77–8.46 which fall within the range reported by Lauber et al. (2009).

3.5.2 Changes in soil bacterial community structure as mediated by application method

Visualization using volcano plots to understand the effects of different biochar materials on the community structure as regards to the application methods revealed that RS increased the OTU count of *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, and *Gemmatimonadetes* respectively (Figure 3-S2i). These taxa that were largely promoted on the surface application of RS had been noted for their function to colonize nutrient rich environment, and also ecologically important in the turnover rate of organic matter (Xu et al. 2016). Also, the

increase in the relative abundance of the microbes is related to the C content and higher biomass C found on the topsoil (Chen et al. 2021). In addition, *Chloroflexi* and *Euryarchaeota* were influenced by application rate which demonstrated that their abundance was sensitive when biochar amount was increased in RS treatment (Figure 3-S2ii). However, CM+RS treatment promoted the increase in relative abundance of *Chloroflexi* but not *Euryarchaeota*. Overall, surface application of RS facilitates microbial growth compared to when mixed in the soil and its rate dependent, however, this could be monitored in a field application to understand its effect on the bacterial abundance.

3.6 Conclusions

Microbial diversity was increased when biochar was applied on the soil surface particularly due to the increase in *Actinobacteria* and *Proteobacteria*. This study suggests that different charred organic materials and application methods should be considered based on their contrasting impacts on soil microbial community composition. This is because of the different pore sizes and nutrient distribution of the charred organic materials which influences their interaction with the soil microbes.

Supplementary

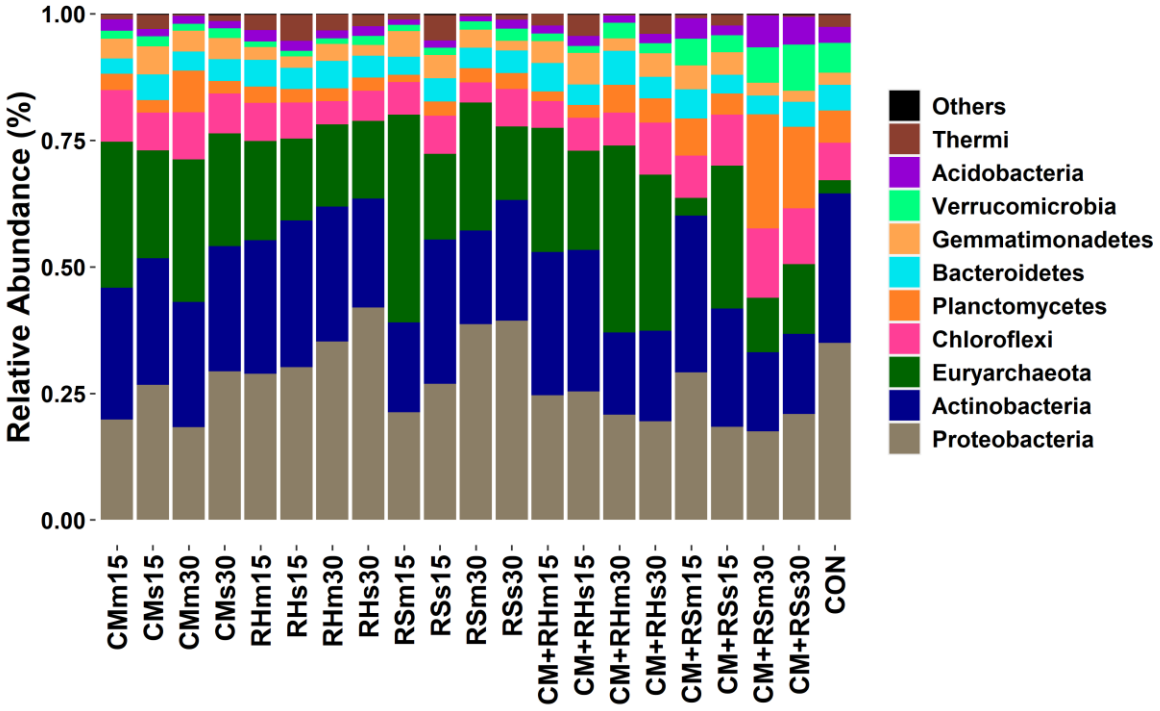


Figure 3-S 1 Community structure with relative abundance of top 10 taxa representing the whole treatments. CM – chicken manure, RH – rice husk, RS – rice straw, CM+RH – chicken manure and rice husk, CM+RS – chicken manure and rice straw, and CON – control with two methods of application; mixed and surface. Two application rates 15 and 30 g kg−1 soil. Results from PERMANOVA showed interaction effects of materials and method, materials, and rate ($P < 0.001$).

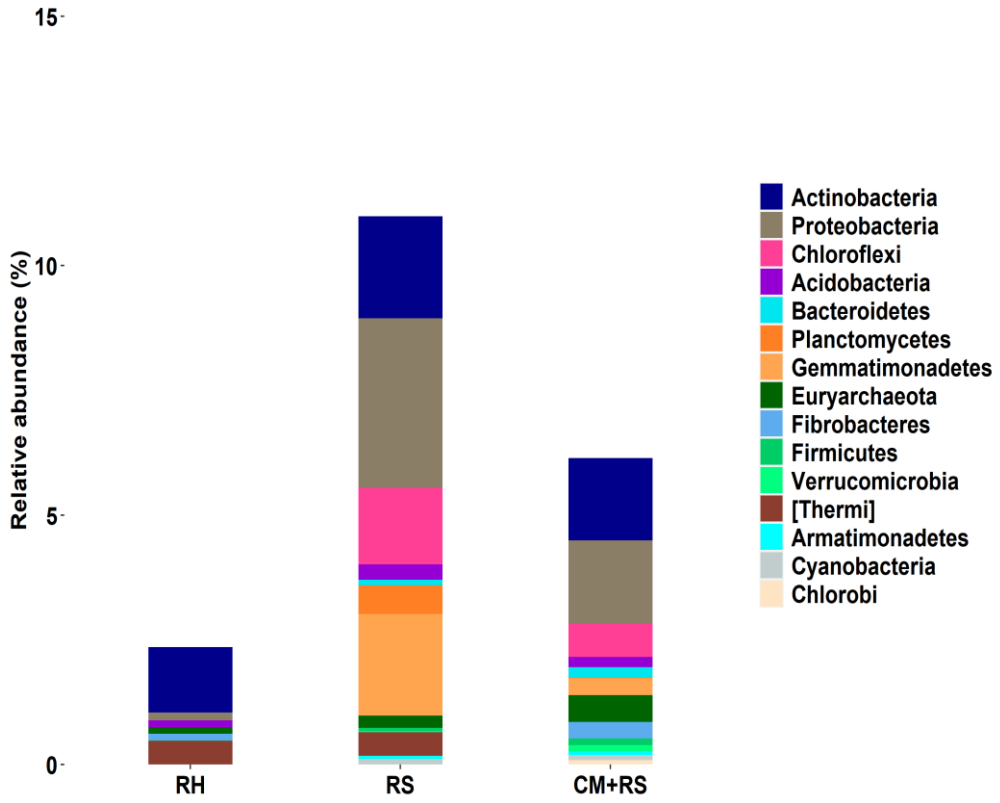


Figure 3-S 2i Different OTUs that were significantly impacted by the surface application.

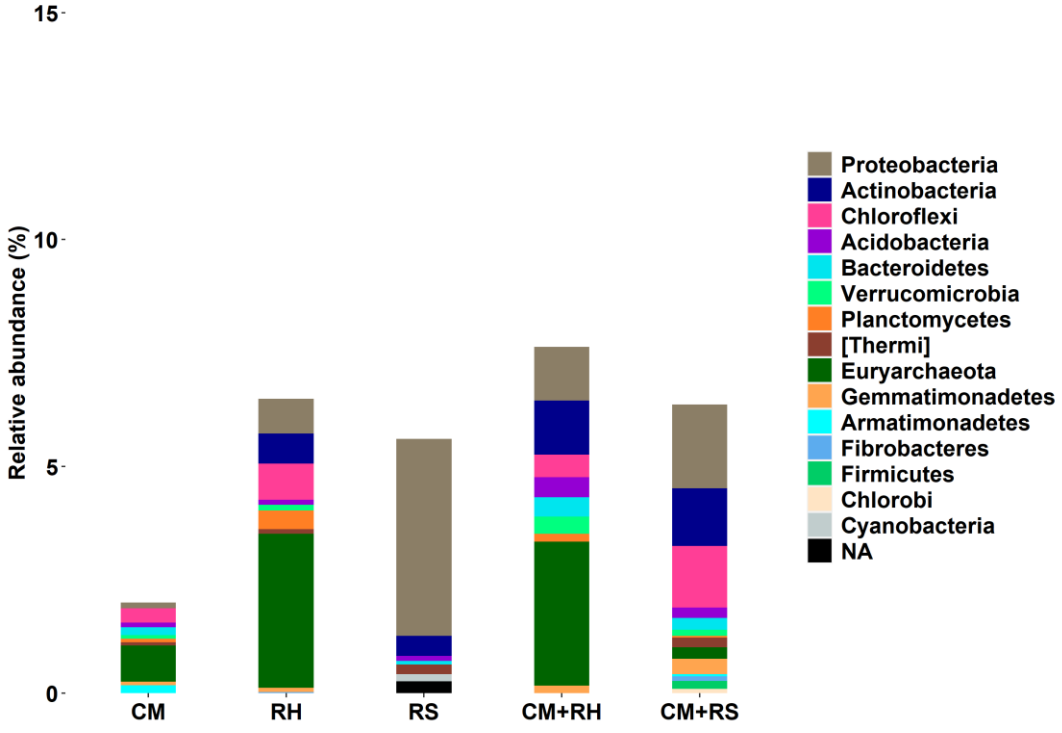


Figure 3-S 2ii Different OTUs that were significantly impacted by the application rate.

Chapter 4 Revealing the effects of different biochar feedstock on the microbial communities using network analysis

4.1 Abstract

The use of biochar as an amendment can improve the soil physicochemical properties and thus create a favorable environment for the microbial interactions. This effect can also be altered regarding the biochar materials and the rate applied to the soil. Therefore, this study investigated the changes in the microbial communities following the application of biochar using network analysis. Using Gephi for data visualization, the results showed that *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* were the phyla consistently predominating in the biochar treated soils. Further, RH biochar increased the abundance of *Euryarchaeota* compared to other treatments while CM+RS increased *Planctomycetes*. Therefore, variabilities of biochar feedstocks should be considered when choosing biochar type for soil amendment because of their different impact on the microbial community structure.

4.2 Introduction

Biochar has gained recognition due to its impact to the soil. Particularly, using biochar as soil amendment can improve the soil nutrient content, physical and biological properties in the soil (Blanco-Canqui, 2017; Hossain et al. 2020; Liu et al. 2018). Also, biochar can improve soil fertility through increase in the cation exchange capacity, surface area and water retention in soil pores which aids to decrease nutrient leaching from the soil (Rutigliano et al. 2014). Thus, the use of biochar has been beneficial, a way to properly recycle and dispose organic waste and represent an effective method to increase the soil nutrients (Galvez et al. 2012).

Biochar can improve the microbial population and activities (Luo et al. 2017), however, there has been variations in the soil microbial communities due to biochar application. Biochar could provide a habitat which serves as a refuge for soil microorganisms like bacteria ranging from 0.3 to 3µm (Gul et al. 2015). Quilliam et al. (2013) found that biochar macropores provides the safest place for microbial habitat. These biochar pores are dependent on the temperature of pyrolysis where higher temperature biochar results in larger pores (Gul et al. 2015). Additionally, the volatile fraction of biochar contributes to the C sources utilized by the microorganisms in a fresh applied biochar (Stewart et al. 2013). Furthermore, studies have shown an increased (Domene et al. 2014) and no effect (Rutigliano et al. 2014) of microbial biomass in soil after biochar amendment. Notwithstanding, biochar favors the growth of microorganism in the soil, however, there are still needs to further study its interactions with the microbes.

Biochar material is an important factor to consider due to their variability in adjusting the soil microbes. Related studies have recorded that biochar efficiency in the soil are feedstock dependent. More precise, Huang et al. (2017) found that the bacterial abundance increased when rice straw biochar pyrolyzed at 600°C was applied at 10 mg kg^{-1} biochar. Also, using chicken manure biochar have been reported to improve the microbial habitat in the soil (Meier et al. 2017). Therefore, understanding the effects of different biochar materials on the microbial community changes is needed for further study. Previous study found that network analysis of co-occurrence can be used to investigate complex microbial communities regarding their correlations between microbial taxa abundance which will provide more insight beyond sample level comparisons (Qiu et al. 2019).

Because microbial community is an important aspect of ecosystem services and thus, supports functioning of the soil when organic materials are applied. Therefore, this study investigated the effects of different biochar feedstock on the microbial community changes using network analysis approach. Among the three different biochar materials used for this study, the hypothesis was that each material would influence the microbial community differently.

4.3 Materials and method

This study was solely based on analytical methods using the sequenced data obtained from chapter 2 experiment. The treatment structure comprised of chicken manure biochar (CM),

rice straw biochar (RS), rice husk biochar (RH), chicken manure with rice straw biochar (CM+RS), chicken manure with rice husk biochar (CM+RH), and control without biochar. Two application method which is mixed and surface application. In the previous experiment, different biochar materials reacted differently in the soil. Therefore, network analysis would enhance more insight into the microbial communities in each biochar materials.

4.3.1 Statistical analysis

The biochar data at OTU level were analyzed to see the treatments interactions considering the application methods and rate using network analysis. Further analysis investigating the co-occurrence of microbial taxa at the phylum level were also evaluated. At first, phyloseq was prepared using table.qza, rooted-tree.qza, taxonomy.qza obtained from QIIME 2 analysis and the experimental metadata. The phyloseq was converted to microbial ecology object and Spearman was used to determine the correlation coefficients. SparCC which required SpiecEasi was used to estimate the correlations and the network was constructed using igraph package (Csardi & Nepusz, 2006). The network was saved with rgexf package. All analysis were performed in R software and the network visualizations and were carried out in Gephi software (Bastian et al. 2009; Grandjean, 2015). In the Gephi software, the network was visualized with Fruchterman-Reingold and the phylum level displayed with the betweenness centrality.

4.4 Results

4.4.1 Application method of biochar materials

There was a clear interaction between the biochar materials using network analysis (Figure 4-1). From this analysis, the distance 0.24, 0.28, 0.32, and 0.36 designate strong, moderate, fair, and weak correlations respectively. CM mixed showed a strong correlation with the RH mixed and a fair correlation with CM+RS and no correlation with CM+RH (Figure 4-1).

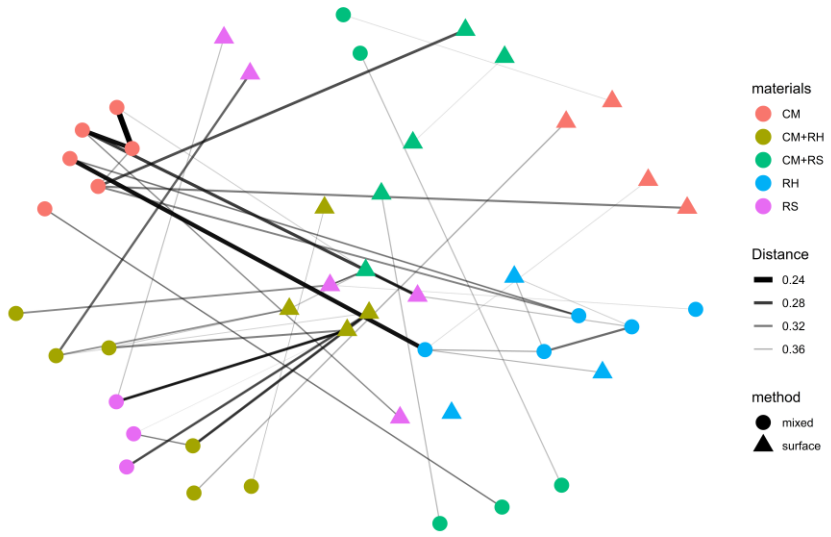


Figure 4-1 Network analysis showing different biochar materials and their application methods

4.4.2 Microbial interactions among all biochar materials

The microbial interactions showed variations in the correlation after network analysis when viewed with all the biochar materials. At phylum level, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Euryachaeota*, *Bacteroidetes*, *Acidobacteria*, *Gemmatimonadetes*, *Fibrobacteres*, *Verrucomicrobia*, and [*Thermi*] occupied 37.61, 20.51, 10.26, 5.13, 4.27, 4.27, 4.27, 3.42, 2.56, 2.56, and 1.71% respectively (Figure 4-2). Strong correlation existed among *Proteobacteria*, *Actinobacteria*, and *Chloroflexi*.

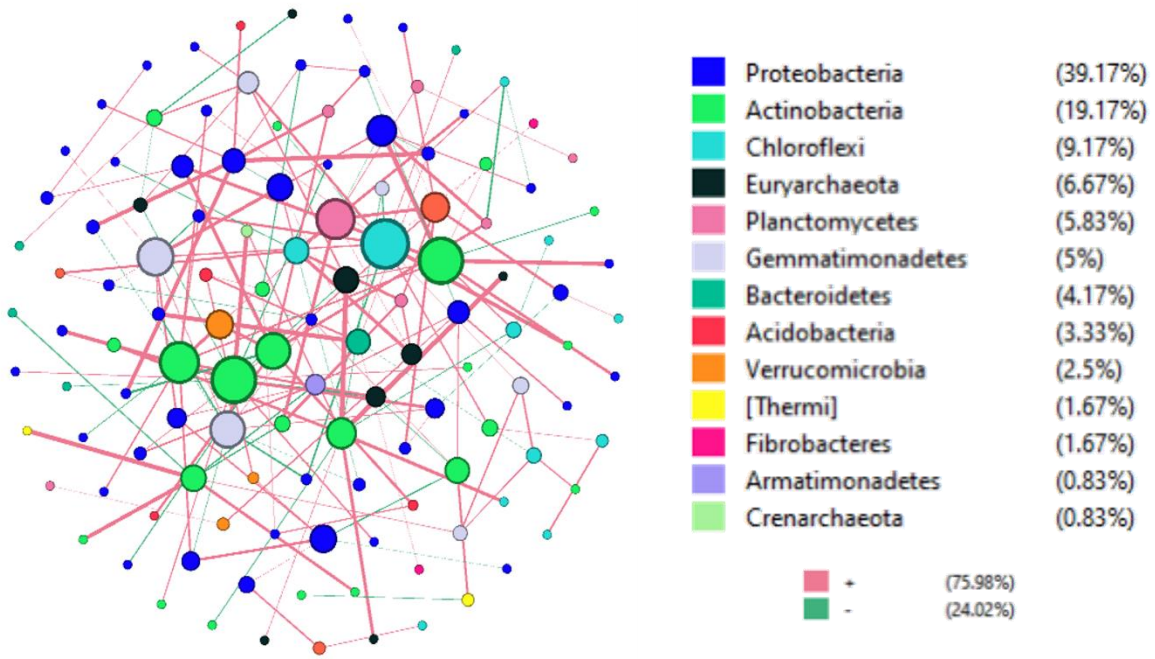
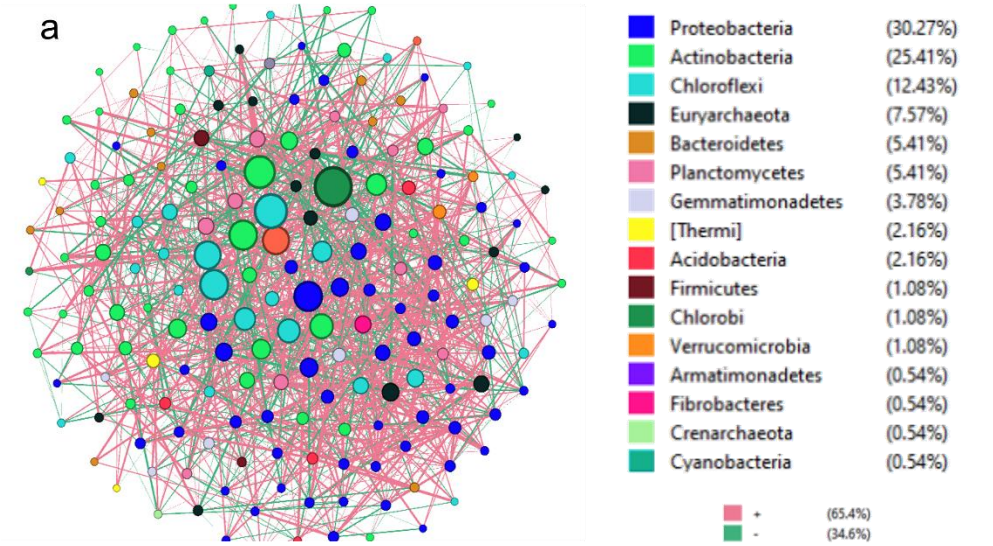
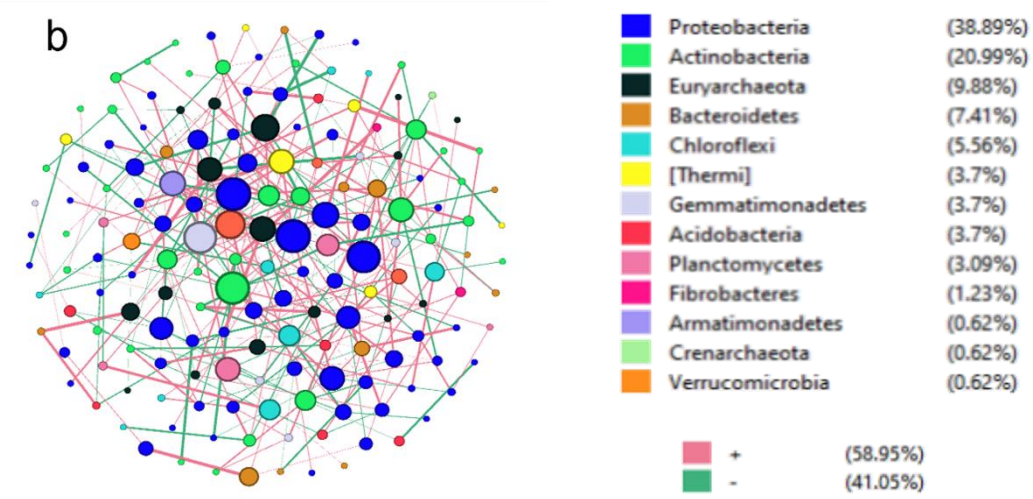
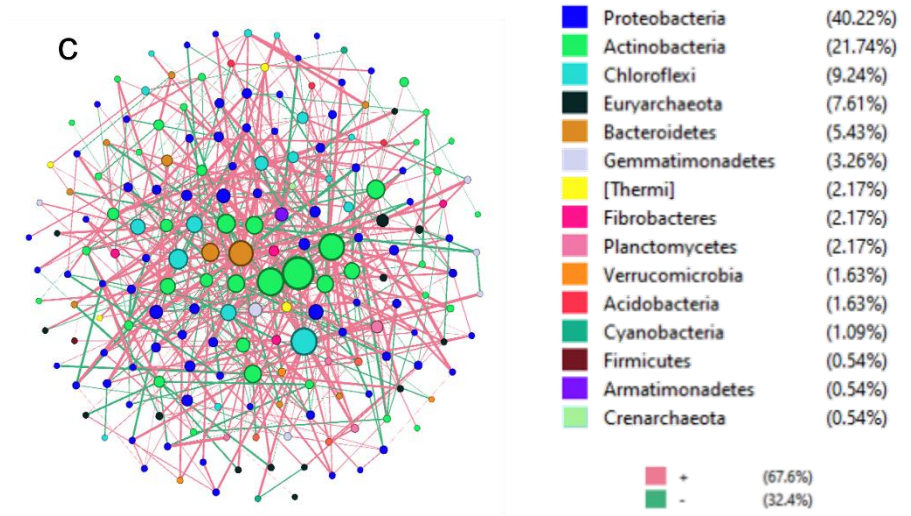
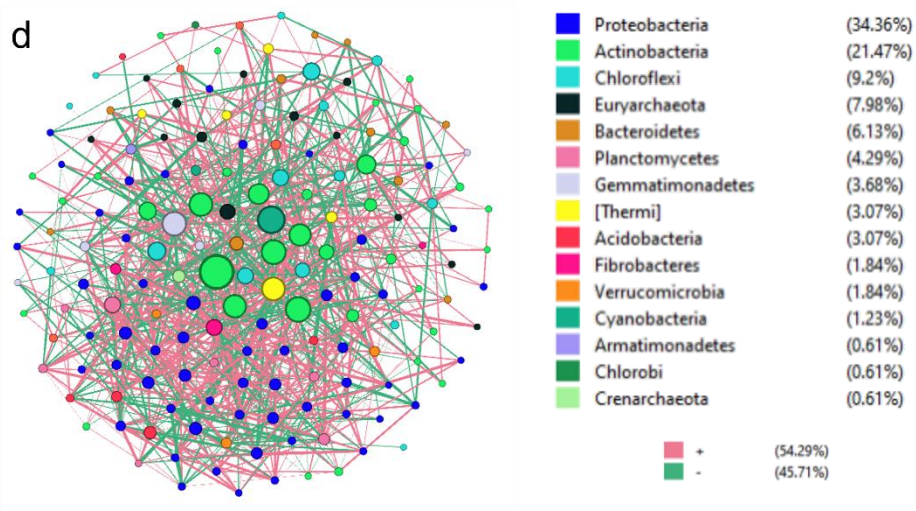


Figure 4-2 Network analysis visualization of the top abundant microbes with all the biochar treatments. The nodes were colored at phylum level and the connections represent Spearman correlations. The strength of correlation is defined by the color label with red indicating positive and green negative correlations respectively.


a
Proteobacteria (30.27%)
Actinobacteria (25.41%)
Chloroflexi (12.43%)
Euryarchaeota (7.57%)
Bacteroidetes (5.41%)
Planctomycetes (5.41%)
Gemmatimonadetes (3.78%)
[Thermi] (2.16%)
Acidobacteria (2.16%)
Firmicutes (1.08%)
Chlorobi (1.08%)
Verrucomicrobia (1.08%)
Armatimonadetes (0.54%)
Fibrobacteres (0.54%)
Crenarchaeota (0.54%)
Cyanobacteria (0.54%)
+ (65.4%)
- (34.6%)


b
Proteobacteria (38.89%)
Actinobacteria (20.99%)
Euryarchaeota (9.88%)
Bacteroidetes (7.41%)
Chloroflexi (5.56%)
[Thermi] (3.7%)
Gemmatimonadetes (3.7%)
Acidobacteria (3.7%)
Planctomycetes (3.09%)
Fibrobacteres (1.23%)
Armatimonadetes (0.62%)
Crenarchaeota (0.62%)
Verrucomicrobia (0.62%)
+ (58.95%)
- (41.05%)


c
Proteobacteria (40.22%)
Actinobacteria (21.74%)
Chloroflexi (9.24%)
Euryarchaeota (7.61%)
Bacteroidetes (5.43%)
Gemmatimonadetes (3.26%)
[Thermi] (2.17%)
Fibrobacteres (2.17%)
Planctomycetes (2.17%)
Verrucomicrobia (1.63%)
Acidobacteria (1.63%)
Cyanobacteria (1.09%)
Firmicutes (0.54%)
Armatimonadetes (0.54%)
Crenarchaeota (0.54%)
+ (67.6%)
- (32.4%)


d
Proteobacteria (34.36%)
Actinobacteria (21.47%)
Chloroflexi (9.2%)
Euryarchaeota (7.98%)
Bacteroidetes (6.13%)
Planctomycetes (4.29%)
Gemmatimonadetes (3.68%)
[Thermi] (3.07%)
Acidobacteria (3.07%)
Fibrobacteres (1.84%)
Verrucomicrobia (1.84%)
Cyanobacteria (1.23%)
Armatimonadetes (0.61%)
Chlorobi (0.61%)
Crenarchaeota (0.61%)
+ (54.29%)
- (45.71%)

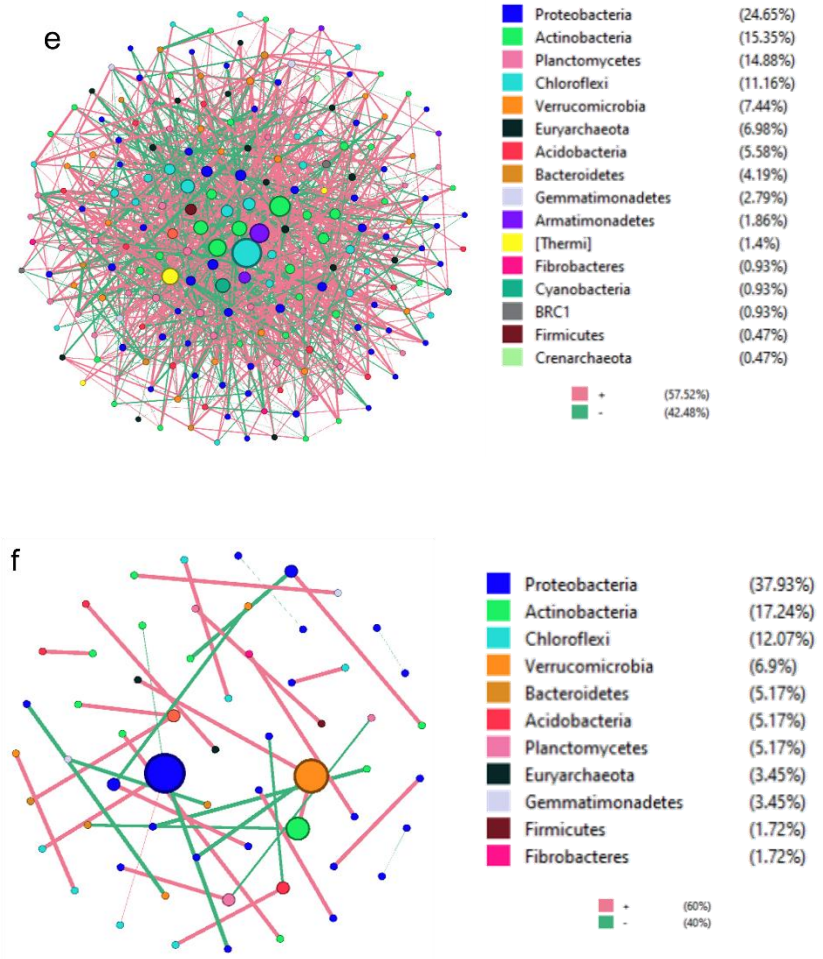


Figure 4-3 Network analysis visualization of the top abundant microbes with each biochar material a) chicken manure CM, b) rice husk RH, c) rice straw RS, d) chicken manure with rice husk CM+RH, e) chicken manure with rice straw CM+RS f) control. The nodes were colored at phylum level and the connections represent Spearman correlations. The strength of correlation is defined by the color label with red indicating positive and green negative correlations respectively.

4.4.3 Microbial interactions among each biochar materials

The network analysis revealed that in CM, the predominant phyla are *Protobacteria*, *Actinobacteria*, *Chloroflexi* with 30.27, 25.41, 12.43% abundance, while the total positive and negative correlations are 65.4 and 34.6 respectively (Figure 4-3a). In RH, the abundant phyla are *Proteobacteria* (38.89%), *Actinobacteria* (20.99%), and *Euryarchaeota* (9.88%) with total and negative correlations as 58.95 and 41.05% (Figure 4-3b). *Proteobacteria* (40.22%), *Actinobacteria* (21.74%), and *Chloroflexi* (9.24%) showed strong influence in RS (Figure 4-3c). In the mixed combined biochar materials CM+RH and CM+RS, *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* with 34.36, 21.47, and 9.21 were the phyla dominating within each material (Figure 4-3de). The abundant phyla in control are *Proteobacteria* (37.93%), *Actinobacteria* (17.24%), and *Verrucomicrobia* (6.9%) (Figure 4-3f).

4.5 Discussion

The network analysis showed topmost abundant phyla controlling each biochar materials. Three phyla were consistently dominating in all the biochar materials. Generally, biochar increased *Chlorofexi* compared to the control, unamended biochar. Following the study by Lu et al. (2020) they demonstrated that using peanut shell and wheat straw biochar pyrolyzed at 500°C enriched the abundance of *Chloroflexi* compared with the no biochar soil. This indicates that *Chloroflexi* are more adapted in a nutrient enriched conditions and its abundance can be enhanced with biochar application. Among the biochar treatments, RH

increased the amount of *Euryarchaeota* compared to other treatments (Figure 4-3b). This is in agreement with previous study (Yang et al. 2021). They found that rich husk biochar increased *Euryarchaeota* by 6.9% compared to other samples. Also, CM+RS increased the abundance of *Planctomycetes* in this study. A recent study reported that chicken manure biochar applied under velvet beans mixed with maize increased the abundance of *Planctomycetes* in the soil (Kimura & Uchida, 2021).

4.6 Conclusions

This study highlighted that the abundance of *Chloroflexi* can be enhanced through biochar amendment. Also, different biochar materials impacted the microbial community structure differently. Particularly, RH increased the amount of *Euryarchaeota* while CM+RS increased *Planctomycetes* in the soil. Therefore, these results suggest that biochar feedstock should properly be considered when selecting biochar as a soil amendment.

Chapter 5 Bacterial communities and soil properties influencing the variations in dung decomposition and gas emissions among Japanese dairy farms

5.1 Abstract

Dung decomposition in dairy farms provides an important microbial ecosystem service to recycle nutrients to the soil. Contrastingly, the emissions of greenhouse gases (GHGs) occurring during dung decomposition are ecological disservices. The decomposition rates of dung and the rates of GHGs produced during dung decomposition can be varied depending on dung and soil characteristics. This study investigated the soil and dung properties that contributes to decomposition rates of dung and their emission patterns under different farms in Hokkaido, Japan. In this study, we incubated dung and soil sampled from 15 different grazed dairy farms within Hokkaido, Japan. Soil and dung DNA were extracted at 0-, 100-, and 200-days during incubation and sequenced targeting changes in bacterial communities. Changes in C dioxide (CO_2) and nitrous oxide (N_2O) were also monitored. The results showed a positive correlation of soil C and nitrogen to increased CO_2 emissions. Bacterial community analysis indicated that during dung decomposition, *Firmicutes* and *Bacteroidetes* were impacted and *Proteobacteria* and *Actinobacteria* were more involved in the decomposition process. This study clearly identified the effects of soil properties on the decomposition of dung and concluded that variability in soil nutrient status is an important factor to consider during dung decomposition in a grazed dairy farms especially within Hokkaido, Japan.

5.2 Introduction

Globally, there is an increasing attention in dairy farms regarding their impact on greenhouse gas emission (GHG). The increasing rate of GHGs is detrimental and cattle production contributes to the largest GHG source which is about two-thirds of total livestock emissions (Gerber et al. 2013). Cattle dung deposited on a pastoral soil is an important source of GHG emissions such as carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4). More specifically, dung patches produce significant amount of CO_2, N_2O, and CH_4 when they are deposited in a soil in Kenya, and the amount of the gases are partly correlated with the quality (cation to nitrogen (CN) ratio) of the dung (Zhu et al. 2018). Also, gas emissions from dung deposited on soils can be influenced by the forage type (Lombardi et al. 2022). Additionally, the deposited dung can change the soil chemical, physical, and biological properties during decomposition (Yoshitake et al. 2014) and this might have a differing effect on the gas emissions. Different soil type impact on gas emissions has been reported (Ogle et al. 2019) and investigating its effect on dung decomposition is strongly needed.

Consequently, grassland ecosystems contribute to carbon (C) balance, store 10–30% of soil organic C, and also serves as C sinks (Yoshitake et al. 2014). Thus, the accumulation and loss of this C from the soil affects GHGs emissions. In grazed grasslands, animals deposit about 60–99% of the nutrients they consume to the soil as dung or urine (Cai et al. 2017). Generally, dung decomposition is an important process for C and nutrient cycling and thus, influences long-term sustainability and productivity of grasslands (Menéndez et al. 2016). This is because, a significant amount of dung nutrients when decomposed is being recycled

back to the soil to improve soil quality and as a result, provides available nutrients for plant uptake. During dung decomposition, microbial respiration releases a significant amount of dung C (Chen et al. 2011). The process of cow dung decomposition has been well studied mostly using dung beetles to initiate dung decomposition and GHG emissions (Evans et al. 2019; Menéndez et al. 2016; Slade et al. 2016). However, studies that monitored the decomposition rates of dung without any additional amendments especially under various dung and soil types together with the bacterial contributions are limited.

Dung contributes to the major source of nutrient, organic matter, and microbes that are provided to the grassland ecosystem (Slade et al. 2016). While microbes play an important role in C and nitrogen cycling in the soils which influences GHG (Li et al. 2018). Consequently, the microbial community controls the release of stored C in the soil, and such activity is dependent on the available nutrient present in the soil. That is, the abundance, activity, and composition of microbes are intently affiliated to the soil organic C mineralization processes (Guo et al. 2019; Sjögersten et al. 2016). Particularly, bacteria play a significant role in the decomposition and mineralization of organic matter (Qiao et al. 2019), and it has been reported that microbial communities differ during the decomposition process of organic matter (Liu et al. 2020). Therefore, the differences in bacterial composition and functions regulating different varieties of dung decomposition need to be understood.

The deposited dung suppresses the grass underneath which makes growth and recovery difficult (Evans et al. 2019), even though plants use the dung nutrient for growth. On the

other hand, dung can stimulate microbial activity in the soil directly under the dung which initiates C lost (Menéndez et al. 2016). Previous study reported that dung decomposes slowly, however, the bacterial compositions and functions contributing to the decomposition process have not properly been documented. This study aimed to identify whether dung or soil bacterial properties and/or soil type strongly determine the dung decomposition rates and GHGs. To achieve this, we incubated soil separately as control and dung applied on the soil surface to achieve field practical condition. The samples were taken from 15 different grazed dairy farms. We hypothesized that gas emissions will be varied among the farms, and the soil bacterial community will most likely influence the decomposition process.

5.3 Methods

5.3.1 Sampling sites and preparation

Soils and dung were sampled from the North (44.716–44.870°N, 141.780–142.261°E), East (43.205–43.367°N, 145.015–145.234°E) and Center (42.455–42.970°N, 141.776–143.231°E) of Hokkaido, Japan to achieve a range of varieties. In each farm, four replicates of soil and dung were collected separately from 15 different grazed dairy farms. Soil samples were sampled from the topsoil 0–20 cm from each farm, sieved to >2mm, and stored in a cold room (5°C) together with the dung. Total C and N from soils and dung were analyzed using CN coder (Perkin Elmer 2400).

We incubated fresh soil with an estimated 70 g dry based in a cup with 85cm^3 surface area. Fresh dung estimated at 1 g dry weight was added after the soils attained 60% WFPS (water

filled pore space). The samples were stored inside an incubator (Eyela SLI-1201) at 25°C throughout the incubation period. Each farm comprised of two different treatments, soil only as control, and soil amended with dung still maintaining 4 replicates of soil and dung from each farm. Thus, a total of 120 samples were produced from the 15 farms, respectively. The WFPS was calculated by

$$\mathrm{WFPS} = (\mathrm{VWC}) / \left(1 - \frac{\mathrm{BD}}{\mathrm{PD}}\right) 100$$

WFPS (%), VWC = volumetric water content (%), BD = soil bulk density (g cm^{-3}), and PD = particle density (2.65 g cm^{-3}).

5.3.2 Gas sampling and measurement

Gas sampling started when the soils attained 60% WFPS and was recorded as day -4. Henceforth, dung was applied on the soil which was regarded as day 0 and the measurement continued for 200 days. Gas was sampled under three different timings 0, 30 and 60 minutes. Before sampling, the cups were inserted into a known volume of glass bottles with headspace of 1.6 L, outside air was passed through the bottles for an average of 50 seconds to eliminate traces of other sources of gas, and then air tightened with the lids to avoid entrance of air. The bottles were allowed to stand for 15 minutes to stabilize the air inside and a volume of 30 ml gas was taken with a string, and then inserted into a 20 ml vacuumed vial.

The concentrations of CO_2 and N_2O, and in the samples were analyzed using gas chromatograph (GC-2014, Shimadzu Co., Kyoto, Japan). The gas chromatograph has Flame Ionization Detector (FID) used for CO_2 measurement and Electron Capture Detector (ECD) for N_2O measurement. Using gas equation and standards, the concentration for daily emissions were calculated and expressed in mg kg^{-1} day^{-1}. The cumulative CO_2-C respired (CO_2-C mg C kg^{-1} soil) after 200 days was calculated by averaging the daily respiration rate, then multiply the average with the interval between measurements and sum the total.

5.3.3 Soil sampling for DNA analysis

DNA was extracted from the soil and dung at three different stages during the experiment. The first was before incubation, after 100 days, and at the end of the incubation to monitor the bacterial contributions to gas emissions. During incubation, samples for DNA extraction were taken from the dung on top of the soil (dung) and from the control (soil) in each farm. Soil and dung DNA were extracted with DNeasy® PowerSoil® DNA Kit (QIAGEN Group) following the manufacturers protocols.

The DNA extract was amplified and barcoded through PCR with a sequence Ion Xpress Barcode Adapters 1–63 kit (Life technologies) for sequencing. The PCR sample contained 17.75 µl of platinum, 1.5 µl of specific forward primer, 0.75 µl of reverse primer (806F attached with the sequence of Ion P1 adaptor, Ion Torrent; Life Technologies) and 5 µl of the extracted DNA. The final volume was 25 µl. The PCR cycle was 94°C for 2 min, then 35 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min. The final step was 4°C for 1 min.

The PCR products were purified with Agencourt AMpure XP (Beckman Coulter) and the DNA concentration measured using Promega Quantus^{TM} Fluorometer (TM396J, USA). The length and concentration of the amplicons were determined through Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Palo Alto, CA, USA). The library was diluted to 25 pM using nuclease-free water. The library was loaded to the Ion PGM Hi-Q Chef Kit using Ion 318 chip (Ion Torrent Life Technologies, USA). The obtained sequence was further analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) software package version 2019.7 (Bolyen et al. 2019).

5.3.4 Measurement of bacterial abundance

The extracted DNA was purified and diluted 10 times with nuclease-free water for qPCR analysis. For the measurement, samples were prepared with 10.4 µl of SYBR ROX, 0.8 µl of forward primer, 0.8 µl of reverse primer and 2 µl of DNA extract. Nuclease-free water was added to make up the final volume to 20 µl. The amplification temperature protocol was set-up as 95°C as the initial temperature for 30 s, annealing temperature 95°C for 35 cycles and the extension conducted for 30 s at 58°C. The final temperature was 72°C for 1 min with melting curve at 95°C for 1 min (Oraegbunam et al. 2022).

5.3.5 Statistical analysis

A one-way analysis of variance (ANOVA) was used to determine the effect of CO_2 and N_2O on the farms. For microbial analysis, the sequenced data were analyzed using QIIME 2 software package version 2019.7. The effect of soil and dung properties, bacterial community

structure, diversity and abundance on gas emissions were determined through Pearson correlation analysis. All statistical analysis were carried out in R software version 3.6.2.

5.4 Results

5.4.1 Cumulative greenhouse gas emissions

In general, dung application on soils increased the cumulative GHGs emissions (CO_2 and N_2O) over 200 days of gas measurement (Figure 5-1a). The cumulative CO_2 emissions in treatments with dung ranged from 4876–10541 mg C kg^{-1} while control treatments (soil alone ranged from 1939–8377 mg C kg^{-1} respectively. When compared among the farms, there was no significant difference between dung treatments, but control showed a significant different ($P < 0.05$). Furthermore, the cumulative N_2O emission in dung treatment ranged from 1500–52761 N_2O µg N kg^{-1} while control had a ranged of 1097–8743 N_2O µg N kg^{-1} (Figure 5-1b). Additionally, there was no significant difference found when compared across the farms.

5.4.2 Influence of selected soils and dung properties on gas emissions

Among the farms used for the incubation study, 6 farms were selected for further analysis. Two farms (one low and one high CO_2 emissions) from each location were selected to investigate the differences in CO_2 gas emissions. Correlation analysis by Pearson showed that soil C, nitrogen, and CEC positively correlated ($P < 0.001$) with high CO_2 gas emissions while dung C, nitrogen, soil pH and bulk density negatively correlated ($P < 0.05$) with the

high CO_2 emissions (Table 5-1). There was no correlation found in low CO_2 gas emissions ($P > 0.05$) except in bulk density ($P < 0.05$) (Table 5-1).

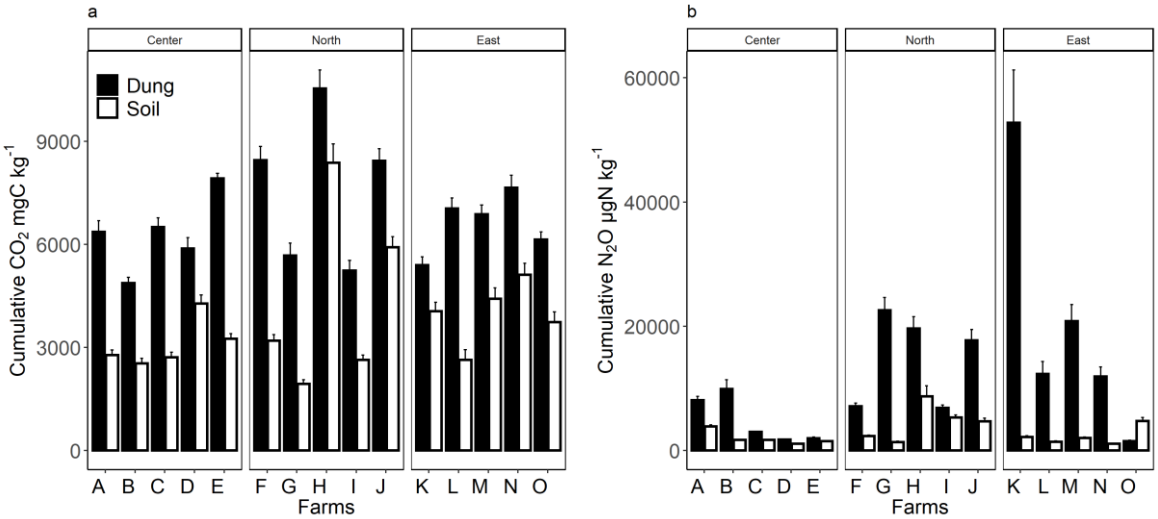


Figure 5-1 Cumulative (a) CO_2 and (b) N_2O emissions from soil and dung treatments among different farms. Means ± SD of four replicates.

Table 5-1 Pearson correlation coefficient between CO_2 emissions, soil, and dung properties

	Soil carbon	Soil nitrogen	Dung carbon	Dung nitrogen	Soil pH	Bulk density	CEC
High CO_2	0.93***	0.94***	−0.68*	−0.65*	−0.66*	−0.74**	0.93***
Low CO_2	−0.35	−0.30	0.05	0.14	0.24	0.65*	−0.11

*, ** and *** indicates significant levels at $P < 0.05$, 0.01 and 0.001

5.4.3 Microbial community structure analysis

Regarding the differences in CO_2 emissions, treatments with dung showed a separation between high and low CO_2 while there was no separation in the control treatments (Figure 5-2). In dung treatments, low CO_2 had a higher unique number of OTUs (3750 OTUs) than high CO_2 (3438 OTUs) (Figure 5-3a). However, control had higher number of OTUs in high CO_2 (5340 OTUs) than low CO_2 (4974 OTUs). At phylum level, unique OTUs in dung treatments at high level CO_2 were dominated by *Proteobacteria* (21.1%, 996 OTUs), *Actinobacteria* (21.4%, 366 OTUs), *Firmicutes* (26.8%, 354 OTUs), and *Bacteroidetes* (9.5%, 243) respectively (Figure 5-S2a). While unique OTUs in dung treatments at low level CO2 were dominated by *Proteobacteria* (23.8%, 1059 OTUs), *Firmicutes* (30.3%, 445 OTUs), *Actinobacteria* (23.6%, 289 OTUs), and *Bacteroidetes* (6.9%, 220 OTUs) (Figure 5-S2b).

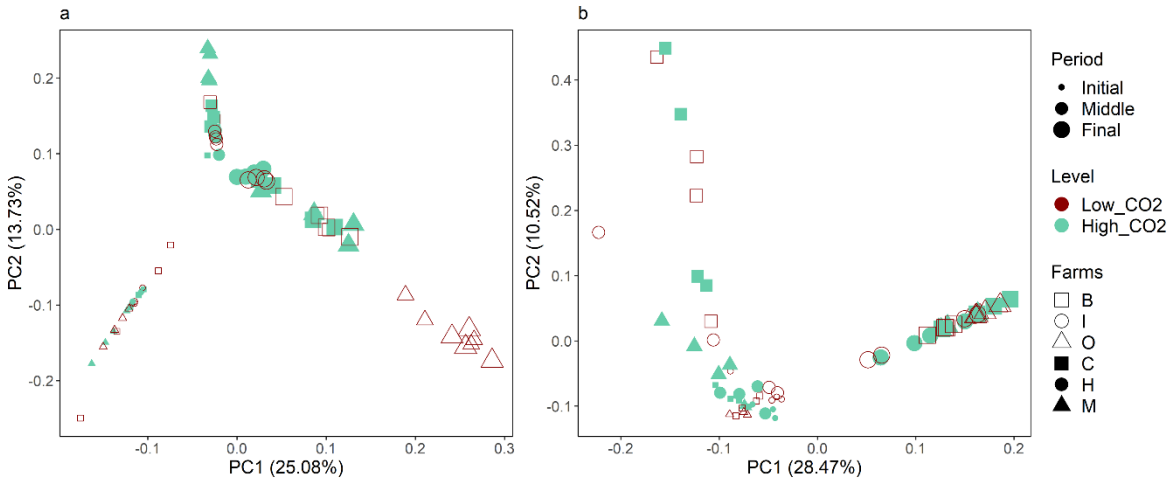


Figure 5-2 Principal Component Analysis (PCA) a) dung and b) soil at OTU level based on the differences in emissions. Period: initial, middle, and final represents different sampling timing. Farms B, I, and O have low CO2 while C, H, and M have high CO2 emission.

In the control, high CO_2 were dominated by *Proteobacteria* (26.2%, 1100 OTUs), *Actinobacteria* (16.3%, 551 OTUs), *Acidiobacteria* (13.3%, 425 OTUs), *Planctomycetes* (10.8%, 631 OTUs), and *Chloroflexi* (9.8%, 658 OTUs) (Figure 5-S3a). While low CO_2 were influenced by *Proteobacteria* (22.9%, 1071 OTUs), *Actinobacteria* (17.9%, 436 OTUs), *Chloroflexi* (15.6%, 679 OTUs), *Acidiobacteria* (15.5%, 428 OTUs), and *Planctomycetes* (8.9%, 480 OTUs) respectively (Figure 5-S3b).

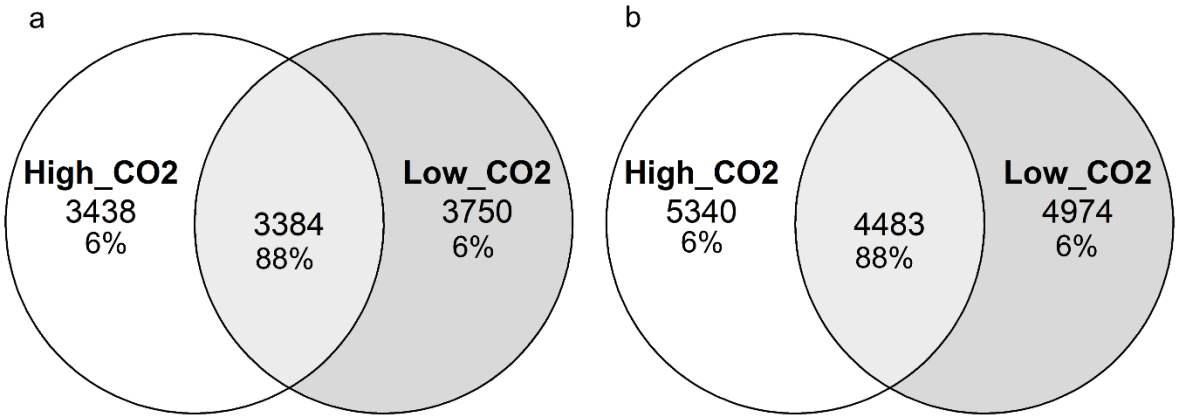


Figure 5-3 Venn diagram showing the number of shared and unique OTUs in a) dung and b) soil based on the high and low CO_2 emissions. Percentage values represent relative abundance of OTUs in each section.

5.4.4 Changes in bacterial communities during dung decomposition

During dung decomposition, there was a clear decrease and increase of certain taxa. Among them include *Firmicutes* and *Bacteroidetes* which significantly decreased as dung decompose (Figure 5-4). The relative abundance of *Proteobacteria* increased during decomposition. Also, *Nitrospirae*, *Gemmatimonadetes*, *Planctomycetes*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria* increased their relative abundance from middle to final dung decomposition (Figure 5-4).

The relationship between CO_2 emissions and bacterial communities were expressed using Pearson correlation analysis. Among the selected taxa, CO_2 had strong negative correlations with Proteobacteria, *Nitrospirae*, *Gemmatimonadetes*, *Planctomycetes*, *Chloroflexi*, *Acidobacteria*, *Actinobacteria* ($P < 0.01$), and a strong positive correlation with *Bacteroidete*s and *Firmicutes* ($P < 0.001$) respectively (Table 5-2).

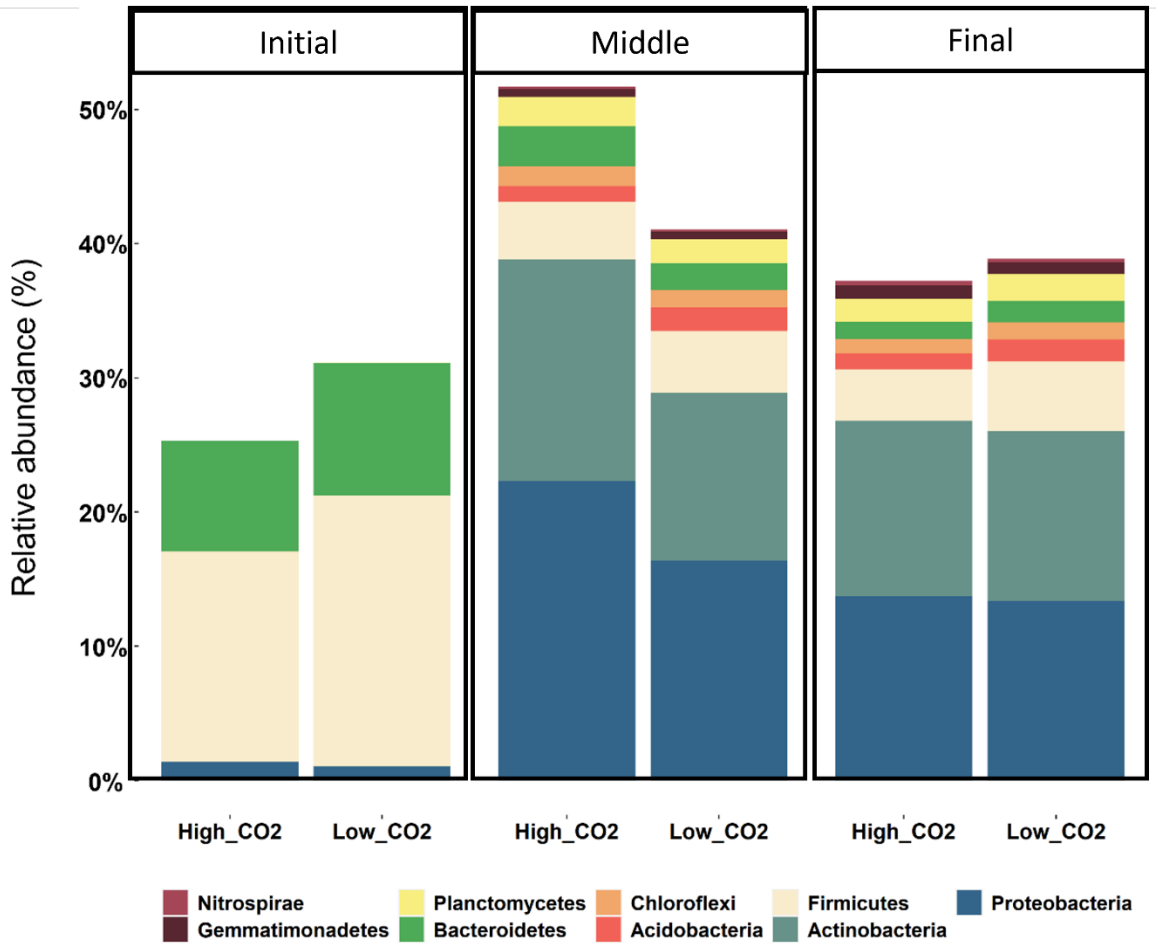


Figure 5-4 Relative abundance of the dominate taxonomic groups at phylum level under high and low CO_2 and their changes during dung decomposition.

Table 5-2. Pearson correlation coefficient between CO_2 emissions and relative bacterial abundance

	High CO_2	Low CO_2
Nitrospirae	−0.40***	−0.40***
Gemmatimonadetes	−0.42***	−0.39***
Planctomycetes	−0.45***	−0.38**
Bacteroidetes	0.69***	0.76***
Chloroflexi	−0.32**	−0.42***
Acidobacteria	−0.42***	−0.46***
Firmicutes	0.52***	0.74***
Actinobacteria	−0.31**	−0.22
Proteobacteria	−0.29*	−0.25*

*, ** and *** indicates significant levels at $P < 0.05$, 0.01 and 0.001

5.4.5 Relationship between evenness, richness, bacterial diversity, and abundance

Pearson correlation revealed that bacterial diversity and observed OTU numbers negatively correlated ($P < 0.05$) with the high CO_2 emissions while the bacterial abundance showed positive correlation ($P < 0.01$) (Table 5-3). Bacterial evenness had no correlation with the high CO_2 emissions ($P > 0.05$) (Table 5-3). Further, bacterial diversity was negatively correlated ($P < 0.05$) with the low CO_2 emissions while bacterial abundance correlated

positively ($P < 0.001$). No correlation was found in evenness and OTU numbers ($P > 0.05$) in low CO_2 emissions (table 5-3).

Table 5-3. Pearson correlation coefficient between CO_2 emissions, evenness, richness, bacterial diversity, and abundance

	High CO_2	Low CO_2
Shannon	−0.28*	−0.23*
Evenness	−0.10	−0.21
OTU	−0.3**	−0.20
Abundance	0.36**	0.48***

*, ** and *** indicates significant levels at $P < 0.05$, 0.01 and 0.001

5.5 Discussion

5.5.1 Differences in gas emissions

The cumulative GHG emissions differ among farms and locations. Generally, dung application increased gas emissions across the farms, but there was no significant difference found among dung applied treatments. Contrastingly, control treatments (soils without dung applications) were found significantly different when compared among the farms. This corresponds with the positive correlation found between soil C and nitrogen content with the cumulative CO_2 emission. Previous study found that soil organic C positively correlated with CO_2 emissions and organic matter decomposition (Yang et al. 2017). It is worth mentioning that gas emission is a clear indication of microbial decomposing activity (Pereira et al. 2018). Therefore, the C and nitrogen content in the soils contributes to their difference in the decomposition rate. Further, Paul (2016) mentioned that C losses are mostly related with the availability of labile C and high microbial activity, which could explain the CO_2 emission pattern found in this study. This means that in low CO_2 treatments, the C content might not be accessible by the microbial interactions which slows its decomposition process.

Because CO_2 emission was the main gas emitted during decomposition with a clear difference found among the locations during the experiment (Figure 5-S1), we selected two farms with the highest and lowest CO_2 emission from each location for further analysis. The farms were categorized as high and low CO_2 emissions and high CO_2 gas emissions correlated with the soil and dung properties. Soil C, nitrogen and CEC showed positive correlations with the high CO_2 emissions from dung treatments, which demonstrated that soil

physical properties were strongly contributing to the differences in the emission patterns. The effect of soil properties on gas emissions have been consistent with previous studies (Bogunovic et al. 2020; Kalu et al. 2021; Mukherjee et al. 2014).

5.5.2 Bacterial community structure and gas emissions

Considering the PCA plot from dung applied soils, there was a separation between the high and low CO_2 emissions (Figure 5-2), and this could mean a difference in their bacterial communities. Also, the Venn diagram showed a higher number of unique OTUs in dung treatments with low CO_2 emissions compared to the high CO_2 (Figure 5-3). Among those OTUs were *Bacteroidetes* and *Gemmatimonadetes* which was reported by Sheng and Zhu (2018) to contribute to CO_2 emissions. This corresponds with the present study due to the larger amount of *Bacteroidetes* (9.5%) and *Gemmatimonadetes* (2.8%) found in high CO_2 treatments. They also noted decrease in *Acidobacteria* which was observed in this study was responsible for increasing CO_2 emissions.

During dung decomposition, the bacterial communities changed starting from the initial to final sampling. Specifically, at the initial stage before dung decomposition, the major bacterial phyla were *Bacteroidetes* and *Firmicutes* and, this was in agreement with previous studies that cattle manure application increased their abundance (Bello et al. 2020; Sun et al. 2019). These phyla were significantly decreased later on which indicate that they are the major phyla strongly affected during dung decomposition, and this result was similar to other reports (Awasthi et al. 2017; Lv et al. 2015; Meng et al. 2019). In the middle and final stage

of the dung decomposition, the abundance of *Proteobacteria* and *Actinobacteria* increased significantly (Figure 5-4). Following the report by Zhang et al. (2020), they found that *Proteobacteria* increased in abundance over other phyla across seasons from 30.3 to 33.2% when cattle manure was used as soil amendment under tea plantation. Also, at the middle stage of decomposition, the abundance of *Proteobacteria* showed a higher trend in the high CO_2 treatments compared to the low CO_2 treatments. Previous study by Ma et al. (2017) found a CO_2 increase in concentration when the proportion of *Proteobacteria* increased from 28.9% to 67.9%. Thus, Proteobacteria could be modified in an environment with increased resources (Kuramae et al. 2012).

5.5.3 Bacterial diversity and abundance impact on dung decomposition

Bacterial diversity negatively correlated with the CO_2 emissions following Pearson correlation analysis (Table 5-3). Maron et al. (2018) have reported that changes in the microbial diversity strongly influenced the CO_2 emissions which affects the C storage in the soil. Our study showed that bacterial diversity was neither supporting the increased or decreased CO_2 emissions. Going further into the results, the OTU numbers were found to negatively correlate with the high CO_2 emissions while there was no correlation in low CO_2. This indicated that there might be decrease in bacterial diversity in the low CO_2 treatments which contributed to the decrease in emissions. This agreed with the findings by Maron et al. (2018) where they found that decreased microbial diversity affected the decomposition of autochthonous plant residue and allochthonous organic matter with reduced CO_2 emissions by 40%.

Bacterial abundance correlate with the two CO_2 emissions pattern similar to the results of Gao et al. (2020). Also, Qiu et al. (2020) mentioned that C mineralization rate can be used to predict bacteria abundance, meaning that increased C mineralization is as a result of higher abundance of bacterial phyla. It is noteworthy that bacterial abundance did not contribute to the differences in emissions found in this study. A similar result was found by Martins et al. (2017). They found that using structural equation modelling to analyze CO_2 emissions after 5 years of experimental warming, the difference in CO_2 flux were caused by abiotic factors such as temperature and moisture rather than bacterial abundance. Due to the correlations observed from the emissions patterns with the bacterial abundance, we suggest that the differences in the emission might be caused by the unique bacteria attributed to each group sourcing from autotropic and heterotrophic (Nielsen et al. 2011).

5.6 Conclusions

This study revealed some specific bacterial communities strongly influenced during dung decomposition. At the phylum level, *Firmicutes* and *Bacteroidetes* were decreased significantly as the dung decompose while *Actinobacteria* and *Proteobacteria* increased in abundance during the decomposition process. The decrease and increase in these bacterial phyla were found in the emission pattern observed in the present study. Furthermore, soil properties such as soil C, nitrogen and CEC strongly contributed to the increased emissions during dung decompositions, therefore soil type and nutrient status is very important to quantitatively estimate dung decomposition and gas emissions in dairy farms.

Supplementary

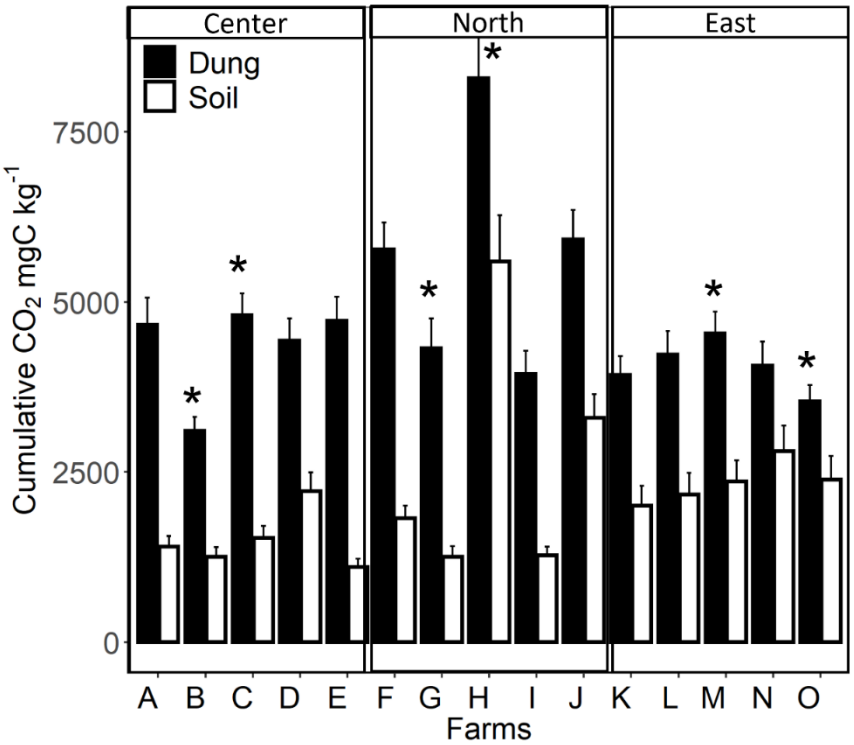


Figure 5-S1. Cumulative CO2 emissions at 100 days of measurement from soil and dung treatments among different farms. Means ± SD of four replicates. Bars with * represents the farms selected for further analysis.

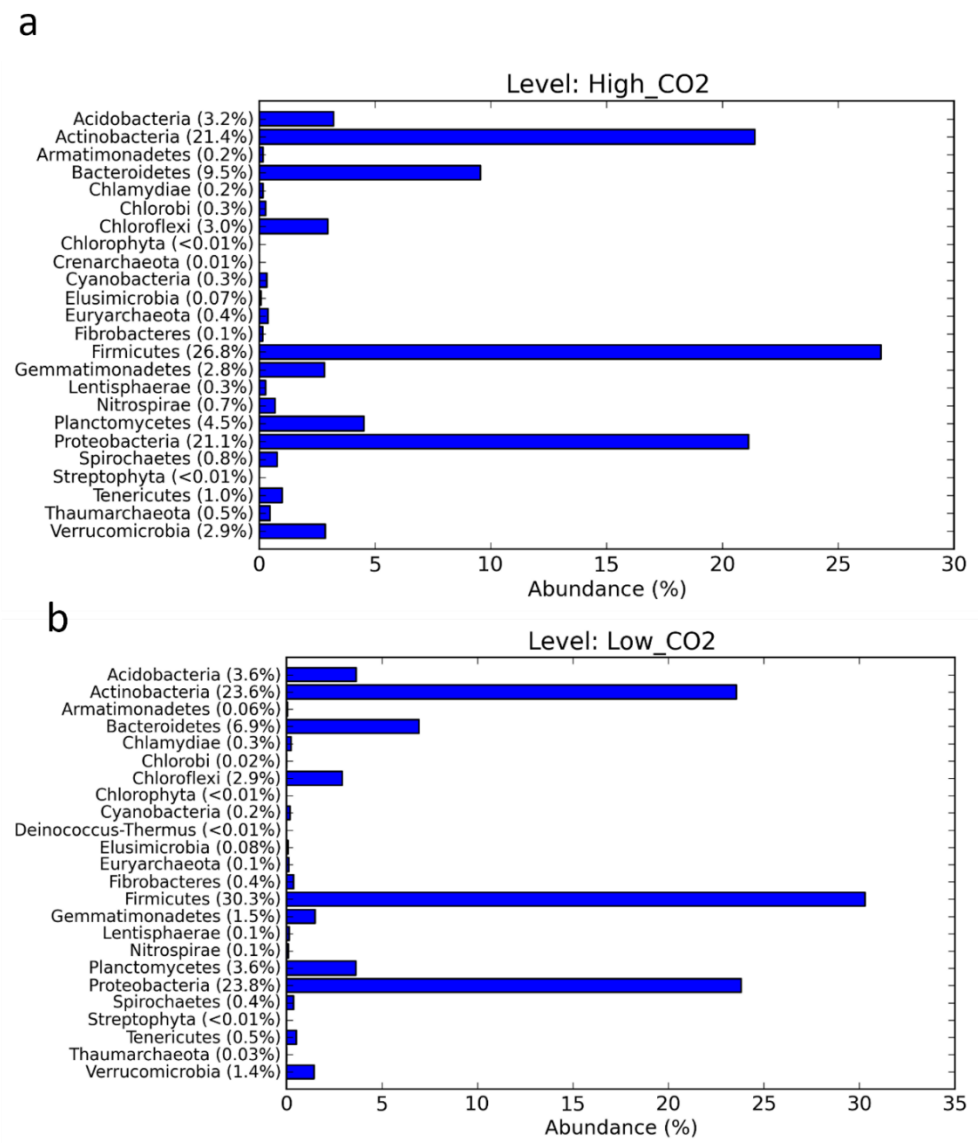


Figure 5-S2i. Unique OTUs that are specific to the a) high CO2 and b) low CO2 emissions under dung applied treatments.

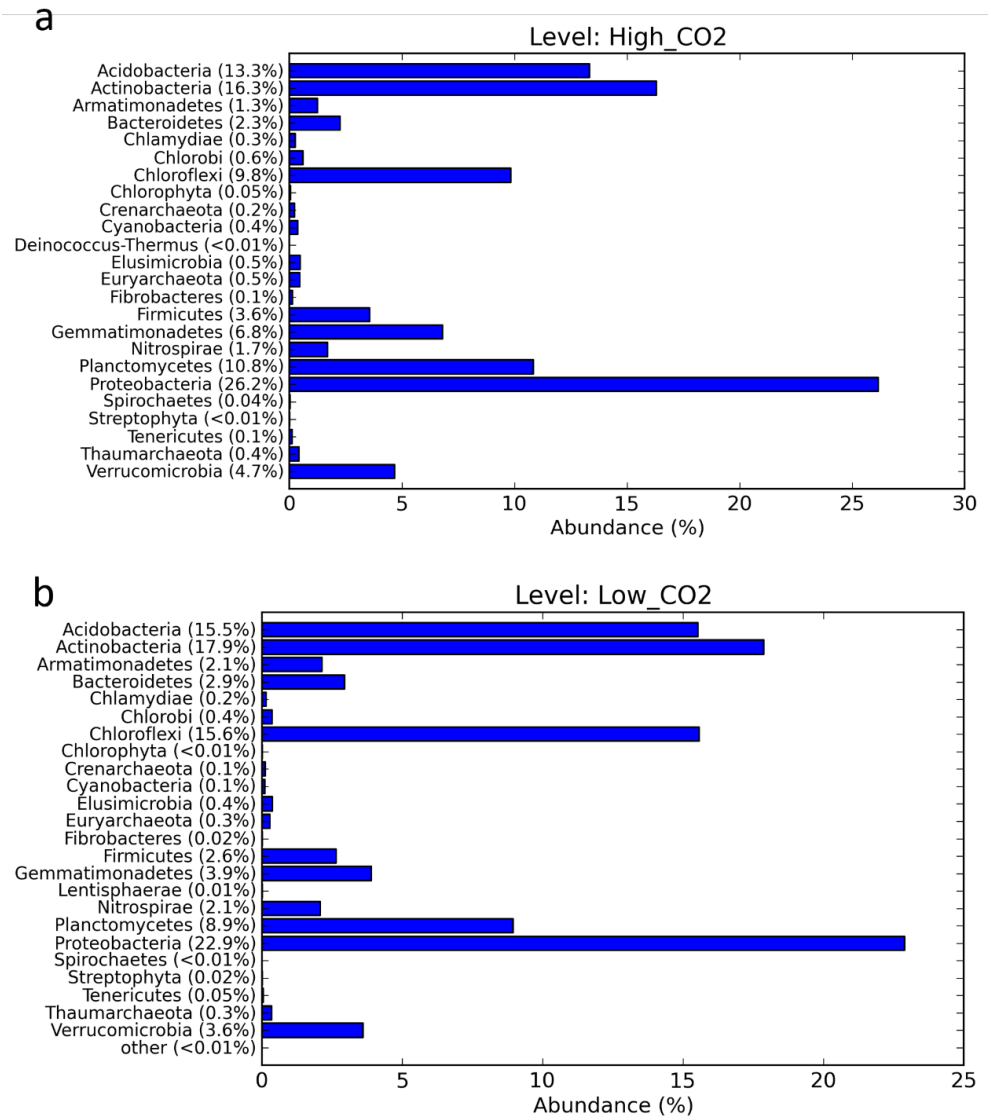


Figure 5-S2ii. Unique OTUs that are specific to the a) high CO2 and b) low CO2 emissions under control treatments.

Chapter 6 Summary and recommendations

This chapter summaries the above research and provide further need for future research.

6.1 Overall summary and future research recommendations

6.1.1 Response of bacterial diversity to different application methods of charred organic materials in sandy soil

In this study, we investigated different application methods of biochar on the bacterial community structure under sandy soil. Three different biochar materials (chicken manure, rice straw and rice husk) were pyrolyzed to produce biochar at 350°C. Samples were taken from the soils after the growth of dent corn, DNA was extracted and sequenced. The result showed an increased microbial diversity at the soil surface. This increase was attributed to the increased numbers of OTU such as *Actinobacteria* and *Proteobacteria* at the phylum level. Also, RS treatments impacted the microbial richness, and evenness under surface application. Thus, the effect on microbial diversities found in this study depends on the feedstock biochar, therefore biochar materials should be considered when interpreting its impact on the microbial community.

This study was conducted under sandy soil and previous research has shown that the response of bacteria abundance to biochar application is soil dependent (Xu et al. 2021). Therefore, future research should be targeted on the evaluation of application methods of biochar on different soil types. Additionally, the biochar was pyrolyzed at 350°C in this study. Research has shown that increased biochar pyrolysis temperature more than 600°C results to biochar

larger pores. These pores serve as habitat for diverse soil microbes (Palansooriya et al. 2019). Future research should aim at incorporating different temperature effects in investigating biochar application methods.

6.1.2 Revealing the effects of different biochar feedstock on the microbial communities using network analysis

This analytical study investigated the changes in the microbial communities following the application of biochar using network analysis. Using Gephi for data visualization, the results showed that *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* were the phyla consistently predominating in the biochar treated soils. Further, RH biochar increased the abundance of *Euryarchaeota* compared to other treatments while CM+RS increased *Planctomycetes*. Therefore, variabilities of biochar feedstocks should be considered when choosing biochar type for soil amendment because of their different impact on the microbial community structure.

Network analysis was used to provide insight into the microbial community among different biochar materials. The current study applied microbial co-association network and previous study highlighted the impact of microbial metabolic modeling as an additional tool to study microbial community structure (Cardona et al. 2016). Therefore, incorporating this tool in this study might provide more insight into the biochar interactions with the microbes.

6.1.3 Bacterial communities and soil properties influencing the variations in dung decomposition and gas emissions among Japanese dairy farms

An incubation experiment was conducted to investigate the soil and dung properties influencing the decomposition rate of cow dung and gas emissions among 15 different farms within Hokkaido, Japan. Gas emissions was also measured. During the incubation, samples were taken at three different timings (before, middle, and final incubation) for microbial analysis. Results showed that *Firmicutes* and *Bacteroidetes* were significantly decreased while *Proteobacteria* and *Actinobacteria* increased during dung decomposition. Also, in each location, there are differences in CO_2 emissions pattern which were categorized as high and low CO_2 emissions. Following this trend, higher numbers of OTUs were found in low CO_2 (3750) compared to the high CO_2 (3438). Further insight revealed that soil properties strongly influenced the emissions pattern based on the positive Pearson correlation coefficient between high CO_2 emissions and soil properties. These results indicate that soil properties were the strong determinant of dung decomposition and gas emissions.

In our study, we applied dung on the soil surface and allowed it to decompose. Previous research reported that dung deposited on the pasture loss 22% up to 80% of its nitrogen to volatilization within 60 days (Pecenka & Lundgren, 2018). Also, study has proved that soil contact with dung promotes faster decomposition (Evans et al. 2019b). Even though our study was carried out in an incubator machine, we believe that nitrogen volatilization might occur during and after gas sampling. Thus, we suggest dung incorporation into the soil for further studies.

6.2 General comments

- In chapter 3 we considered one pyrolysis temperature to produce biochar and soil type. Research has shown that biochar efficacy in the soil depends on the pyrolysis temperature and the type of soil used. Considering these two measures in the evaluation of biochar application method might result in different effects on the bacterial communities.

- In chapter 4 microbial co-association network was used to investigate the impact of biochar materials on the soil microbial community structure. It has been reported that microbial metabolic modeling can provide detailed mechanistic understanding of species interactions. Therefore, applying this method in this study might infer different understanding.

- In chapter 5 we applied dung on the soil surface and monitored its decomposition and gas emissions. Previous research reported nutrient loss and decrease in decomposition when dung was deposited on the soil surface. Therefore, dung incorporation into the soil can enhance microbial activities and dung decomposition which will efficiently supply nutrients to the soil to improve grass recovery after grazing in dairy farms.

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