The microbial dynamics in natural farming rice paddy

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

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The microbial dynamics in natural farming rice paddy

By

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Microbes play a considerable role on the growth of rice crop in a natural farming system, and there are various factors influencing the microbial community dynamics in such system. Hence the objectives of this thesis were (1) to investigate what microbes are responsible for rice yields in Japan and Taiwan, (2) to figure out either perform inter-tillage or no-tillage is beneficial to a natural farming rice paddy, (3) to determine whether adding different weed species can accelerate the decomposition of rice straw and stimulate priming affect.

The first study investigated what bacteria predominated in high yield rice paddies in Japan and Taiwan. Then we tried to find the biological nitrogen fixation "hot spot" in Japan rice paddies. Our results found the class of *Acidobacteria Planctomycetes* and *Deltaproteobacteria* had lager relative abundance in the more production rice paddy, and the δ15N natural abundance had a gradient decreasing from lower temperature spot to higher temperature spot.

The second study aimed to confirm whether inter-tillage impact microbial dynamics in natural farming rice paddy. The soils were sampled at rice proximity, soil surface and 10 cm depth location from five times inter-tillage (5T) and no-tillage (NT) natural farming rice paddies, owing to look at with and without disturbance on microbial abundance and microbial community. The result showed there was no significant difference between the rice yield from

NT (2.3 ± 0.7 t ha−1) and 5T plots (2.7 ± 0.9 t ha−1), however, the 5T might have negatively impacted soil bacterial abundances but not the community structure of the bacteria. In the third study, we added low C/N ratio clover, Rumex, and mixture of clover and Rumex residues as extra nitrogen source to observe whether incorporated rice straw decomposition rate could be accelerated. Our study suggested input plant residues after harvesting potentially improve higher F : B ratio as well as increasing nitrogen nutrient.

Our studies found NF system has the potential to produce as many rice production as chemical fertilizer relied farming system, although the yield was not significant difference in 2019 between the two different managed rice paddies in Japan, and the yield was much lower in the Taiwan NF rice paddy. Our results indicated the microbial community markedly controlled the nutrient serving mechanisms to plant growth, thus how to increase the beneficial microorganisms for rice yield is needed further research.

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Chapter 1

Introduction

1.1 Background

Rice is one of the most important crops in the world, especially in Asia. There are more than 135 million hectare are used for rice cultivation worldwide (International Rice Research Institute World Rice Statistics; http://ricestat.irri.org:8080/wrsv3/entrypoint.htm). Worldwide nitrogen fertilizer use by rice accounted for 15.2% which is about 15.6 million tons in 2014-2015 (IFA, 2017), the rice nitrogen fertilizer consumption is second only to wheat. Natural farming is an alternative agricultural system to grow food instead of conventional farming, which is highly supported by microbial functions rather than utilization of various types of fertilizer (Fukuoka, 1975; Hirano et al., 2001; Xu, 2006; Liao et al., 2019).

Inter-tillage and no-tillage (NT) are both performed with NF rice system. The main disadvantages of performing no–tillage are the risk of crop failure due to weed invasion and less availability of nutrients. Previous study concluded that conducting five times inter-tillage (5T) in a natural farming rice paddy during the rice growing period can increase the yield as high as that of the conventional farming about five years later (Kasubuchi et al., 2019). However, there is a gap in the role of microorganisms that need to be filled.

In a natural farming rice paddy, where relies on organic residue incorporation, rice straw plays an essential role. Rice straw incorporation has the potential to improve soil quality and maintain sustainable soil productivity by increasing soil organic carbon sequestration and nutrients

deposition. Thus, a study to complete the rice straw decomposition one step earlier than rice transplanting in a natural farming rice paddy, is profitable to agronomic.

1.2 Research Objectives

The objectives of this project were to:

- Chapter 3: To analyze the microbial community under conventional and natural farming managed rice paddies in Japan and Taiwan; (2) to evaluate what are the predominant microbes at high yield rice paddies in the two countries; (3) to investigate the biological nitrogen- fixation (BNF) “hot spot” by measuring delta 15N natural abundance in Hokudai rice paddies under two different farming system in spring and summer.

- Chapter 4: To compare the dissimilarity of soil bacterial abundance and diversity under 5T and NT treatment within a paddy farm managed using the natural farming style.

- Chapter 5: To investigate whether the surrounding weeds can improve soil microbial activities. We hypothesized that incorporated weeds would increase bacteria and fungi abundance and lead to a higher rice straw decomposition rate.

Chapter 2

Literature Review

2.1 State of modern agriculture

Since "Green Revolution" started from 1960s, population had doubled and the crops had tripled over the past 50 years (Pingali, 2012), crop yields also have increased steadily. One of the reasons is due to rely on utilizing of chemical fertilizers (Tilman et al., 2002, Boli et al., 2011, Yinghua et al., 2016). Global nitrogen fertilizer application has increased approximately 10 fold between 1950 and 2008 (Robertson and Vitousek, 2009). Although increasing input of chemical fertilizers contributes to food security, however, every cloud has a silver lining, chemical fertilizer has been causing soil deterioration, greenhouse gas emissions, and water contamination (Wen-yuan and Rhona, 1993; Tilman et al., 2001; Johanna et al., 2006; Han and Zhao, 2009; Ju et al., 2009; Wauters et al., 2010; Liu et al., 2013a,b; Stuart et al., 2014; Wei-feng et al., 2013; Sierra et al., 2015; Smith and Siciliano, 2015; Norman and Dazzo, 2016). Studies have shown that crops can take up only 30-50% of chemical fertilizers, the other 50% of nitrogen remains in soils or leaves cropping systems through air, surface water, or groundwater pathways (Follett and Delgado, 2002; Eickhout et al., 2006; Robertson and Groffman, 2007; Norse, 2005, Zsófia et al., 2012). For example, the chemical fertilizer saturation is leading to the decrease of nitrogen use efficiency, which has been causing the excess nitrogen being releasing to the environment, mainly ammonia volatilization, denitrification, leaching and runoff losses (Cho, 2003; Zsofia et al., 2012; Carter et al., 2012). Volatilization and denitrification cause atmospheric pollution through the emission of nitrous oxide (N2O), nitric oxide (NO), and ammonia (NH3)(Azam et al. 2002; Reeves et al. 2002). Nitrous oxide absorbs infrared radiation contributing to the greenhouse warming and the depletion of the stratospheric ozone layer (Bohlool et al. 1992). Nitric oxide contributes to the

formation of tropospheric ozone, a major atmospheric pollutant that affects human health, agricultural crops, and natural ecosystems (Chameides et al. 1994). The deposition of nitric oxide and ammonia in terrestrial and aquatic ecosystems can lead to acidification, eutrophication, shifts in species diversity (Reeves et al. 2002; Vitousek et al. 1997). Nitrate leaches to groundwater causes toxicity harming ecology system and human health (Shrestha and Ladha 1998).

2.1.1 The nitrogen cycle in rice paddy

Rice is one of the most important crops in the world, especially in Asia. There are more than 135 million hectare are used for rice cultivation worldwide (International Rice Research Institute World Rice Statistics; http://ricestat.irri.org:8080/wrsv3/entrypoint.htm). Worldwide nitrogen fertilizer use by rice accounted for 15.2% which is about 15.6 million tons in 2014-2015 (IFA, 2017), the rice nitrogen fertilizer consumption is second only to wheat. The nitrogen cycle in rice paddy has substantial effect on global environment sustainable. Nitrogen is the most limiting nutrient for rice production as well as an essential element for all living organisms, and is required for the biosynthesis of key cellular components, such as proteins and nucleic acids. The largest natural source of accessible nitrogen is atmospheric dinitrogen, but most of the nitrogen in the atmosphere is unavailable for use by organisms. This is because the strong triple bond between the N atoms in N2 molecules makes it relatively inert, or unreactive, whereas organisms need reactive nitrogen to be able to incorporate it into cells. In order for plants and animals to be able to use nitrogen, N2 gas must first be converted to more a chemically available form such as ammonium (NH4+), nitrate (NO3-), or organic nitrogen (e.g., urea, which has the formula (NH2)2CO). This bioavailable nitrogen is controlled primarily by

biochemical processes. In rice paddy soils, various biochemical processes occur regarding nitrogen cycling, including nitrification, denitrification, and N2–fixation (Fig 2.1).

Except input chemical nitrogen fertilizer for rice growth, the process of N2–fixation is the reduction of N2 to a biologically inorganic ammonia. The equation for the reaction is as follows:

$$N \equiv N + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Bacteria and Archaea have been conducting N2–fixation from the air for hundreds of millions of years. This mechanism accounts for much of the nitrogen input to natural environment more than rock weathering or lightning. N2–fixation prokaryotes, also called diazotrophs, can be free-living or exist in symbiotic associations with eukaryote, for example, fungi, termites and plants. Some N2–fixation members of the Rhizobiales order live in specific root nodules of crop legumes or of plants, such as alfalfa, beans, peas, soy and clover. (Burris and Roberts., 1993; Kneip et al., 2007; Hartmann and Barnum., 2010; Vitousek et al., 2013). However, how to let the rice grow healthily without relying on chemical fertilizer is the main issue in this study.

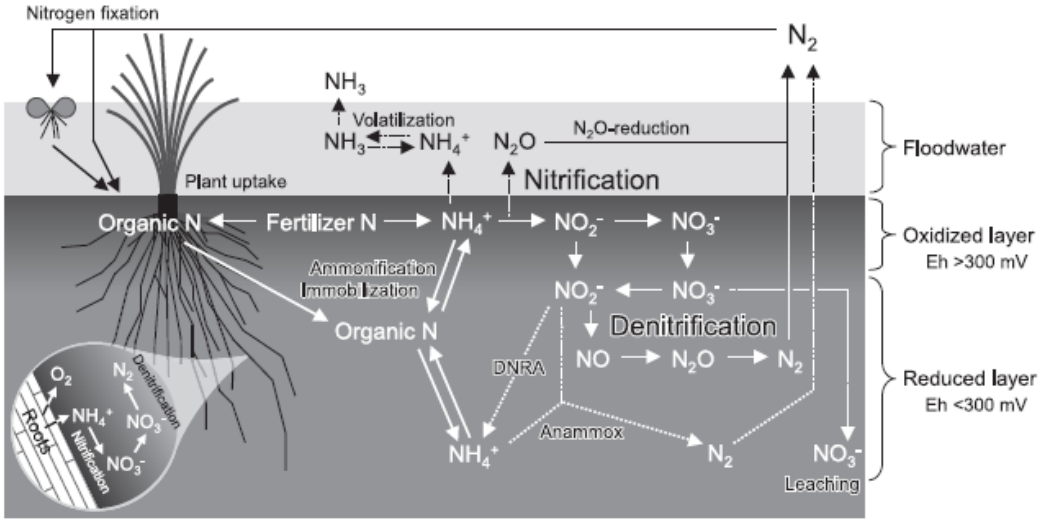

Figure 2.1 Overview of nitrogen cycle in rice paddy (Source: Ishii et al., 2011)

2.1.2 Measurement of N2 fixation

Isotopes are atoms whose nuclei contain the same number of protons but a different number of neutrons. About the N atoms on earth, 99.6337 % of them are the lighter 14N with the heavier 15N (0.3663 %), and the ratio between the two stable N isotopes (15N/14N) is expressed as 15N atom% (Mariotti, 1983). The 15N atom% varies in the biosphere as a result of isotope fractionation during physical, chemical, and biological processes, and the atmospheric N2 (0.3663 atom %) is accepted as the standard (Junk and Svec, 1958; Mariotti, 1983). In natural ecosystems, the 15N atom% usually varies from 0.355 to 0.377 atom % (Macko and Ostrom, 1994; Nadelhoffer and Fry, 1994). Because the variation in the absolute abundance of 15N is small, N isotope composition is expressed using the δ notation in parts per thousand (‰) as:

$$\delta^{15}N(‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 1000$$

where Rsample and Rstandard are the atom % of the sample and the standard (atmospheric N2, 0.3663%), respectively. This equation indicates that the δ15N of atmospheric N2 is 0 ‰ by

definition and that the more 15N-enriched a sample is, the more positive its δ15N and vice versa. Most N compounds found in agricultural ecosystems have δ15N between –30 and +30m that are equivalent to 0.355 and 0.377 atom%, respectively (Robinson, 2001). Advantages and disadvantages of current techniques and their appropriate use are described here briefly.

2.1.2.1 Use of $^{15}N_2$ Gas as a Direct Measure of N2 Fixation

The only direct measurement of N2 fixation is the 15N2 labelling technique in which a rice–soil system is placed in a gas-tight growth chamber with an atmosphere enriched in 15N2 (Warembourg, 1993). This method can be sensitive, accurate and provide absolute proof of N2 fixation and has been used to demonstrate N2 fixation associated with cereals and grasses (Giller et al; 1987; Gupta et al., 2014) and in soils (Roper, 1983; Azam et al., 1988]. It is a useful method for calibrating other measures of N2 fixation.

2.1.2.2 C_2H_2 Reduction Assay

The C2H2 reduction assay (based on the reduction C2H2 to C2H4 by nitrogenase) is a rapid, sensitive, simple and low cost method which if used with appropriate controls and calibrations can be useful for evaluating nitrogenase activity in time and space (Witty, 1979]. Under controlled conditions it can be extremely useful for comparative purposes where absolute values of N2 fixation are not critical. Hardy et al (1968) found a direct correlation between N2 fixation (N2→2NH3) and C2H2→C2H4 in pure cultures of diazotrophs and in legumes, and calculated that the theoretical relationship of C_2H_2 reduced to N2 fixed was 3. App et al (1986) calculated the amount of N fixed by blue green algae (BGA) was estimated to be 10–80 kg N ha^{-1} $crop^{-1}$, averaging 30 kg N ha^{-1} $crop^{-1}$ by C2H2 Reduction Assay.

2.1.2.3. ^{15}N Isotope Dilution and Natural Abundance ($\delta^{15}N$) to Measure Associative N_2 Fixation

15N isotope dilution and natural abundance methods both rely on differences in isotopic composition of the sources of N used for plant growth, for example, atmospheric N, soil N and fertiliser N. Both methods require a non-N2-fixing reference plant, the reference plant and the test plant with associative N2 fixation have a similar root architecture and can extract N from the soil at the same rate in space and time (Boddey et al., 1983).

The 15N isotope dilution technique involves supplying a 15N enriched (or depleted) source of N to the soil so that it is significantly different from the natural abundance of the atmospheric N2. For accurate measurement, the spatial and temporal availability of the isotope should be uniform (Chalk, 1985).

The ability of the natural abundance method to measure associative N2 fixation depends on N2 fixed by associative microorganisms being predominantly taken up by the plant rather than going into the soil N pool (Shearer and Kohl, 1988). However, some factors can affect δ15N in plants, such as N from precipitation (NOx, NH3), the depths in the soil from which N is taken up and the form of soil N that is used (organic N, NH4+ or NO3-) (Högberg, 1997).

2.1.2.4 N Budget (N_2 Fixed by Difference)

Studies indicated that the ultimate test of the contribution of N from fixation is to measure net inputs of N over long periods (>10 years) in the field (Giller and Merckx, 2003). However, this may be difficult as it requires measuring all inputs and outputs of N over this period, including inputs from fertilizer, wet N deposition, dry N deposition, run-on and uptake from lateral flow, outputs from crop/animal removal, gaseous losses, N leaching and soil erosion. To achieve a

reliable N balance it is necessary to have a very high repeatability and accuracy of N measurements through strict sampling protocols and extremely high sample numbers to enable the mean soil N to be precise enough to determine statistically significant changes in soil N (Vallis, 1973; Chalk, 1998). Despite App et al (1986) estimated about 7 kg N ha-1 crop-1 fixed by heterotrophic BNF from N balance studies in unfertilized planted pots covered with black cloth, however, major uncertainties still exist in extrapolating short-term N balance results to estimates of N fixation over a whole cropping period due to the lack of full account of N sources and losses (Unkovich et al., 2008).

2.1.3 Microorganisms in rice paddy

Various soil microorganisms have been playing different important roles in nutrient cycling, decomposition of organic matter, improving crop health in rice paddy, particularly flooded rice paddy. Flooded rice paddy soil can be considered as several phases (oxidized and reduced layer, oxic surface soil, anoxic bulk soil, and rhizosphere), where provides a complicated environment interacting with microbes, to look at the soil microbial diversity and community dynamics over the rice growing period. After irrigation in a rice paddy, the bulk soil is predominant by anaerobic microorganisms because of depletion of oxygen, for example, methanogenic archaea, which use carbonate as final electron acceptor to produce methane (Liesack et al., 2000; Thauer et al., 2008).

The composition of microbial community differ from farming system including conventional farming (adding chemical fertilizer), organic farming (adding organic fertilizer), natural farming (no-tillage, inter-tillage, returning rice straw or nothing added) as well as rice growth stage. Chemical fertilizers are likely to stimulate the growth of gram-positive bacteria in rice soils, while organic amendments increase the relative abundance of bacteria and fungi and

decrease the abundance of Actinomycetes (Zhang et al., 2007; Zhang et al., 2012). In regard to conventional farming, nitrogen addition increases the relative abundance of Actinobacteria, Firmicutes, Bacteroidetes (Ramirez et al., 2002; Nemergut et al., 2008; Hou et al., 2018), and decreasing the relative abundance of Acidobacteria and Verrucomicrobia (Ramirez et al., 2002). Pittol et al (2018) indicated Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria were abundant in the vegetative stage; Gammaproteobacteria, Bacilli and Clostridia were the more dominant in the reproductive stage. They also found fertilizer application during the vegetative stage might benefited bacterial richness. Betaproteobacteria, Gammaproteobacteria and Actinobacteria had the highest relative abundance among organic farming rice paddy (Hou et al., 2018). Farming systems make a difference on microbial community, diverse soil microorganisms are responsible for specific tasks in soil. For example, Actinobacteria and Bacteroidetes are associated with the decomposition of organic matter in agriculture soils, and genus Streptomyces of Actinobacteria makes its contribution for promoting plant growth and secretion of antibiotics against the plant pathogens in soil (Thomas et al., 2011; Reddy, 2014). Betaproteobacteria, a subgroup of Proteobacteria, has been studied for nitrogen fixation ability (Gyaneshwar et al., 2011), and the genus Burkholderia are able to promotes plant growth (Reddy, 2014). Studies also found in no-tillage farming system, where is more related to nitrogen fixation microorganisms (Wei et al. 2008; Wang and Zhang, 2010; Ma et al., 2011). There are many studies trying to figure out what role soil microorganisms acting in a conventional and an organic rice paddy, nonetheless, the response of microbial community composition to the no-tillage rice paddy at a lower taxa level is still unclear.

2.1.4 Analysis of soil microorganisms

Sequencing analysis of 16S rRNA subunit is the most widely used method to recognize the community of bacteria and archaea in soil and all environments (Whon et al., 2018). The 16S rRNA gene encodes the small subunit ribosomal RNA molecules of ribosomes, in charge of converting genetic messages to functional cell components via the translation of mRNA to proteins. Since ribosomal RNA is a component of all self-replicating systems, it is readily isolated, and its sequence changes slowly with time, able to detect among very distant species (Woese & Fox, 1977). All eukaryotic and prokaryotic cells contain ribosomes, which function as the sites of protein synthesis. Ribosomes are composed of two subunits, each of which consists of protein and a type of RNA called ribosomal RNA (rRNA). The letter S refers to Svedberg units, for example, 16S, which indicate the relative rate of sedimentation during ultra-high-speed centrifugation. Sedimentation rate is a function of the size, weight, and shape of a particle (Tortora et al., 2018). 16S rRNA is a highly conserved and ubiquitous gene, its sequence is about 1,500 basepair long and consists of nine hypervariable regions (V1–V9) separated by conserved segments, nine hypervariable regions are used as target sequences for primer design (Baker et al., 2003). Numerous 16S rRNA primers have been designed to analyze microbial community through the next-generation sequencing (NGS) technologies platforms such as Ion PGM Sequencer (Klindworth et al., 2013). After acquiring sequencing data from NGS, the QIIME 2 (Quantitative Insights Into Microbial Ecology) analysis is performed to analyze data. QIIME 2 is a microbiome analysis package for analyzing microbial community marker gene such as 16S or 18S rRNA genes (Bolyen et al., 2019).

2.2 The concept of natural farming

Natural farming is an alternative agricultural system to grow food instead of conventional farming, which is highly supported by microbial functions rather than utilization of various

types of fertilizer (Fukuoka, 1975; Hirano et al., 2001; Xu, 2006; Liao et al., 2019). The concept of natural farming is similar to conservation agriculture (often involves minimal tillage or no tillage and rotation) (Pittelkow et al., 2015; Hou et al., 2018; FAO, 2020), yet differs from organic farming (still in need of plowing, tilting, spreading organic fertilizer and weeding). The philosophy of natural farming could be elaborated well with 6th century BC ancient Chinese wisdom Laozi's Tao Te Ching"There is something that is perfect in its disorder, which is born before Heaven and Earth. So silent and desolate! It establishes itself without renewal and functions universally without lapse. We can regard it as the Mother of Everything. I don't know its name. Hence, when forced to name it, I call it 'Tao'. When forced to categorize it, I call it 'great'. Greatness entails transcendence. Transcendence entails going-far. Going-far entails return. Hence, Tao is great, Heaven is great, the Earth is great and the human is also great. Within our realm, there are four greatness and the human being is one of them.

Human beings follow the Earth;
Earth follows Heaven;
Heaven follows the Tao;
The Tao follows the way things are".

2.2.1 Introduction of "No-tillage"

No-tillage might match Laozi's philosophy. No-tillage has been used since ancient age for the cultivation of crops. The most indigenous cultures around the world such as the Incas in the Andes of South America have used a stick to make a hole in the ground, put seeds in the soil by hand and cover the seeds with the foot. This method has been using in central and South America until today. The ancient Chinese on the loess plateau and the ancient Egyptians has been conducting similar farming as well. 20 century's no-tillage or reduced tillage concept were introduced by Masanobu Fukuoka in his book the "One Straw Revolution" in 1975 (Kassam et

al., 2015) and Edward Faulkner in his book "Ploughman's Folly" in 1945. However, the no-tillage expansion globally has occurred since the mid- to late-1990s, was due to the dust bowl of the 1930s in the united states, where severe erosion of degraded soils occurred over large areas of agricultural land because of over using herbicides, therefore prompting a shift toward reduced tillage practices (Six et al., 2002; Derpsch et al.,2010). The advantages of no-tillage farming are considered as 1) labour and time are reduced, 2) costs for machines and fuels are saved, 3) soil tilth, moisture retention, and soil water infiltration are improved, 4) soil erosion, gas release and water pollution are reduced or prevented, 5) biodiversity in soils and fields are enriched, and 6) long term productivity and sustainability are maintained (Xu, 2006). No-tillage reduces mineralization rate of soil organic nitrogen because decomposition of organic matter by soil microorganisms often temporarily keep nitrogen in its body, making nitrogen in organic form which is less available for rice uptake. Although the main disadvantages of performing no–tillage are the risk of crop failure due to weed invasion and less availability of nutrients. However, the risks will be minimal if suitable weed removal tools are used, as well as adequately incorporating crop residue into the rice paddy.

2.2.2 Weed issue in natural farming rice paddy

Rice is grown over a widely divergent environments, however, in natural farming rice paddies, crop yields declined in the first 1–2 years following no-tillage, then reached conventional farming over time after 3–10 years (Pittelkow et al., 2015). This conclusion is consistent with our interview with rice natural farming farmers. Overall, no-tillage yields were reduced by 12% without N fertilizer addition and 4% with inorganic N addition (Pittelkow et al., 2015). In addition to the insufficient nutrients, weeds are one of the major issues that affect the rice yields. Weeds not only directly compete for sunlight, nutrients, and water with rice, but also increase

production costs and reduce grain quality and price. Cyperaceae and Echinochloa are the most prevalent varieties in rice paddy. The average yield losses in rice due to weed competition are estimated to vary between 40% and 60% which may go up to 94%–96% with uncontrolled weed growth (Chauhan and Johnson, 2011; Paul et al., 2014). Weed management is crucial to achieving the potential yield gains implementing natural farming. There are several weed management conducting in natural farming rice paddy. In this study, we focused on five–times inter-tillage.

2.2.3 What is "Inter-tillage"?

Tillage is a common management practice used worldwide by arable and horticultural land managers. Soil tillage aim to improve the physical environment by facilitating seed germination, root growth, plant establishment and minimizing competition with weeds (Sosbai, 2016). However, studies have shown that intensive rice cultivation conducting tillage every year, will reduces soil organic matter. Such a reduction in soil organic matter which is highly related to soil microbial activity and nutrient availability, leads to severe negative effects on rice yield. Over time, successive soil mobilization also tends to produce deleterious effects on soil drainage and promotes subsurface compaction, hampering water movement and aeration along the soil profile (Bado et al., 2010; Pedrotti et al., 2001; Beutler et al., 2014; Buarach et al., 2014). Inter-tillage is an alternative tillage system using in natural farming rice paddy, which is performed by a small machine with chain–weeder (photo 1) to control weeds amounts, enhance soil and plant root room aeration and water infiltration (Ahmad et al., 2000; Kasubuchi et al., 2019). Kasubuchi et al (2019) concluded that conducting five times inter-tillage (5T) in a natural farming rice paddy during the rice growing period can increase the yield as high as

that of the conventional farming about five years later. However, there is a gap in the role of microorganisms that need to be filled.

Photo 1. The inter-tillage machine used to remove weed in five-times inter-tillage plots.

2.2.4 Importance of rice straw incorporation

In a natural farming rice paddy, where relies on organic residue incorporation, rice straw plays an essential role. Rice straw incorporation has the potential to improve soil quality and maintain sustainable soil productivity by increasing soil organic carbon sequestration and nutrients deposition. Rice straw consists of easily degradable compounds, for example, polysaccharides such as cellulose and hemicellulose, which serve as valuable carbon source for different soil microorganisms. Lignin is a more complex compound, which is only degraded by specialized microorganisms (Watanabe et al., 1993). Previous studies indicated that the crop straw

incorporation resulted in an increase on the soil microorganism abundance (Zhang et al., 2016; Zhao et al., 2016). Bacterial taxa like Clostridium, Proteobacteria, Bacteroidetes, Chlorobi, Acidobacteria, Actinobacteria, Sphingobacteria, Cyanobacteria and Bacilli, are involved in the straw decomposition in flooded rice soils (Rui et al., 2009). Nevertheless, organic acids such as formic, acetic, propionic and butyric acids, derive from organic matter during decomposition stage under reduced layer of flooded rice paddy (Fox 1995; Jones 1998), may reduce crop yields if phytotoxin accumulation to a toxic level (Angeles et al. 2006; Jones and Darrah 1994). Thus, a study to complete the rice straw decomposition one step earlier than rice transplanting in a natural farming rice paddy, is profitable to agronomic.

2.2.5 Other practices of natural farming in rice paddy

2.2.5.1 Rice–duck and rice–fish farming system

The Aigamo rice–duck is a rice farming system that relies on ducks to eat insects and weeds has been practiced in Japan (Asano et al., 1999; Isobe et al., 2005), which has now been widely using in Korea, China, Vietnam, Philippine, Thailand, India and middle east. This farming system is particularly accepted by farmers who weeding by hand or performing natural farming. One or two weeks after the seedlings are planted, the ducks are released to rice paddy, and kept in rice fields during the growing season (Furuno, 2001).Releasing ducks to rice paddies have been practiced back to 1000 years ago in ancient China, and introduced to Japan about 500 years ago. In 1989, Japanese farmer and agronomist Takao Furuno established Aigamo Farming, in which domestic “Aigamo” ducks (hybridized mallards and Pacific black ducks Anas superciliosa). The Aigamo rice–duck riceduck farming also be able to help reducing methane emissions from paddy paddies, is due to increased oxidation of sediments by bioturbation. Sesser et al (2014) suggested the potential impacts of after harvest agricultural practices that

reduce GHG emissions on waterbirds. Duck excreta are rich in nitrogen that could reduce both fertilizer costs for farmers, one duck produces 150 g per day (Subramanian et al., 1996). However excessive concentration of duck excreta might result in over dose of nitrogen nutrient and cause contamination to water and plants (Roh et al., 2009).

Rice-fish mutualism system is a traditional agriculture practice, has been practiced for more than 1700 years in China and other Asian countries. (Guo, 1985; Li, 1992). The studies indicated the rice–duck mutualism system has potentials for control of aquatic weeds, pests, plant diseases, improving the soil fertility (Noorhosseini-Niyaki and Bagherzadeh-lakani, 2013). In this system, before releasing fishes into flooded rice paddies, the fries are raised in flooded rice paddies before transplanting, in rice growing fields and in nearby ponds, planting rice on the ridges while raising fish in the furrows, and raising fish in the small channels prepared in the paddy fields (Xu, 2006).

There are some disadvantages that farmers hesitating to adopt such farming. A large amount of irrigation water is necessary to apply to a deeper depth field for fishes living, the shortage of water either cause by climate or human demand, will greatly reduce the effect on rice growing, and fish may damage and eat seedlings (Coche, 1967). Overall, a well-managed is considerable to perform a successful rice–duck or rice–fish farming system otherwise side effect might occur.

2.2.5.2 Rice–soybean rotation

Crop rotation is an agricultural practice that two to three different economic crop species sequentially planted on the same field or plot, which has been used since the Middle Ages (Bruns, 2012). Rice–soybean rotation system possessing multiple functions, has been mainly

employed in southern America, southern and eastern east, northern Japan owing to meet variety of requirements. (Nishida et al., 2013; Carroll et al., 2020). Rice–soybean rotation system rotations is proved to increase nitrogen in the soil through nitrogen fixation (Peoples et al., 2009; Scherner et al., 2018) and rice yield compared with rice–rice succession (Cheng and Pai, 1995). Water resource for irrigation could be reduced if rice–soybean system planting dates are planned optimally (Popp et al., 2005). This system is also able to reduce seed bank and weed of troublesome weed species, because the different types of crops and different cultivation methods, the crop rotation system changes the pressure of weed competition, resulting in changes in the amount of weeds and the weed phase (Cheng, 1997; Scherner et al., 2018). Studies also reported that crop rotation with soybean significantly show a higher microbial diversity and richness (Venter et al., 2016; Chamberlain et al., 2020). In eastern Taiwan, there are a few young farmers have been performing rice–soybean rotation with natural farming for three to five years. They found some of most common rice diseases “Rice blast” and pests is decreasing, probably because of without fertilizing thus less nitrogen residue in the soil, as well as the interruption of insect host by crop rotation. Despite rice are healthier, young farmers are still concern about the rice yields. The yield of rice–soybean rotation with natural farming is far below to conventional farming in the next county, which is 1500 kg / ha to 2400 kg / ha, only about 60 % of conventional farming yield.

Soil microorganisms are a main role for maintaining the sustainability of an agricultural production system (Bending et al., 2004). Studies reported soil biological activity can be enhanced as evaluated by soil microbial biomass, soil dehydrogenase, urease, β-glucosidase, phosphatase and arylsulfatase activities with green manure, as well as improved the microbial biomass and activity and changed the soil microbial community significantly (Stark et al; 2007; Tejada et al., 2008; Piotrowska and Wilczewski, 2012;). Zhang et al (2017) found Acinetobacter and Pseudomonas accumulated in rhizosphere of green manure treatments with

molecular analysis. In spite of the fact that many evidences have been proved, but indirect methods such as biomass and enzyme activities or low throughput methods such as DGGE and clone library were mainly used, few studies discussed microbial community and composition. In addition, the impact of soybean as green manure under natural farming on bacterial population dynamics and crop yields remains unclear.

Chapter 3

Identifying the microbes communities in conventional and natural farming rice paddies in Japan and Taiwan

3.1 Abstract

"Natural farming" (NF) practices (farming systems without any use of chemicals) have the potential to decrease the use of chemical fertilizer. Microbial community are one of the keys to conduct a successful natural farming rice paddy. In order to understand the microbial community dynamics in a high yield rice paddy, we investigate conventional (C) and natural farming managed system respectively in Japan and Taiwan, as well as the delta 15N natural abundance in Japan. Our results indicated there were no significantly different microbial community between C and NF rice paddies in Japan. In contrast to Japan, most of the phylum were significantly different in Taiwan between two different system rice paddies. In terms of microbial diversity, the Shannon diversity indices was not significantly higher in NF rice paddy where had higher yields (5.6 ± 0.7 t ha−1) compared with C rice paddy (3.3 ± 0.7 t ha−1) in Japan. In Taiwan, the Shannon diversity indices was significantly higher in C where produced more rice (6.5 t ha−1), the yields in NF (1.5–2.4 t ha) were only about one third of C. The 16S sequencing results showed rice paddy had higher rice production in Japan was more influenced by class of Acidobacteria and Planctomycetes, Deltaproteobacteria was the predominant class in Taiwan. The δ15N natural abundance was decreasing from water inlet to outlet, and was lower in summer (July) compared with spring (June). In conclusion, specific microbes influenced rice yields, and N-fixing microorganisms might prefer to habitant in warmer temperature spot.

3.2 Introduction

The widespread utilize of chemical fertilizer in rice crop system in order to keep up with production stability. Global nitrogen fertilizer application has increased approximately 10 fold between 1950 and 2008 (Robertson and Vitousek, 2009). However, studies have shown that crops can take up only 30–50% of chemical fertilizers, The other 50% of nitrogen remains in soils or leaves cropping systems through air, surface water, or groundwater pathways (Eickhout et al., 2006; Robertson and Groffman, 2007), thus a great amount of the applied fertilizer is lost in the soil where it pollutes groundwater (Norse, 2005; Zsofia et al., 2012), and the efficiency of chemical fertilizer use has decreased because of fertilizer saturation (Zsofia et al., 2012; Carter et al., 2012).

Alternative strategies to improve soil health and fertility, conserve soil resources, and establish a sustainable agricultural model, such as natural farming system, have been developed, including crop rotation, intercropping, reduced tillage, without the use of agrochemicals. Natural farming system is highly rely on soil microbes which play key roles in the decomposition of organic matter, nutrient cycling and altering the availability of nutrients to plants (Larsen et al., 2014), so it has been used as a sensitive indicator to predict soil biological conditions and the effect of agricultural practice in soil ecosystem (Hartmann et al., 2015). Studies indicated that conventional with chemical fertilizer and natural farming without chemical fertilizer are likely to affect the composition of microbial community, furthermore, high crop production rice paddy are predominant by specific microbes (Zhang et al., 2012; Luo et al., 2016).

Biological nitrogen fixation (BNF) by free-living bacteria in soils or associated with the rhizosphere has the potential to meet the lower input cropping farming systems particularly natural farming rice paddy, although their ability of providing nitrogen can be disputed varies

widely range from 0 to 60 kg N ha−1 yr−1 (Gupta et al., 2006), the contribution of N2 fixation microorganisms to sustainable cropping system is undoubtable. The degree in BNF often controls the successfulness of the natural farming rice paddy. However, where is the BNF “hot spot” in a rice paddy is not well understood.

Thus, the aims of this study were: (1) to analyze the microbial community under conventional and natural farming managed rice paddies in Japan and Taiwan; (2) to evaluate what are the predominant microbes at high yield rice paddies in the two countries; (3) to investigate the biological nitrogen- fixation (BNF) “hot spot” by measuring delta 15N natural abundance in Hokudai rice paddies under two different farming system in spring and summer.

3.3 Materials and methods

3.3.1 Field sites and agricultural management

3.3.1.1 Hokudai

The study was conducted on flooded rice paddy research fields of the Field Science Centre of Hokkaido University, Sapporo, Japan (N43° 04′ 39"151, E141° 20′ 03"634). The trial investigates the dynamic of in a conventional and a natural farming rice paddy. The conventional and natural managed rice paddies are 40 x 25 m in size. The conventional managed rice paddy was with chemical fertilizer, herbicide, pesticide and insecticide application. The natural managed rice paddy was divided in nine plots with 13.34 x 8.33 m in size each, where alongside with no fertilizer, herbicide, pesticide and insecticide application was established in 2017. Before 2017 there were three years maintained by flooded conditions during the cropping seasons with no crop growth and herbicide application. The treatment of the nine plots is divided in 0–, 2– and 5–times inter-tillage (three plots each).

3.3.1.2 Taiwan

The natural farming rice paddies "GA" (N23° 35′ 53.5, E121° 23′ 07.2) and "GB" (N23° 38′ 02.0, E121° 26′ 46.8) are located in Guanfu, Taiwan. "GA" and "GB" are conducted rice and soybean rotation, without using fertilizer, herbicide, pesticide and insecticide. The GA rice paddy area is 0.29 ha, where planting rice form March to July followed by planting soybean as green manure from August to November, no crop was planting from November to coming February; the GB rice paddy area is 0.39 ha, where planting soybean form March to July, followed by planting rice from August to December, rapeseed as green manure planted in January. The conventional farming rice paddy "Yuli" (N23° 20′ 13.9, E121° 19′ 25.0) is located in Yuli, Taiwan. Yuli rice paddy is 1.02 ha, conducted double crops a year, with chemical fertilizer, herbicide, pesticide and insecticide application. The first crop was grew in early February; the second crop was grew in July. No green manure is used in this rice paddy.

3.3.2 Soil sampling procedure

In frame of the study plots from the conventional and natural farming rice paddy were sampled in Hokudai and Taiwan. In Hokudai, for the determination of the δ15N natural abundance, three replicates of the soil samples were collected from soil surface (0–0.5 cm depth) and rice root (2–5 cm depth) from the two differently managed rice paddies in June and July, the two rice paddies were neighboring. After collected the soil samples were stored at 4 ℃. For microbial community analysis, three replicates of the soil samples were collected from 0−5 cm soil depth from the same locations in August, collected soil samples were stored at−80 ℃. In Taiwan, the GA location with no flooded situation was due to the preparation of growing soybean, the two replicates of the soil samples were collected at soil surface (0–2 cm depth). The GB and

Yuli soil samples were collected from 0−5 cm soil depth. All the soil samples in GA, GB and Yuli were collected in 27th August, and sent to Hokkaido, Japan in September. The soil samples arrived at November and were stored at−80 °C for microbial community analysis.

3.3.3 16S sequencing library preparation

The DNA of the sampled soils was extracted using a NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol,and buffer SL2 was used. A negative extraction control was performed using empty extraction tubes with the first and last extraction. The DNA extract was quantified using Qubit 2.0 Fluorometer (Invitrogen, Waltham, United States) and a Qubit dsDNA BR Assay Kit. The V4 region of the 16S rRNA gene was amplified using PCR primer F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′). An AmpliTaq Gold® 360 Master Mix (Applied BiosystemsTM, Carlsbad, USA) and 5 ng input DNA were used for the PCR. The PCR program included initial denaturation at 95° C for 10 min followed by 20 cycles of denaturation at 95℃ for 30 sec, annealing at 57℃ for 30 sec and elongation at 72℃ for 1 min and ended with final elongation at 72℃ for 7 min. A positive control using E. coli DNA control (Applied BiosystemsTM, Carlsbad, USA) and a negative control were performed with the same conditions. The amplicons were checked on a 1.5% agarose gel. Purification of PCR products was performed using an Agencourt AMPure XP kit (Beckman Coulter Inc., Webster, United States) according to the manufacturer protocol. Ten nanogram of amplified DNA per sample were barcoded using the IonA–barcode[i]-F515 forward and IonP1–R806 reverse primers. The PCR program included the same conditions than above but with 5 cycles. The barcoded amplicons were purified and quantity checked as described above. The quality was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, USCA)

using an Agilent DNA 1000 Kit. The library was diluted to 50 pM and loaded into the Ion 314 Chip (Thermo Fisher Scientific K.K., Japan) using the Ion Chef Instruments (Thermo Fisher Scientific K.K., Japan) with the Ion PGM™ Hi–Q Chef Kit. DNA sequencing was conducted on the Ion PGM Sequencer (Thermo Fisher Scientific K.K., Japan) using the Ion PGM 400 Kit.

3.3.4 Analysis of the 16S rRNA based bacterial community structures

The barcoded 16S rRNA gene sequences were de–multiplexed, quality–filtered, and assessed using the Quantitative Insights Into Microbial Ecology (QIIME 2) workflow. Operational Taxonomic Units (OTUs) were prepared by eliminating all the OTUs that matched the GreenGenes 13_5 reference sequence with 97% similarity. To analyze the changes in microbial community and their interactions with other environmental factors, Principal Component Analysis (PCA) was performed, the PCA plots were separated into three part with same variation.

3.3.5 Statistical analysis

One-way ANOVA and Tukey–Kramer multiple comparison tests was performed for the analysis of significant differences among samples on duration or for each treatment.
Statistical analysis were performed by R 3.6.1 and set the significance level at $P < 0.05$.

3.4 Results

3.4.1 Bacterial community in Taiwan and Hokudai

The microbial taxonomic composition showed a total of 64 phyla and 186 classes in Hokudai; that of 71 phyla and 229 classes in Taiwan (Archaea and Bacteria domains). There was only one different phylum in top 10 (Fig 1), they were Bacteroidetes in Hokudai and Nitrospirae in Taiwan. Consists of the top 5 phylum were the same in Hokudai and Taiwan. The top five bacteria in both countries were Proteobacteria, Acidobacteria, Planctomycetes, Chloroflexi and Crenarchaeota. Proteobacteria predominated C, GA and Yuli. Acidobacteria and Chloroflex were highest in GB and NF, respectively. One–way Anova results (Table 1) showed that there were no significant difference among bacteria at C and NF rice paddies in Hokudai. However, at GA, GB and Yuli rice paddies in Taiwan, apart from Chloroflexi, Crenarchaeota and Nitrospirae, the bacteria were significantly different between conventional and natural farming rice paddies (Table 2). The Shannon diversity indices (Fig 2a) was higher at NF rice paddies in Hokkdai, but not significant difference. In contrast, it was significantly higher ($p < 0.02$) at C rice paddies in Taiwan (Fig 2b).

Table 3.1 Relative abundance of the top 10 bacteria at the phylum level of taxonomy, and results of tests for significant differences among sites in Hokudai (Japan). The right side shows F- and P- values from one-way ANOVA testing for significant differences among sites. Lowercase letters identify significant pairwise differences between sites for each bacteria group (Tukey test).

	C	NF	F	P
Chloroflexi	a	a	2.296	0.168
Proteobacteria	a	a	1.23	0.3
Planctomycetes	a	a	1.846	0.211
Acidobacteria	a	a	1.253	0.295

Crenarchaeota	a	a	0.189	0.675
Verrucomicrobia	a	a	3.439	0.101
Euryarchaeota	a	a	0.962	0.355
Actinobacteria	a	a	2.09	0.186
Bacteroidetes	a	a	2.08	0.187
WS3	a	a	1.493	0.257
Others	a	a	0.072	0.795

Table 3.2 Relative abundance of the top 10 bacteria at the phylum level of taxonomy, and results of tests for significant differences among sites in Taiwan. The right side shows F- and P- values (*P < 0.05, **P < 0.01, ***P < 0.001) from one-way ANOVA testing for significant differences among sites. Significant values are bolded. Lowercase letters identify significant pairwise differences between sites for each bacteria group (Tukey test).

	GA	GB	Yuli	F	P
Proteobacteria	ab	a	b	4.715	**0.0258 ***
Acidobacteria	b	b	a	12.98	**0.000535 *****
Planctomycetes	b	b	a	10.64	**0.00133 ****
Chloroflexi	a	a	a	0.646	0.538
Crenarchaeota	a	a	a	1.428	0.271
Nitrospirae	a	a	a	0.521	0.604
Actinobacteria	b	a	a	14.52	**0.00031 *****
Verrucomicrobia	b	ab	a	4.598	**0.0277 ***
WS3	b	b	a	14.16	**0.000351 *****
Euryarchaeota	a	b	ab	4.375	**0.0319 ***
Others	b	ab	a	4.01	0.0403 *

Principal components analysis (PCA) analysis of Hokudai showed that the first and second principal components explained 89% and 5% of the total sample variation, respectively (Fig 3 a and b). The microbial community for each of the two treatments clustered into two groups which were C and NF rice paddies, three NF sampling soils were not clustered (Fig 3a). The first and second principal component separated the conventional and natural farming rice paddies. PCA analysis of Taiwan showed that the first and second principal components explained 56% and 25% of the total sample variation, respectively (Fig 3 c and d). The microbial community for each of the three location clustered into two groups which were GB and Yuli rice paddies, GA rice paddy scattered around the diagram. The first and second principal component separated the conventional and natural farming rice paddies.

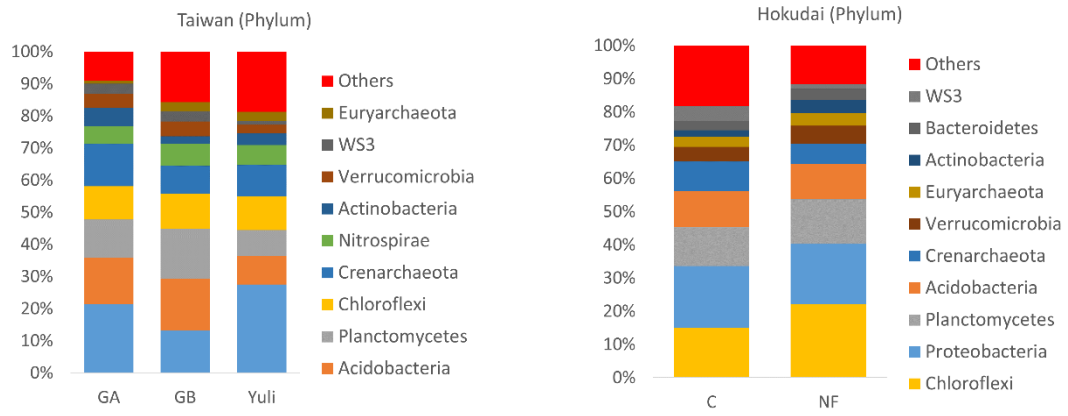

Figure 3.1 Relative abundance of the top 10 bacteria at the phylum level of taxonomy in Taiwan and Hokudai (Japan). The other phylum are categorized as "Others". GA, GB and NF rice paddies were natural farming practice; Yuli and C rice paddies were conventional practice.

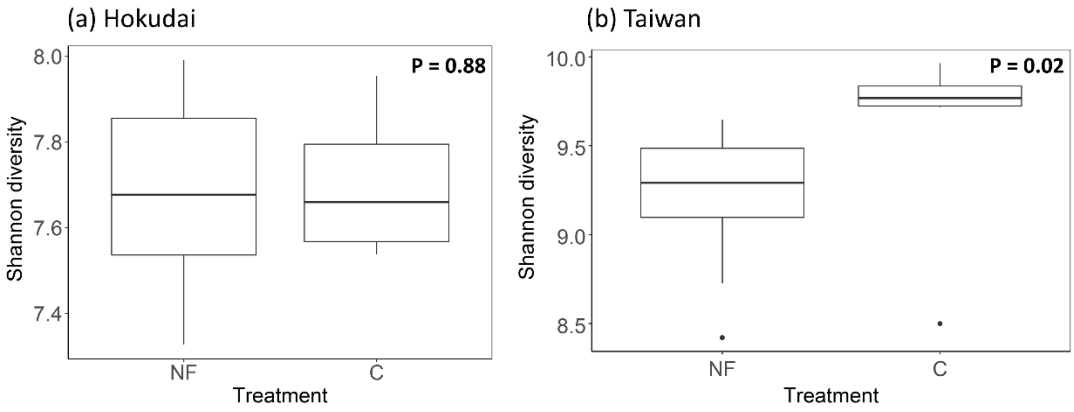

Figure 3.2 The Shannon diversity indices at OTUs level at (a) Hokudai site and (b) Taiwan site. Level of significance was determined by and one-way ANOVA. Error bars represent standard deviation (n = 3). NF: natural farming practice with no chemical fertilizer, C: conventional farming practice with chemical fertilizer.

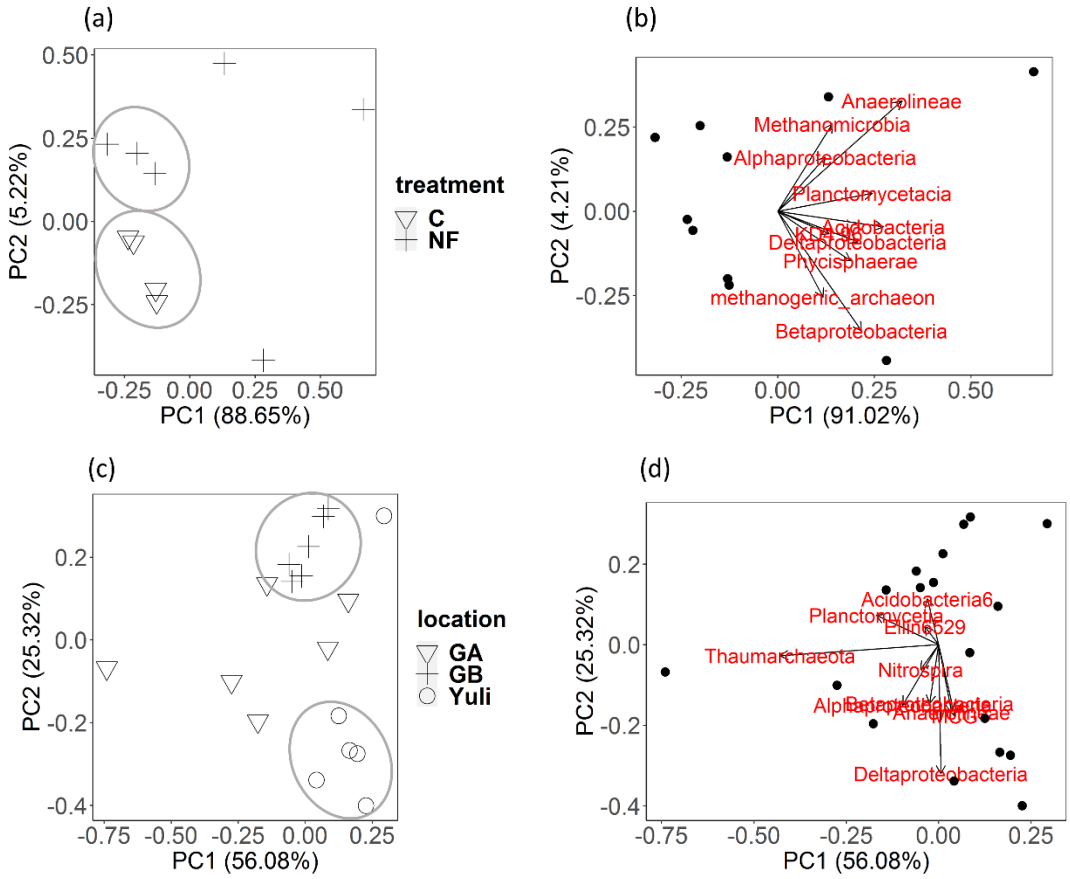

Fig 3.3 Principal component analysis (PCA) on (a) conventional farming practice with chemical fertilizer (C, triangle) and natural farming practice with no chemical fertilizer (NF, cross) and (b) arrows show bacteria on class level in Hokudai. (c) Natural farming practice location with no chemical fertilizer (GA, inverted triangle; GB, cross); conventional farming practice location with chemical fertilizer (Yuli, circle) and (d) arrows show bacteria on class level in Taiwan.

3.4.2 The δ15N natural abundance in Hokudai rice paddies

At conventional and natural rice paddies in spring (June) and summer (July), the δ15N natural abundance was decreasing from water inlet to water outlet in both soil surface and rice root location. In spring, from water inlet to outlet, the δ15N natural abundance value was 7.3 to 7.1 at soil surface, 6.9 to 6.6 at rice root in the natural farming rice paddy; that was 7.7 to 6.4 at soil surface, 7.8 to 6.1 at rice root in the conventional farming rice paddy. In summer, from water inlet to outlet, the δ15N natural abundance value was 7.1 to 6.2 at soil surface, 6.6 to 5.9 at rice root in the natural farming rice paddy; that was 7.7 to 6.3 at soil surface, 7.2 to 5.6 at rice root in the conventional farming rice paddy (F).

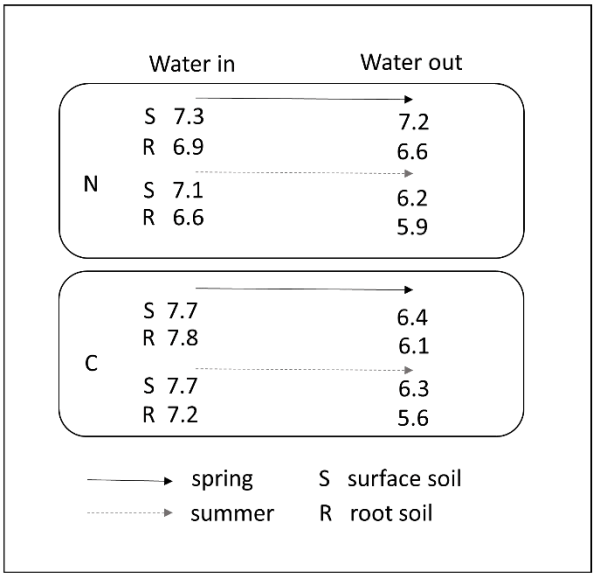

Figure 3.4 The δ15N natural abundance dynamic from water inlet to water outlet at two different seasons in Hokudai rice paddy. N: natural farming practice with no chemical fertilizer; C: conventional farming practice with chemical fertilizer. Solid line: spring; dashed line: summer. S: surface soil; R: rice root soil.

3.5 Discussion

Our study found that there were no significantly different microbial community between C and NF in Hokudai (Table 1). In contrast to Hokudai, most of the phylum were significantly different in Taiwan (Table 2). In terms of CF rice paddies, Hou et al (2018) indicated that bacteria had a very different pattern of relative abundance in less Proteobacteria but more Verrucomicrobia, and higher relative abundance of Bacteroidetes come from conventional rice paddy due to chemical fertilizer utilize (Nemergut et al., 2018). However, we did not observe the consistent results in both conventional rice paddies, the relative abundance of Verrucomicrobia and Bacteroidetes were low in Hokudai, that of Bacteroidetes even not showed up in top 10 phylum in Taiwan. Numerous studies found chemical fertilization influences soil microbial diversity through direct effects on the soil nutrient content, which is due to soil microbial communities are sensitive to fertilization and their responses to mineral fertilizers in soils, repeated overuse or long-term application of nitrogen fertilizer have a negative effect on soil quality and soil microbial community structure, might further impact crop yields (Bell et al., 2015; Martin et al., 2015; Fu et al., 2017; Singh and Gupta, 2018). However, the Shannon diversity indices (Fig 2a) was not significantly higher in NF rice paddy where had higher yields (5.6 ± 0.7 t ha−1) compared with C rice paddy (3.3 ± 0.7 t ha−1) in Hokudai. Interestingly, in Taiwan, the Shannon diversity indices was significantly higher in C where produced more rice (6.5 t ha−1), the yields in NF (1.5–2.4 t ha) were only about one third of C. Thus, in these cases, the rice yields were not influencing by utilize of chemical fertilizer, besides, the microbial diversity indices was not capable of explaining rice yield in Hokudai rice paddies.

A study mentioned that the microbes in a high-yield rice paddy soil was more active and responsive to changes in soil properties, and more effective at modulating soil enzymatic activities (Luo et al., 2016). At a high-yield crop soil, Acidobacteria, Planctomycetes, and Deltaproteobacteria are the most predominant (Masuda et al., 2017; Khan et al., 2017; Wu et

al., 2018). According to the principal component analysis (PCA) in this study, we found NF rice paddy was more influenced by class of Acidobacteria and Planctomycetes in Hokudai (Fig 3b), and the Deltaproteobacteria was predominant at C rice paddy in Taiwan (Fig 3d). Therefore our results also proved that high-yield rice paddies were related to the microbes mentioned above. These microbes are considered to stronger adaptability and resilience, which enable their survival under stressful conditions, and regulated the surrounding environmental attributes through feedback mechanisms (Trivedi et al., 2013), class such as Deltaproteobacteria were associated with contributing NH4+ production via DNRA and nitrogen fixation to rice paddy and increasing plant nutrient use efficiency by solubilization and mineralization of nutrient components particularly mineral P as well as synthesis of phytohormones (Sarkar et al., 2012; Islam et al., 2016; Khan et al., 2016 Masuda et al., 2017).

In general, water temperature and several environmental factors have been suggested to influence the rates of N-fixation in soil (Barron et al. 2008; Hsu and Buckley, 2009; Reed et al., 2011). There was a decreasing gradient of δ15N natural abundance from water inlet to outlet at Hokudai conventional and natural farming rice paddies (Fig 4), the δ15N natural abundance in summer (July) was lower compared with spring (June), potentially due to higher temperature inducing more N-fixation. Montañez et al (1995) found N-fixation ability was lower below 15℃, where explained the exposure to the sun of the irrigation water might improve N-fixation that was close to water outlet area. When looking at the difference between soil surface and rhizosphere, the δ 15N natural abundance in rhizosphere was higher than soil surface. Flooded rice paddies are known to contain two major N-fixing microorganisms, among which phototrophic bacteria (e.g. blue green algae) inhabit flooded waters and soil surfaces; heterotrophic bacteria living in the rhizosphere of rice (Ladha and Reddy, 2003). Phototrophic bacteria generally obtain their energy through photosynthesis while heterotrophs derive energy by utilizing exogenous organic compounds and heterotrophic bacteria are often associated with

plants (Choudhury and Kennedy, 2004). In contrast to our result, Bei et al (2013) measured the proportions of 15N fixed by heterotrophic and phototrophic, they found that heterotroph fixed fewer nitrogen than phototroph, which was 2.15 ± 0.07 and 2.18 ± 0.19 mg pot -1 respectively. Therefore, the N-fixing microorganisms community in soil surface and rhizosphere need more investigations.

3.6 Conclusion

The 16S sequencing results showed rice paddy had higher rice production in Japan was more influenced by class of Acidobacteria and Planctomycetes, Deltaproteobacteria was the predominant class in Taiwan. The δ15N natural abundance was decreasing from water inlet to outlet, and was lower in summer (July) compared with spring (June). In conclusion, specific microbes influenced rice yields, and N-fixing microorganisms might prefer to habitant in warmer temperature spot.

Chapter 4

Inter–tillage during natural farming rice paddy production negatively impacted the microbial abundances in soils but not on diversities

4.1 Abstract

In natural farming rice paddies, inter-tillage (tillage between rows, during rice growth period) is often performed mainly to remove weeds without the use of chemicals. Also, the inter-tillage disturbs soil surfaces, potentially impacting the characteristics of soil microbial communities, such as their diversity and abundance. Natural farming systems aim to maintain biodiversity, but it remains unclear whether the inter-tillage impact soil microbes in rice paddies. Thus, this study aimed to understand to what extent "five-times inter-tillage" treatment (5T) influences on soil bacterial abundance and community structures compared with no-tillage (NT), under a natural farming rice paddy system. Soils were sampled at rice proximity, soil surface and 10 cm depth in a natural farming rice paddy, during the early to late vegetative phase (June to July), in Hokkaido, Japan. The 16S rRNA community structures and abundance were analyzed by next generation sequencing (NGS) and quantitative PCR, respectively. We observed NT had significantly higher bacterial abundances at the soil surface than 5T. However, there was no clear differences between 5T and NT, regarding the bacterial community structures, including their diversity indices. Instead, the sampling timings markedly impacted the bacterial community structures for the rice proximity and soil surface, showing increasing diversity indices at the late vegetative stage, compared to the early vegetative stage, suggesting the interaction between the crop growth and bacterial communities. In this study, we did not observe the significant difference between the rice yield from NT (2.3 ± 0.7 t ha^{-1}) and 5T plots

(2.7 ± 0.9 t ha^{-1}), however, the 5T might have negatively impacted soil bacterial abundances but not the community structure of the bacteria.

4.2 Introduction

Natural farming is an alternative agricultural system to grow food, which is highly supported by microbial functions rather than utilization of various types of fertilizer (Fukuoka, 1975; Hirano et al., 2001; Xu, 2006; Liao et al., 2019). The concept of natural farming is similar to conservation agriculture (often involves minimal tillage or no tillage (NT) and rotation) (Pittelkow et al., 2015; Hou et al., 2018; FAO, 2020), yet differs from organic farming (still in need of plowing, tilting, spreading organic fertilizer and weeding).

Regarding natural farming rice paddy systems, Kasubuchi (2019) concluded that conducting five times inter-tillage (5T) in a natural farming rice paddy during the rice growing period can increase the yield as high as that of the conventional farming about five years later. Multiple inter-tillage method had been widely conducted in rice paddies to control weed growth during Edo period where was from the end of 17^{th}–18^{th} century. Inter-tillage is performed during the rice growth period, using a small machine with chain–weeder to control weeds amounts (Supplementary Photo 1), enhance soil aeration, plant root growth and water infiltration (Ahmad et al., 2000; Kasubuchi et al., 2019).

Generally, tillage can soften soil for plant root to spread over a deeper distance underneath the soil surface; shatter soil organic matter to accelerate the release of nutrients for crop growth; remove weed to minimize its competition with crop; incorporate oxygen which benefits aerobic microorganisms active (Hobbs et al., 2008; Powlson et al., 2012; Fujiwara, 2013; Das et al., 2014). Contrastingly, tillage decreases microbial abundance and activities in a long-term period due to increasing decomposition rate of soil organic matter which contains dead animal body and plant (Mohammadi et al., 2011; Rey Benayas et al., 2012). In contrast to tillage treatment, NT treatment can increase formation of soil organic matter and soil aggregate because without

disturbance on soil thus further improve soil microbial activities (Six et al., 1999; Baker et al., 2007; White and Rice., 2009; Derpsch et al., 2010; Marandola et al., 2019).

However, the difference in microbial abundance and diversity under NT and inter-tillage treated soils in naturally managed rice paddy systems was not well understood. The inter-tillage is performed during the rice growth period, when the field is submerged in water, thus the impact of the tillage in the rice paddy can be different from the impact of tillage for the upland soils. Therefore, better understanding of the impacts of inter-tillage on soil microbial abundance and diversity, specific for the natural farming rice paddy, is required to elucidate the reasons behind the successfulness of this system. The maintenance of soil microbial activities and functions is critically important for natural farming, in general.

Thus, the primary goals of this study were to compare the dissimilarity of soil bacterial abundance and diversity under 5T and NT treatment within a paddy farm managed using the natural farming style. We hypothesized: (1) Soil bacterial abundance and community would sharply change after inter-tillage compare with NT; (2) 5T and NT treatment would be significantly at variance with soil bacterial abundance and community for the duration of vegetative phase.

4.3 Materials and methods

4.3.1 Field sites and agricultural management

The study was conducted on flooded rice paddy research fields of the Field Science Centre of Hokkaido University, Sapporo, Japan (N43°04′39"N / E141°20′03" E) (Fig 4.1). The trial investigates the dynamic of inter–tillage and NT treatments in a natural farming rice paddy. In this study, we focused on 5T and NT treated plots. The natural managed paddy is 40 x 25 m in size and divided in nine plots with 13.34 x 8.33 m in size each. The natural farming rice paddy alongside with no fertilizer, herbicide, pesticide and insecticide application was established in 2017. Before 2017 there were three years maintained by flooded conditions during the cropping seasons with no crop growth and herbicide application. Also, the fields were ploughed at one month before seedlings were transplanted to the paddy fields. The treatment of the nine plots is divided in 0–, 2– and 5–times inter-tillage (three plots each) (Fig 4.2).

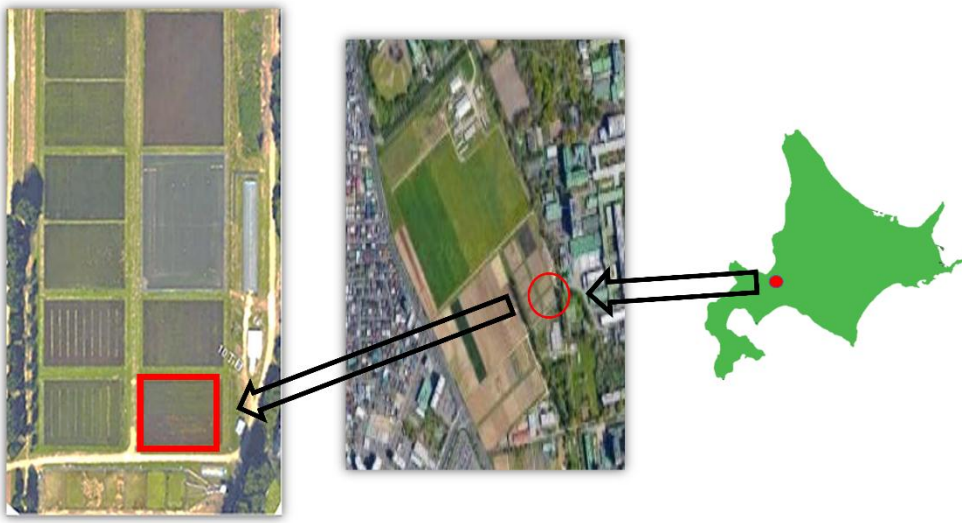

Fig 4.1. The sampling site. the Field Science Centre of Hokkaido University, Sapporo, Japan (N43°04′39"151, E141°20′03"634).

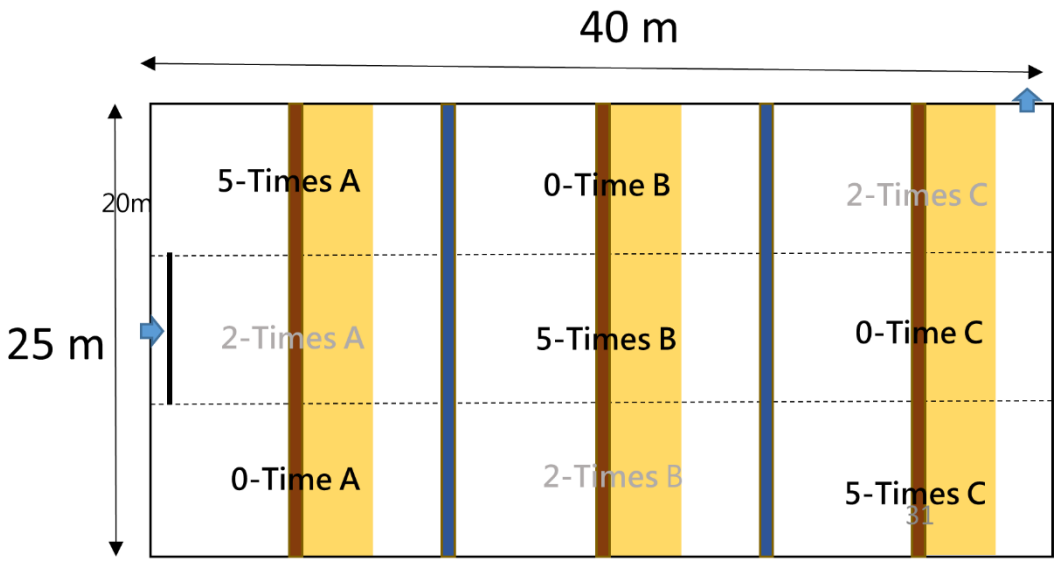

Fig 4.2. The experimental site of natural farming rice paddy. It was divided into nine plots and A, B, C blocks. Panels were set within block (brown) and between blocks (blue). Each plot was separated with polycarbonate board. Each block including 0- (No-tillage; NT), 2- (2 times inter-tillage; 2T) and 5- (5 times inter-tillage; 5T) treatments, where soils were sampled at NT and 5T plots. Each treatment had three replicates.

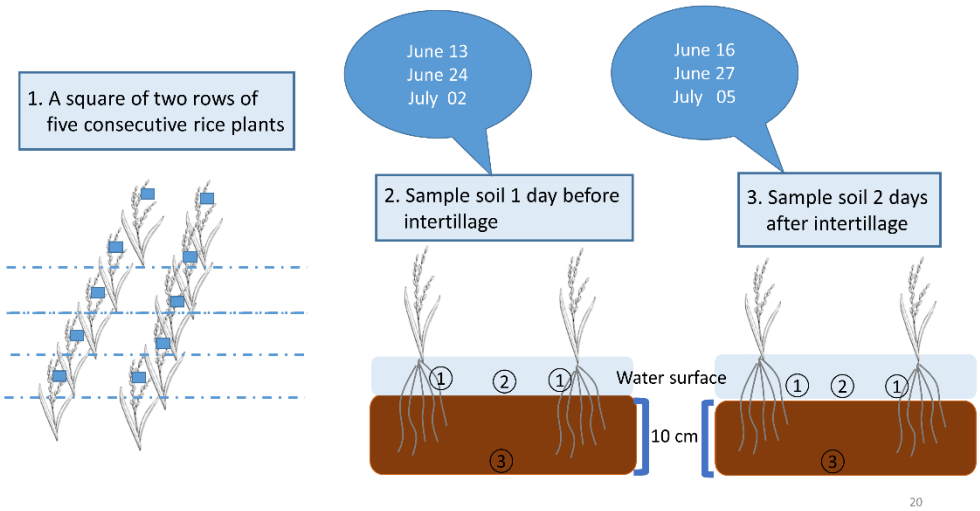

Fig 4.3. Soil samples were taken from: ① rice proximity; ② soil surface; ③ 10 cm depth one day before and two days after management activities for the third, fourth and fifth inter–tillage event in 5T and NT plots.

4.3.2 Soil sampling procedure

In frame of the study plots from the natural farming rice paddy with 5T and NT treatment were sampled. To investigate short–term effects of 5T treatment compared with NT, samples were taken one day before and two days after management activities for the third, fourth and fifth inter–tillage event. The third and fifth inter–tillage events corresponded to early vegetative phase (June) and late vegetative phase (July) respectively. In total six plots were sampled corresponding to three replicates per treatment. From each plot soil samples were taken at three different locations within a square of two rows of five consecutive rice plants. The soil surface samples were taken from soil layer at 0–0.5 cm depth, between plant rows. The rice proximity samples were taken from the rhizosphere zone of the rice plants. A spatula was used to scrape off the soil attached to the rice roots. The rice roots were exposed at the soil surface (in the paddy water) thus the scraping could be performed easily. The 10 cm samples were taken from

between the rice plant rows, similar to the soil surface samples, but at 9–11 cm depth. At 10 cm depth, the soils were not disturbed by the inter-tillage (Fig 4.3).

4.3.3 16S sequencing library preparation

The DNA of the sampled soils was extracted using a NucleoSpin Soil Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol,and buffer SL2 was used. A negative extraction control was performed using empty extraction tubes with the first and last extraction. The DNA extract was quantified using Qubit 2.0 Fluorometer (Invitrogen, Waltham, United States) and a Qubit dsDNA BR Assay Kit. The V4 region of the 16S rRNA gene was amplified using PCR primer F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′–GGACTACHVGGGTWTCTAAT-3′). An AmpliTaq Gold® 360 Master Mix (Applied Biosystems™, Carlsbad, USA) and 5 ng input DNA were used for the PCR. The PCR program included initial denaturation at 95℃ for 10 min followed by 20 cycles of denaturation at 95℃ for 30 sec, annealing at 57℃ for 30 sec and elongation at 72℃ for 1 min and ended with final elongation at 72℃ for 7 min. A positive control using E. coli DNA control (Applied Biosystems™, Carlsbad, USA) and a negative control were performed with the same conditions. The amplicons were checked on a 1.5% agarose gel. Purification of PCR products was performed using an Agencourt AMPure XP kit (Beckman Coulter Inc., Webster, United States) according to the manufacturer protocol. Ten nanogram of amplified DNA per sample were barcoded using the IonA–barcode[i]-F515 forward and IonP1–R806 reverse primers. The PCR program included the same conditions than above but with 5 cycles. The barcoded amplicons were purified and quantity checked as described above. The quality was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, USCA) using an Agilent DNA 1000 Kit. The library was

diluted to 50 pM and loaded into the Ion 314 Chip (Thermo Fisher Scientific K.K., Japan) using the Ion Chef Instruments (Thermo Fisher Scientific K.K., Japan) with the Ion PGM™ Hi–Q Chef Kit. DNA sequencing was conducted on the Ion PGM Sequencer (Thermo Fisher Scientific K.K., Japan) using the Ion PGM 400 Kit.

4.3.4 Gene abundance assay

Abundance of the 16S rRNA gene was assessed by quantitative polymerase chain reaction (qPCR) using a Stratagene Mx3005P cycler (Agilent Technologies, Inc., Santa Clara, USCA). Prior to the experiment, the qPCR assay was optimized using different oligonucleotide concentrations, soil dilutions and annealing temperatures to reach $R^2 > 0.999$ and amplification efficiencies (Eff) between 0.8 and 1. The amplification efficiencies were calculated using the following formula $Eff = 10^{(-1/slope)}-1$. The qPCR reactions were performed in 20 µl using KAPA SYBR green Master Mix (Takara Bio) and 400 nM of each primer F515 and R806. The cycler program was set with an initial enzyme activation step for 5 min at 95℃ and annealing step for 1 min at 58℃. The DNA extracts were 100–fold diluted. The biological replicates were analyzed in technical duplicates. Negative controls and serial dilutions of amplified E. coli DNA (10^2 to 10^6 ng/µl) were included in every qPCR run to calculate standard curves for absolute quantification. Melting curve analyses was performed to check the quality of the generated amplicons.

4.3.5 Measurement of ammonium and pH

The determination of the inorganic-N concentrations was performed following the approaches taken by Silva (1964) and Hatton and Pickering (1990). Firstly, 2 g of the sampled fresh soil was extracted with 2M KCl (10 ml). After shaking the mixture for 60 min, the suspension was

filtered through a filter paper (Grade 5C, <5 mm; Advantec, Tokyo, Japan). The extracted solution was stored at minus 20℃ until measurement. For the measurement, a colorimetric method was employed with a flow injection analyzer (AQLA-700; Aqualab, Tokyo, Japan) (Hamamoto et al.,2015; Oka and Uchida., 2018). To measure soil pH, 5 g of fresh soil was shaken with 25 ml of 10% KCl for 30 minutes. The pH of the extracts was measured by pH meter (AS800, AS ONE Corporation, Osaka, Japan).

4.3.6 Analysis of the 16S rRNA based bacterial community structures

The barcoded 16S rRNA gene sequences were de–multiplexed, quality–filtered, and assessed using the Quantitative Insights Into Microbial Ecology (QIIME) workflow (Caporaso et al., 2011). The next generation sequencing based method expressed the loading density was at 87% and 94%. On average, 58,889 reads were mapped per 16S rRNA sample. Operational Taxonomic Units (OTUs) were prepared by eliminating all the OTUs that matched the GreenGenes 13_5 reference sequence with 97% similarity. To analyze the changes in microbial community and their interactions with other environmental factors, Principal Component Analysis (PCA) was performed, the PCA plots were separated into three part with same variation. Soil microbial community analysis performed with permutational multivariate analysis of variance (PERMANOVA) and the Bray–Curtis distance based on 999 permutations of the raw data using the Adonis function in R.

4.3.7 Statistical analysis

For the pH, concentration of ammonium, two–way and three–way analysis of variance (ANOVA) was performed to investigate the effect of the location and the duration. One-way ANOVA and Tukey–Kramer multiple comparison tests was performed for the analysis of

significant differences among samples on duration or for each treatment. Statistical analysis were performed by R 3.6.1 and set the significance level at $P < 0.05$.

4.4 Result

4.4.1. Bacterial Abundance

At the soil surface, the bacterial abundance in the NT soils was higher than that in the 5T soils ($p < 0.05$), however, the abundance of bacteria in soils were similar between NT and 5T at the rice proximity and 10 cm depth (Fig 4.4). The bacterial abundance was higher at the rice proximity and soil surface, compared with 10 cm ($p < 0.05$). The bacterial abundance was decreasing over time for the soil surface and rice proximity, but it was relatively stable over time at the 10 cm depth soils.

When averaged across the sampling timings, NT soils had a larger amount of DNA, when compared with 5T soils, in the soil surface, but this was not observed for the rice proximity and the 10 cm depth soils (Fig 4.5). The raw soil DNA concentrations increased over time at the rice proximity and soil surface under NT treatment. For the 5T at the rice proximity and soil surface, the raw DNA concentrations peaked at day 32 and did not show increase towards the latter stage of the experiment. At the 10 cm depth, the raw DNA concentrations under NT and 5T treatment had the similar fluctuation and they were lower than the other locations.

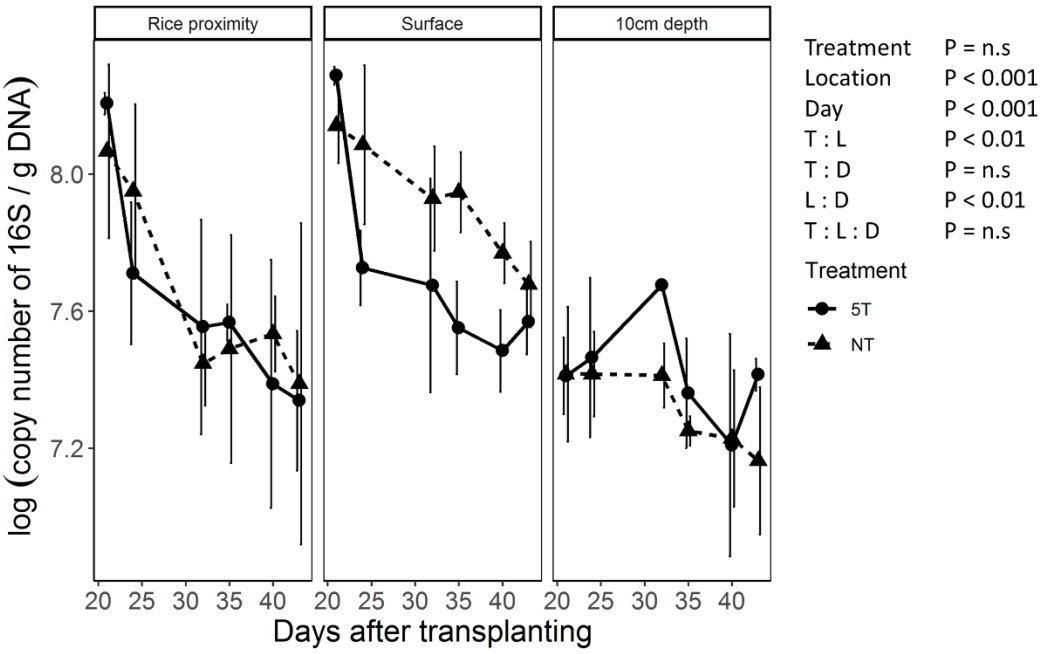

Figure 4.4. The time course changes of 16S bacterial abundance under no–tillage (NT) and 5–times tillage (5T) at three different locations (rice proximity, surface and 10 cm depth) after transplanting. Level of significance was determined by and two–way ANOVA. Error bars indicate standard deviations (n = 3).

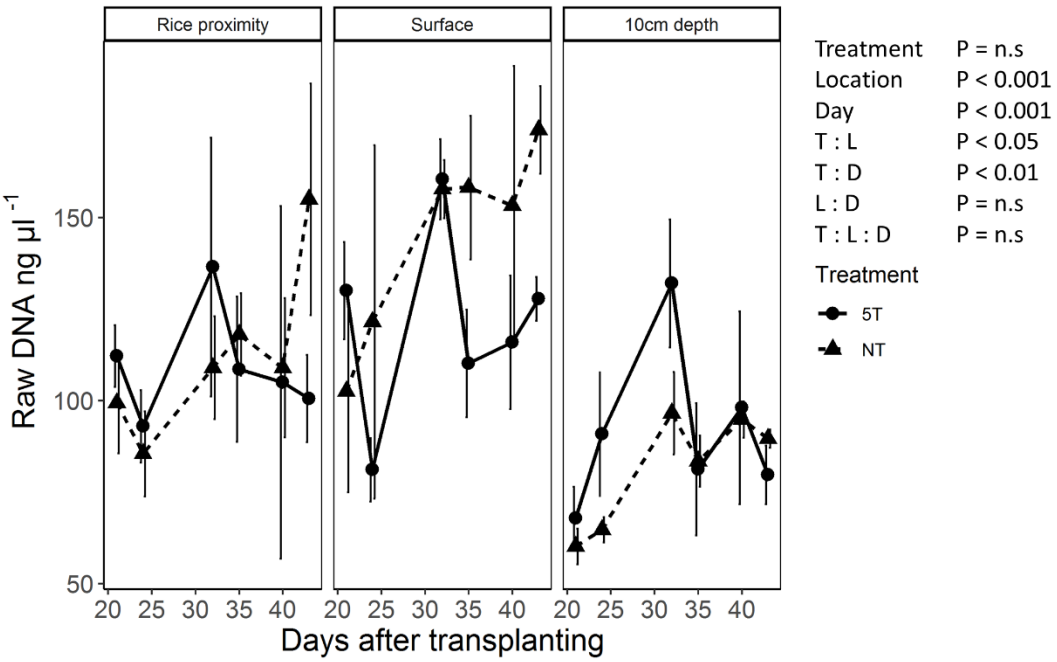

Figure 4.5. The time course changes of raw DNA under no–tillage (NT) and 5–times tillage (5T) at three different locations (rice proximity, surface and 10 cm depth) after transplanting. Level of significance was determined by and two–way ANOVA. Error bars indicate standard deviations (n = 3).

4.4.2. Bacterial Community

On early vegetative phase, the bacterial community structure was similar at the same location regardless of the tillage treatment (Fig 4.6). However, there was a significant difference between 10 cm depth and rice proximity as well as soil surface (Table 4.1). At the rice proximity location, NT treatment had a higher bacterial relative abundance in *Chloroflex*i (9.91%), *Gemmatimonadetes* (6.07%), and *Spirochaetes* (10.66%) compared to the 5T, while *Acidobacteria* (8.33%) and *Betaproteobacteria* (8.63%) were relatively higher in the 5T compared to the NT treatment. At soil surface location, NT treatment had a higher *Spirochaetes* (10.11%) bacterial abundance, but *Chloroflex*i (12.26%) had a higher relative abundance under 5T treatment, compared to the NT. At 10 cm depth location, *Firmicutes* (17.71%) was higher under NT treatment, but *Planctomycetes* (3.37%) and *Spirochaetes* (9.55%) were higher under

5T treatment, compared to the NT. The N_2-fixing genus only appeared at the rice proximity and soil surface, while the relative abundances of *Firmicutes* and *Archaea* predominated 10 cm depth location. Nonetheless, there was no specific bacteria predominated rice proximity and soil surface locations.

On late vegetative phase, the bacterial community structure (Fig 4.6) was alike among three locations. There was no bacteria particularly predominated rice proximity, soil surface and 10 cm depth locations. Nevertheless, we observed several bacterial clusters partly fluctuated from the early vegetative phase to the late vegetative phase. The relative abundance of *Deltaproteobacteria Geobactor* increased obviously at the rice proximity and soil surface. Contrastingly, *Betaproteobacteria*, *Firmicutes and Pseudomonas* had decreased at the two locations. The 10 cm depth was a relatively steady location compared to the rice proximity and soil surface.

The result of PERMANOVA showed soil bacterial community structure was significantly influenced by vegetative phase ($p < 0.001$) rather than treatment (Supplementary Table S1). The Shannon diversity index was also significantly influenced by the vegetative phase ($p < 0.001$) (Table 4.2). Its result showed that the bacterial diversity in the rice proximity and the soil surface increased over time, but they were maintained at the 10 cm. (Table 4.3).

Base on the PCA, there was no clear clusters of NT and 5T (Fig 4.7a), suggesting there was no major impacts of the inter-tillage on the bacterial community structures. For the locations of rice proximity and soil surface, they clustered differently by the two vegetative phases (Fig 4.7b). In contrast to the rice proximity and soil surface locations, the 10 cm depth data made a separate cluster on the PCA, and the NT and 5T showed different clusters for the 10 cm depth.

The PCA on genus level (Fig 4.7c) indicated that genus *Clostridium* predominated the rice proximity and surface under both treatments on early vegetative phase. Stramenopiles (or Heterokonts), a micro eukaryotic community which most were algae [26], predominated rice proximity and surface under both treatments on late vegetative phase. With regard to 10 cm depth location, the uncultured order *pGrfC26* of the Miscellaneous Crenarchaeota group (MCG) was predominant.

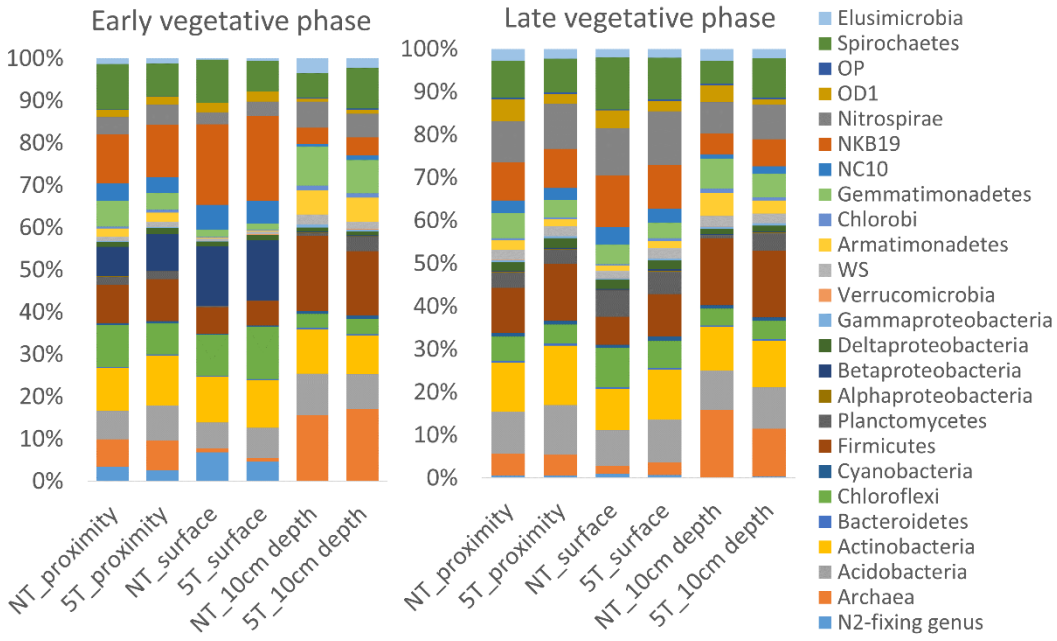

Figure 4.6 Relative abundances for the bacterial community under no–tillage (NT) and 5–times tillage (5T) treatment in three different locations (rice proximity, surface and 10 cm depth) at early vegetative phase and late vegetative phase.; *Proteobacteria* was shown in class level; "N_2 fixing genus" was included *Pseudomonas, Azoarcus, Rhizobium, Anabaena, Azospirillum, Bradyrhizobium*. Other bacteria were shown in phyla level.

Table 4.1. PERMANOVA (Permutational multivariate analysis of variance) tested effect results of vegetative phase, location and treatment on bacterial community composition.

Pairwise comparison	DF	SS	F	R^2	P value
Vegetative phase					
June vs July	1	1.421286	8.02184	0.190897	0.001
Location					
Proximity vs Surface	1	0.156729	0.78602	0.034496	1
Proximity vs 10 cm	1	1.068200	6.84868	0.237400	0.003
Surface vs 10 cm	1	1.305654	7.76662	0.260917	0.003
Treatment					
NT vs 5T	1	0.242106	1.14277	0.032517	0.274

Table 4.2. Results of ANOVA for Shannon index at the OTU level.

	DF	SS	MS	F value	P value
Vegetative phase	1	13.16	13.156	23.47	***
Location	2	3.121	1.5606	1.75	ns
Treatment	1	0.134	0.1338	0.14	ns

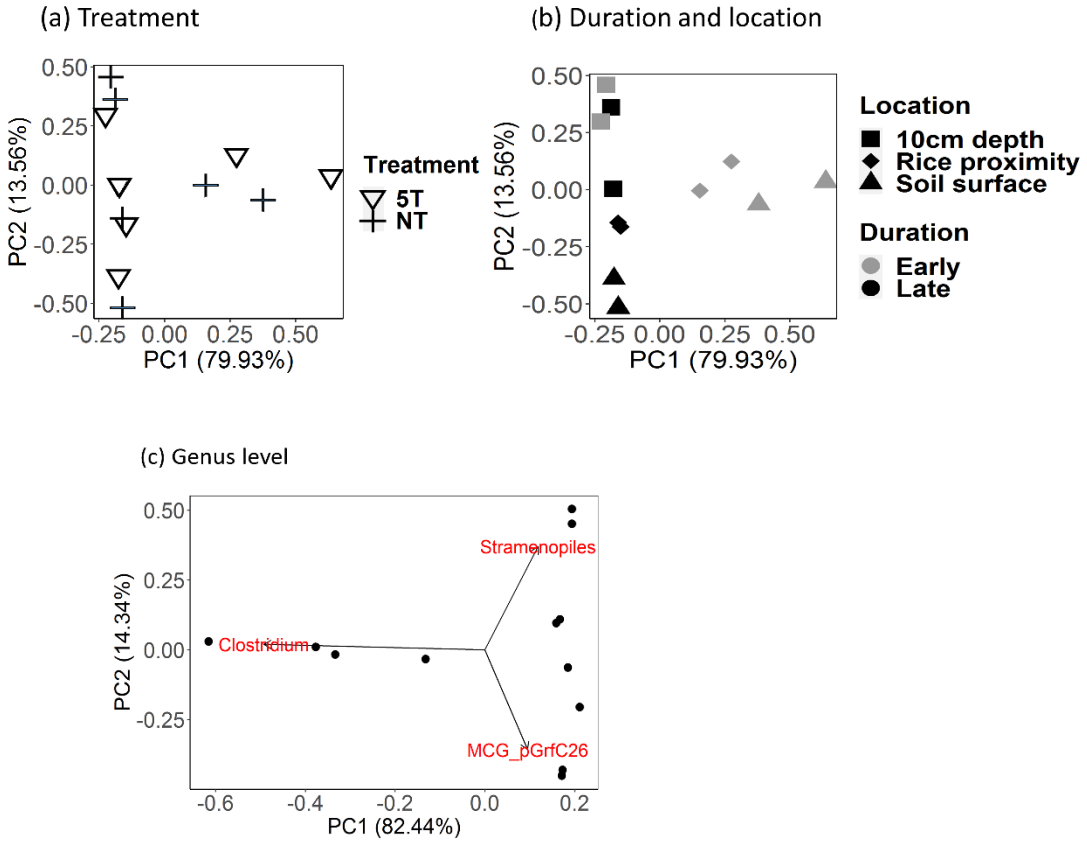

Fig 4.7. Principal component analysis (PCA) on (a) treatment with no–tillage (NT, cross) and 5–times tillage (5T, triangle) and (b) interaction between duration (early vegetative phase (gray) and late vegetative phase (black)) and location (rice proximity, soil surface and 10 cm depth). (c) Arrows show bacteria on genus level. Axis 1 and 2 account for 72 and 16% of the variation, respectively.

4.4.3. Dynamics of pH and ammonium

When averaged across the rice vegetative phase, soil pH was the highest on the soil surface (6.8 ± 0.01), followed by the rice proximity (6.5 ± 0.02) and the 10 cm depth (6.2 ± 0.02). Soil pH decreased from 21 days to 43 days under NT and 5T treatment at both rice proximity and soil surface location. The pH of rice proximity and soil surface under NT aIt nd 5T treatment decreased about 5%. Soil pH at 10 cm depth location under NT and 5T treatment was relatively stable, ranging from 6.25 to 6.08. There was a significant difference between location and

duration (P < 0.001) (Fig 5). However, soil pH whether be disturbed (5T) or not (NT) did not show obvious significantly discrepancy.

The ammonium declined from 21st day at the early growth after transplanting at the rice proximity and soil surface (Fig 4.9). There was a significant difference between day (p < 0.001), but there was no significant impact on the tillage treatments. The amount of ammonium concentration declined at rice proximity (23 to 9 mg kg^{-1}, NT; 19 to 7 mg kg^{-1}, 5T) and soil surface (25 to 11 mg kg^{-1}, NT; 21 to 9 mg kg^{-1}, 5T) from 24 days to 32 days and afterwards slightly increased by 43 days after transplanting. There was a significant difference at the 10 cm depth location between NT and 5T treatment. The ammonium concentration at the 10 cm depth was relatively stable compared with the rice proximity and soil surface.

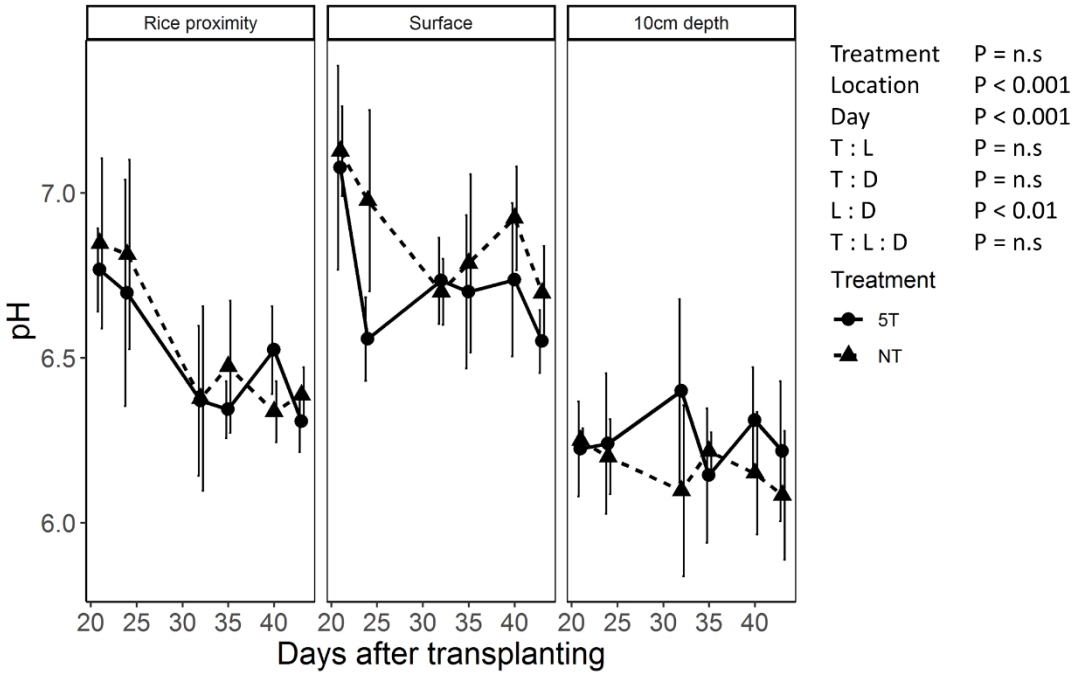

Figure 4.8. Change of soil pH after transplanting at location rice proximity, surface and 10 cm depth under no–tillage (NT) and 5–times tillage (5T) treatment. Level of

significance was determined by three–way ANOVA. Error bars represent standard deviations (n = 3).

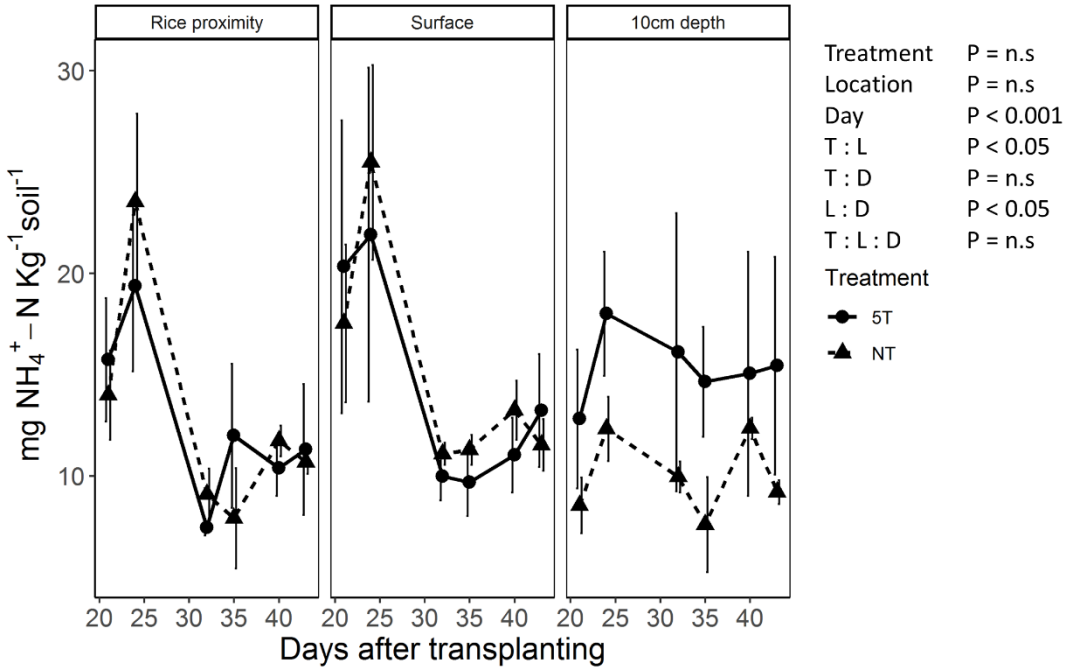

Figure 4.9. Fluctuation of ammonium (NH4 +–N) after transplanting at location rice proximity, surface and 10 cm depth under no–tillage (NT) and 5–times tillage (5T) treatment. Level of significance was determined by three–way ANOVA. Error bars represent standard deviations (n = 3).

4.5 Discussion

Our study found that there was a negative impact of the tillage on the bacterial abundances ($p < 0.05$) at the soil surface (Fig 4.4). However, the tillage treatment did not impact the bacterial abundance in soils at the rice proximity and 10 cm depth. We note that the rice grain yields for the 0- and 5-times inter-tillage treatments were 2.3 ± 0.7 and 2.7 ± 0.9 t ha^{-1}, respectively, without the significant difference between them. The tillage disturbs soil surfaces, and the disturbance must have negatively influenced the soil microbial abundance on the soil surfaces in our study. Previous studies also had shown that a tillage treatment results in lower microbial biomass than NT treatment at the soil surface (Madejón et al., 2009; Liu et al., 2016). Previous studies stated that the negative impact of the tillage on soil microbial biomass was due to the disruption of microbial extracellular enzyme activities. For example, one of the enzymes in soils, Beta-Glucosidase activity is positively related to total soil carbon that is closely associated with soil microbial biomass. In contrary to tillage, NT treatment retain the soil carbon in soils, when compared to the systems with tillage and this allows microorganisms to grow. Schmidt et al. also indicated that at a 0–5 cm soil depth, the NT treatment led to higher copy number of bacteria that was approximately 7.5×10^8 copy kg^{-1} soil (this study was from 4.9×10^7 to 1.4×10^8 copy kg^{-1} soil at the soil surface) when compared to that under the tillage treatment. However, the same study indicated that this was not the case at deeper soil layers. Thus, we conclude that the disturbance of soil microbes occurred in rice paddy soils by inter-tillage occurs, even when the soils were submerged in water, similar to previous reports performed in upland soils.

Also, we found that the 16S rRNA copy number at the location of rice proximity and soil surface under NT and 5T treatment decreased along with rice growth (Fig 4.4), but at the 10 cm depth it was more stable compared to other locations. This was consistent with the previous

studies that 16S rRNA copy number decreased with increasing plant growth. We also observed the highest amount of 16S rRNA copy number appeared on the early vegetative phase. Thus, we assumed that one of the reasons on the decrease of bacterial abundances might be caused by rice plants rapidly consuming nutrients resulting in nutrient deficiency for microbial growth. Therefore, the effect of inter-tillage on the microbial growth may depend on the nutrient availability in the soils, thus the interaction between the soil's nutrient availability and inter-tillage treatments should be further studied, regarding the soil microbial abundance. In the current study, we aimed to compare tillage and no-tillage systems within a natural farming rice paddy. However, future studies should compare the natural farming and conventional systems, regarding the relationships between plant growth and microbial abundance, because we were uncertain whether the use of chemical fertilizers and other practices in the conventional farming systems change the relationship between plant growth and microbial abundance.

As another factor controlling the microbial abundance in the current study, the bacterial abundance peaked at around pH of 7 and decreased with soil pH at the rice proximity and soil surface both under NT and 5T treatments (Fig 4.4 and Fig 4.8). This agreed to a previous study reporting that the bacterial growth was correlated with soil pH and tended to be relatively higher at around neutral soil pH environment. However, unexpectedly, our results showed an increase of the raw DNA concentrations with decreasing soil pH and over the experimental period, particularly for the NT (Fig 4.5). Other biological activities such as fungal, faunal or plant activities might have contributed to the increase of raw soil DNA amount, for example, previous studies reported that acidic environments promoted fungal activities, while bacterial activities decreased along with reducing soil pH. Also, NT systems can improve the soil porosity by increasing 0.5-50 mm macropores, as well as alter water holding capacity (0–10 cm) and the amount of exchangeable ions, compared with tillage systems. Thus, further investigation is needed to identify the factors contributing to the increase of the raw DNA concentrations.

For the community structures of bacteria in the soils, there was no clear impact of inter-tillage treatments, when compared to NT (Fig 4.7(a)). Rather, the sampling timing influenced the community structures, particularly at soil surface and rice proximity (Fig. 4.7(b)). For example, *Clostridium* of *Firmicutes* in rice proximity and surface was present in relatively larger amount during the early vegetative phase compared to the late vegetative phase (Fig 4.7(c)). *Clostridium* is a microorganism responsible of the rice straw decomposition in a rice paddy. Due to low temperatures in Hokkaido limiting the microbial decomposition ability, we often find a large amount of rice straw left beneath the soil surface in spring. Thus, ours results suggested that the activated *Clostridium* might decompose some of the rice straw in spring. The contribution of *Clostridium* to the decomposition of rice straw has to be further studied, especially in relation to their low temperature activities.

Contrastingly, *Stramenopiles* dominated the rice proximity and soil surface soils during the late vegetative phase (Fig 4.7). *Stramenopiles* are an assemblage of eukaryotic organism, including unicellular such as diatoms to large multicellular forms, such as the brown algae, and oomycetes. These *Microeukaryotes* act as grazers which can affect microbial biomass and community as well as releasing ammonium and nitrate. Thus, these microbes might play an important role in natural farming rice paddy systems, providing available nitrogen to rice plants in the latter stages of their growth, although further studies are needed to confirm this.

In the 10 cm depth soils, Class *MCG* of *Crenarchaeota* showed relatively larger amount, when compared to the soils sampled from other parts (Fig 4.7(c)). Miscellaneous *Crenarchaeota* Group (*MCG*) of *Archaea* are widely distributed in terrestrial and marine ecosystem. These microorganisms play an important role in biogeochemical cycles. *MCG* in anaerobic soil ecosystem are related to degrade carbon from plant, reduce nitrite to ammonium, and produce acetate. Undecomposed rice straw which was found while soil sampling at the 10 cm depth

under both treatments, possibly lead *MCG* to be in the majority as well as lower pH and higher ammonium concentration compared to the rice proximity and soil surface.

Overall diversities of the bacterial communities also did not show the difference between the tillage treatments (Table 2). However, the rice proximity and soil surface under NT and 5T treatment showed a significant increase of microbial diversity on late vegetative phase, compared to the 10 cm soils (Table 3). There were previous studies found that NT treatments increased microbial community's diversity when compared to the soils under tillage, but other previous studies concluded that the NT treatment did not influence the microbial community. The microbial diversities' fluctuations over the growing period might have been the reasons behind these contrasting results because microbes are influenced by fluctuating soil chemical properties such as pH and nutrients.

We expected different soil pH and nutrient characteristics at soil surfaces and near rice plants between the NT and 5T treatments because previous studies stated that in natural farming rice paddy systems, inter-tillage can incorporate oxygen to aerate soil therefore promoting microorganism activities to decompose organic matter and crop residues, facilitated nitrogen mineralization and ammonification. For the ammonium concentrations, we observed relatively higher ammonium concentration in 5T than NT only in a deeper soil layer ($p < 0.01$). Possibly, the rapid consumption of ammonium ion by rice plants masked the differences between the NT and 5T, regarding the soils' chemical characteristics. In results, we observed the decrease in soil pH and ammonium in both of the tillage treatments and we conclude that the increase in the diversity indices with time might have caused by increased relative abundances of acid preference bacteria, such as *Acidobacteria* and *Firmicutes*. We note that our natural farming rice paddy experiment was only conducted for one year, thus the significant impact of the five

times inter-tillage treatments (compared to NT) on soils' microbial diversity might appear more in a longer-term, although it needs to be confirmed.

4.6. Conclusion

From this study we draw the following conclusions:

- Bacterial abundance was higher at soil surface with no tillage, compared to the soils under inter-tillage. Soil surface was the most disturbed location due to the tillage, thus we observed the significant decrease in bacterial abundance in this zone, when the tillage was conducted.
- Bacterial abundance was reduced over time, regardless of the tillage treatments, at rice proximity and soil surface. Decreasing nutrients and pH because of the plants' nutrient absorbance limited the bacterial growth therefore led to bacterial abundance reduced.
- The bacterial diversity and community structure were affected by soil pH which was decreasing with ammonium levels in soil. However, the impacts of the tillage treatments were not clear.
- In this study, the grain yields were not influenced by the inter-tillage treatments.

Chapter 5

Weeds incorporation affected on microbial abundance but not on straw decomposition

5.1 Abstract

Incorporation of organic matter such as crop residue is widely performed in agricultural fields. Returning rice straw is the most conducted way to increase soil organic matter especially natural farming rice paddy, but the slow decomposition rate tends to affect rice growth. Therefore, this study aimed to evaluate whether adding extra nitrogen source (clover and Rumex) is possible to stimulate the rice straw decomposition rate. As a result, the amount of respired CO2-C was negatively correlated to the C/N ratio of the added residues. This suggests that the systems were N limited. However, the rice straw decomposition rate was not influenced by the treatments in the current study, but we found Rumex treatment had relatively higher F : B ratio among treatments; Clover treatment had largest ammonium and nitrate concentration. Our study suggested input plant residues after harvesting potentially improve higher F : B ratio as well as increasing nitrogen nutrient. In order to acquire faster rice straw decomposition rate to release a great extent of carbon, nitrogen and mineral nutrient into soil, a long term incubation period and higher plant residue to rice straw ratio, more plant residue varieties, as well as microbial composition are considerable to further confirm what factors most benefit rice straw decomposition rate.

5.2 Introduction

Incorporation of organic matter such as crop residue is widely performed in agricultural fields, especially on natural farming or so call conservation agriculture, they are alternative agricultural systems to grow food, which is highly rely on crop residue incorporation rather than utilization of various types of fertilizer (Fukuoka, 1975; Hirano et al., 2001; Xu, 2006; Liao et al., 2019). Returning rice straw is the most conducted way to increase soil organic matter in natural farming rice paddy. Rice straw decomposition involves the mineralization-immobilization which acts the role in soil nitrogen and carbon nutrient cycle and transformation (Lal, 2004). To apply crop residues not just save fertilizer expense for farmers but also reduce global greenhouse gas emission and promote agricultural sustainable.

Rice straw decomposition process is induced by many forms of microbial activities (Güsewell and Gessner, 2009; Schneider et al., 2012). To measure microbial abundance is an essential pathway to quantify microbial activities. Real time quantitative PCR is a molecular approach to evaluate microbial copy number (Smith and Osburn., 2009). Soil microbial biomass carbon (MBC) is a soil management indicator and performs a basic role in soil organic C dynamics (Fierer et al., 2009; Liang et al., 2011). Soil respiration defined as soil microbes release CO2 in per unit soil surface during a specific time, which is also an index of estimating soil microbial abundance (Bentham et al., 1992; Phillips and Nickerson, 2015).

Rice straw decomposition involves two process because it consists of less recalcitrant polysaccharides (i.e cellulose, oligosaccharides, organic acids, hemicellulose, cellulose) which are easily available carbon source, bacteria are mainly predominant in this decomposition stage; then followed by the recalcitrant compounds such as lignin or suberin, in this decomposition stage fungi has higher abundance because they are major lignin decomposers (Watanabe et al., 1993; Moore et al., 2005; Paterson et al., 2008). Due to returning rice straw to a natural farming

rice paddy is an indispensable step to input nutrients for rice growth, thus to accelerate rice straw decomposition in order to shorten the rice paddy soil restoration period, not only reduce the effect of seedling growth issue by undecomposed rice straw in the coming rice crop (Khaliq et al., 2011), but also enhance the soil microbial activities.

Carbon to nitrogen (C/N) ratio is regarded as a critical index to evaluate in what extent the incorporated organic matter influence soil microbes whether conduct mineralization or immobilization, in other words, the nutrients such as inorganic nitrogen and carbon release to soil or stock in microbes. In general, the C/N ratio of microorganisms is about 10, to synthesize 1 gram of microorganisms, 0.1 gram of nitrogen is needed. Therefore, the C/N ratio of organic materials needs to be maintained below 30, and the nitrogen contained in it can be fully supplied for the growth of microorganisms after being decomposed by microorganisms. For example, if the organic material C/N ratio is higher than 30, the nitrogen becomes insufficient for microbial proliferation, thus results in immobilization. (Green and Blackmer, 1995; Moritsuka et al., 2004). Pervious study suggested that the incorporated organic material C/N ratio between 10 to 20 is able to induce soil microbial activities as well as maintaining available nitrogen in the soil for crop uptake (Wang et al., 2013). Fungal to bacteria (F : B) ratio has been widely used to assess agricultural soil management, which is related to organic material C/N ratio. Higher C/N ratio (over 30) usually results in higher F : B ratio, thus lead to more C sequestration in soil (Strickland and Rousk, 2010).

Generally, weeds near rice paddy are either ignored, or killed by herbicide. However, few studies have investigated whether add weed residue around rice paddy can stimulate soil microbial proliferation and further increase available nutrients for crop. In this study, we sampled two most predominated weeds which are Trifolium

Pretense (red clover) and Rumex obtusifolius (dock) around natural farming rice paddy after harvested in early October in Hokkaido, Japan. Clover can fix nitrogen from atmosphere and release crop available nitrogen into soil when they are being decomposed; low C/N ratio (13-16) makes itself to rapid decomposition for microbes (Bruulsema and Christie, 1987; Kadziuliene, 2004; Moyo et al., 2015; Xie et al., 2018). Dock has similar C/N ratio with clover, which contains potassium, zinc and magnesium, these micronutrients profit plant growth, increase plant photosynthesis and disease resistance, as well as help enzyme activity (Courtney, 1972; Fao, 2015). Our objects was to investigate whether the surrounding weeds can improve soil microbial activities. We hypothesized that incorporated weeds would increase bacteria and fungi abundance and lead to a higher rice straw decomposition rate.

5.3 Materials and methods

5.3.1 Soil and plant residue sampling

The soil was sampled from the natural farming rice paddy in Hokkaido University, Field Science Center for Northern Biosphere, Experimental Farm, in Sapporo, Hokkaido, Japan (N43°04′39"151, E141°20′03"634) (Supplementary Fig S1). The soil classified into grey lowland soil (Gleysol; FAO/UNESCO) was collected from the surface to depth of 10 cm before transplanting in 2019. The pH of the soil was 6.01 ± 0.07 (s.d., n = 3), and ammonium and nitrate were 4.22 ± 0.3 mg kg^{-1} and 3.14 ± 0.63 mg kg^{-1}, respectively. To measure soil pH, 5 g of dry soil was mixed with 25 ml Milli-Q water and shaken for 30 min, followed by pH measurement using a pH meter (AS800, AS ONE Corporation, Osaka, Japan). Ammonium and nitrate content of the soil samples were determined using a flow injection analyzer (AQLA-700; Aqualab, Tokyo, Japan).

The dominated species were *Trifolium Pretense* (red clover) and *Rumex obtusifolius* (dock) after harvesting in early October, 2019. Clover and *Rumex* were used as plant residues for this experiment. Each plant including leaf, stem and root was sampled around the natural farming rice paddy in Hokkaido University where we sampled the soil in early October, 2019. The collected plants were dried at 60℃ for two days and cut into less than 1 cm in length; the rice straws were collected after harvesting immediately and cut into 1 cm length and being dried at 60℃ for two days. Both plant residues and rice straws were immediately stored at 4 ℃ for inorganic nitrogen and C/N ratio analysis and stored at −40 °C for molecular analysis. The C and N content of each plant residue was measured by CN analyzer (EA 2400 Series; Perkin Elmer, Foster City, CA, USA). The property of them is shown in Table1.

Table 5.1. The property of white clover and maize. Each value is based on air-dried weight and shown in mean (SD) (n=3).

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	C/N ratio
Red clover	39.17 (4.45)	6.04 (0.63)	2.89 (0.98)	14.69 (6.55)
Rumex	38.72 (1.87)	6.37 (0.13)	2.23 (0.13)	17.46 (1.90)
Rice straw	35.59 (0.71)	4.70 (0.11)	0.83 (0.06)	42.83 (2.60)

5.3.2 Incubation setup

To make soil cores, 745 g of wet soil (equivalent to 514 g dried soil. Hereinafter, unit "kg soil" or "g soil" means oven dried soil based) was placed to glass bottles (9.5 cm in diameter and 18 cm in depth). Milli Q water was applied to the soils to make their water-filled pore space (WFPS) = 60%, this value represents an optimal condition where microorganisms are fully functioning (Groffman & Tiedje, 1991). The density of the soils were adjusted to 1 g $cm3^{-1}$. The glass bottles were covered with metal lids which have 2 holes made by pin to avoid contamination and excess evaporation. Three replicates for each treatment were prepared. In total, 12 (= 4 treatments (control/ *Rumex* / clover /mix * 3 replicates respectively) cores were arranged. The cores were then pre-incubated for one week at room temperature (22 ± 3℃).

The straw decomposition experiment was based on the litter bag method. After the pre-incubation period end, 48 litter bags were prepared for incubation experiment. The amount of rice straw was adjusted to 3000 mg-C kg dry $soil^{-1}$ which is 1.088 g. 1.088 g of dried rice straw was placed into one litter bag (4.7 cm in length and 10 cm in width).

After the pre- incubation, plant residue clover, *Rumex* and mix were applied to the soils except control treatment. The amount of applied plant residue was adjusted to 3000 mg-C kg dry soil^{-1}, and make the rice straw to plant residue ratio is 1 to 1.5. The weight of red clover and *Rumex* were 26.8 mg and 11.7 mg respectively. The "mixture" treatment was the mixture of red clover and *Rumex* (30% of red clover and 70% of *Rumex*), adjusted to be the same C amount, which was 16.25 mg. During the application, the soils and the residues were mixed well with stainless spoon. Four litter bags were separately buried in a vertical position into the each glass bottle. Four bags were collected from each replicate at 7, 14, 21, 28 days.

5.3.3 The analysis of rice straw C/N ratio and microbial abundance

Carbon and nitorgen contents of rice straw residues in each collected litter bag were determined by combustion on elemental analyzer (EA 2400 Series; Perkin Elmer, Foster City, CA, USA).

Microbial abundance of the 16S and ITS rRNA gene were assessed by quantitative polymerase chain reaction (qPCR) using a CFX96 (Bio-Rad) real-time PCR System. On each sampling day, straw DNA was extracted from 0.2 g fresh straw with NucleoSpin Soil kit (TAKARA BIO INC., Shiga, Japan) according to the manufacturer's instructions. The extracted DNA was purified with Agencourt AMPure XP (Beckman Coulter) according to a predetermined protocol. The concentration of the purified DNA was measured with Qubit dsDNA HS Assay Kit

(Invitrogen, USA). The purified DNA was then diluted 50 times with nuclease-free water for qPCR. For measuring 16S gene copy number, F515 and R806 primers were chosen. A standard curve was prepared with a serial dilutions of amplified E. coli (10^{-3} to 10^{-9} ng/µl). The initial denaturation temperature was 95 ℃ with an annealing temperature of 95℃, and the extension was conducted for 1 min at 58 ℃ for 30 cycles. The final extension was done at 72℃ for 1

min. The threshold line was calculated by R, and the result was shown as log copy number of 16S rRNA gene g soil^{-1}. For measuring ITS gene copy number, ITS1 and ITS2 primers were chosen. A standard curve was prepared with a serial dilutions of the highest concentration of raw DNA sample (10^{-3} to 10^{-9} ng/µl). The initial denaturation temperature was 95 ℃ with an annealing temperature of 95℃, and the extension was conducted for 1 min at 55 ℃ for 35 cycles. The final extension was done at 72℃ for 1 min. The threshold line was calculated by R, and the result was shown as log copy number of ITS rRNA gene g soil^{-1}.

5.3.4 The analysis of soil pH, soil respiration, inorganic nitrogen and microbial biomass carbon

Soil pH of four treatments were measured before incubation. 5 g of fresh soil was shaken with 25 ml of 10% KCl for 30 minutes. The pH of the extracts was measured by pH meter (AS800, AS ONE Corporation, Osaka, Japan).

Soil respiration (CO_2 emission) was measured at 7th day, 14th day, 21th day and 28th day from the first day of incubation, there were four measuring times in total. Each soil pot was sealed with a screw lid. At 1, 6, 11 minutes after closing the lid, 250 ml of the gas sample was collected from the septa. The collected gas sample was injected to Carbon Dioxide Isotope Analyzer (CCIA-38-EP, Ros Gatos Research, CA, USA) to measure CO_2 concentration. The data which showed more than 0.7 of correlation index was used for the calculation of soil respiration. The soil respiration rate was calculated based on the increase of CO_2 concentration during the 11 minutes and the headspace within the bottle. The result was shown as mg CO_2-C m^{-2} s^{-1}.

For the determination of inorganic N (NH_4^+ and NO_3^-) concentration, from the three replicates of each treatment, 2 g of the 28th day incubation soil was extracted with 2M KCl (10 ml). After

shaking the mixture for 60 minutes, the suspension was filtered through a filter paper (Grade 5C, <5 mm; Advantec, Tokyo, Japan). The extracted solution was stored at -30℃ until measurement. For the measurement, a colorimetric method was employed with a flow injection analyzer (AQLA-700; Aqualab, Tokyo, Japan) (Hamamoto et al.,2015; Oka and Uchida., 2018). To measure soil pH, 5 g of fresh soil was shaken with 25 ml of 10% KCl for 30 minutes. Briefly, NH_4^+ was detected by the indophenol blue method and NO_3^- was detected by the cadmium reduction and hydrochloric acidic naphthylethylenediamine method. The result of NH_4^+ and NO_3^- were shown as mg NH_4^+− N mg L^{-1} or NO_3^- − N mg L^{-1}.

Microbial biomass carbon (MBC) was determined by chloroform fumigation-extraction method. For each treatment, three replications of 5 g fresh soils were taken from soil cores after 28 days incubation. 48 soil samples were placed in a 6.4 cm in diameter and 1.7 cm in height glass petri dishes and fumigated with chloroform for 24 hours. The fumigated and non-fumigated soils were shaken with 0.5M K_2SO_4 for 30 minutes followed by filtration through a filter (Grade 5C, <5 mm; Advantec, Tokyo, Japan). The extract was stored at -30℃ until the measurement. The carbon concentration in the extract was measured using combustion catalytic oxidation method by TOC (TOC-5000A, Shimadzu, Kyoto, Japan). The amount of MBC was calculated as MBC = Ec / Kec, where Ec is (carbon contents in fumigated soil) minus (carbon contents in non-fumigated soil) and Kec is 0.45 (Vance et al., 1987). The result of MBC is shown as mg kg dry $soil^{-1}$.

5.3.5 Statistical analysis

For the result of pH, inorganic N, MBC, soil respiration, two-way analysis of variance (ANOVA) was performed to investigate the effect of the treatment and the sampling date. For the result of microbial abundance, two-way ANOVA and Tukey's test was performed to see

the effect of treatment. Statistical analysis was performed using R ver. 4.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

5.4 Results

5.4.1 Rice straw decomposition rate

Rice straw decomposition was significantly influenced by time ($P < 0.001$, Fig 5) but not by the plant type treatments. When averaged across the treatments, approximately 30% of the total weight of rice straw was decomposed within 28 days (Fig 5.1).

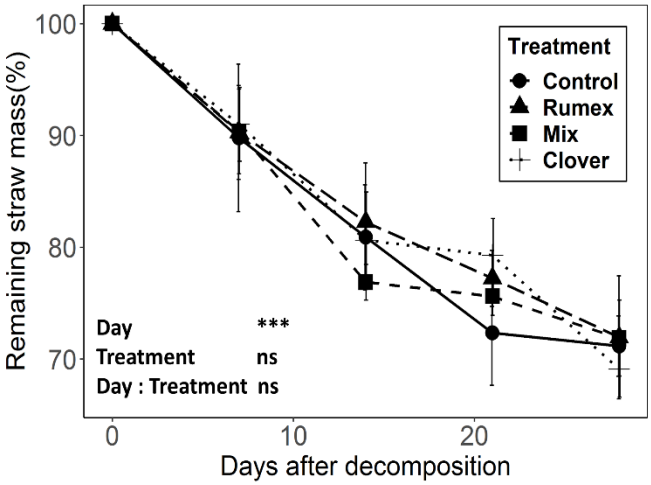

Fig 5.1. The rice straw decomposition rate at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as *** p < 0.001; ns = not significant. Error bars represent standard deviations (n = 3).

5.4.2 Rice straw C/N ratio

There was a significant effect of different treatments on rice straw CN ration after decomposition ($P < 0.001$, Fig 4). Under the Clover treatment, the rice straw showed the lowest CN ratio (26 ± 3) across the incubation period. Rice straw CN ration was relatively steady in control treatment and remained relatively higher than other treatments towards the end of the experiment. We observed that the release of nitrogen from the rice straw under *Rumex* and mix treatment resulted in an increase on C/N ratio during 0 to 7 days (Fig 5.2).

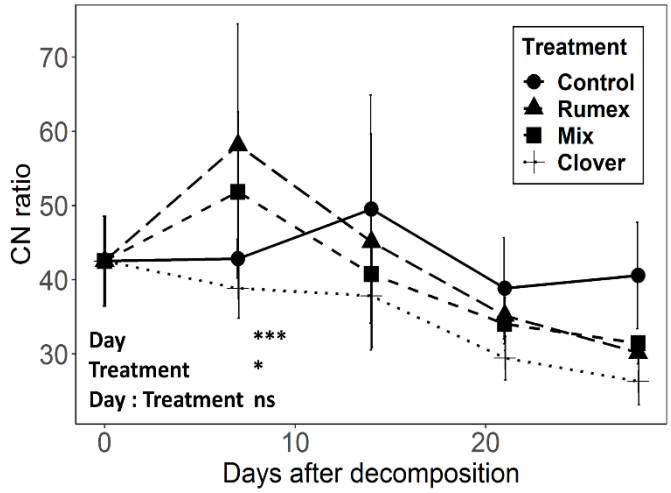

Fig 5.2. Change of rice straw CN ratio at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as * $p < 0.05$; *** $p < 0.001$; ns = not significant. Error bars represent standard deviations (n = 3).

5.4.3 Soil respiration CO_2 – C, accumulated CO_2 respiration and accumulated Carbon released from rice straw

Both time and treatment had significant effect on soil respiration. The soil respiration from Clover, *Rumex* and Mix treatments was relatively higher than control in 7 days after the start of the experiment. Control maintained the lowest soil respiration rates throughout the incubation period. The soil respiration rates from the Clover treatment were the highest throughout the incubation period (Fig 5.3).

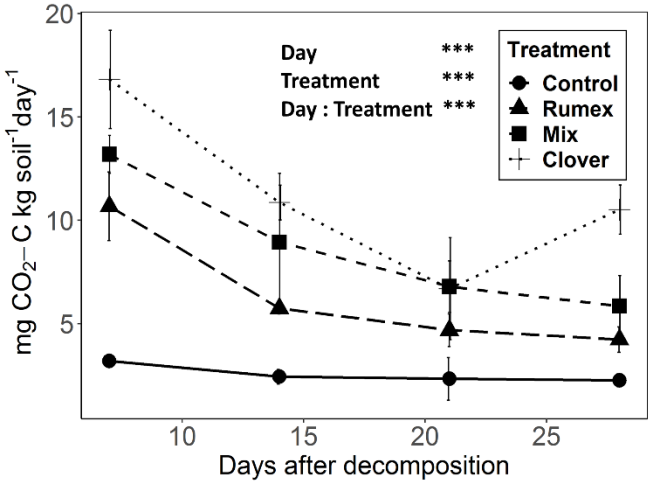

Fig 5.3. The soil respiration at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as *** p < 0.001. Error bars represent standard deviations (n = 3).

Compared with CO_2 soil respiration, the accumulated CO_2 respiration was significant among treatments. Control treatment appeared the lowest accumulated CO_2 (52 ± 7) and the highest was clover (219 ± 21). As for accumulated C released from rice straw there was no significance

among treatments, the concentration of accumulated C was between 304 to 332 mg, control and mix treatments both had the relatively larger concentration (Fig 5.4).

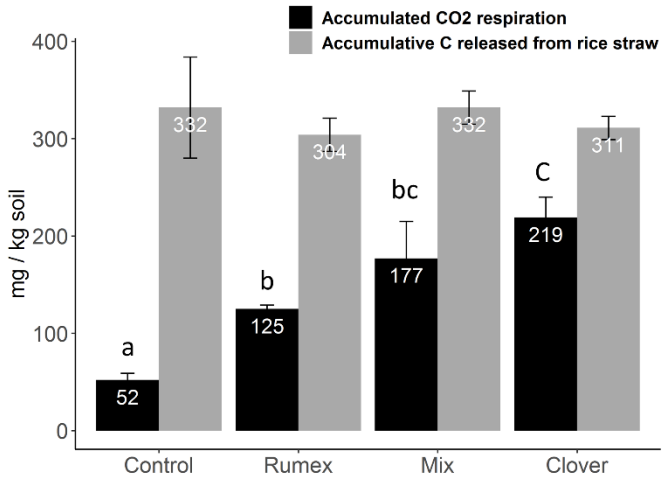

Fig 5.4. The accumulated CO_2 and cumulated C released from rice straw over 28 days incubation under Control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Different lowercase letters indicate significant differences between treatments ($p < 0.05$). Error bars represent standard deviations ($n = 3$).

5.4.4 Microbial abundance (16S + ITS)

Control treatment microbial copy number had a decreasing trend on bacteria, and increasing trend on fungi and F:B ratio, which might when without extra amendments fungi and bacteria competed with single nutrient from rice straw (Fig 5.5c). Other than control, there were no regular appearance on clover, *Rumex* and mix microbial copy numbers (Fig 5.5a). Among treatments, control and *Rumex* had higher F : B ratio, clover had lower F : B ratio, which indicated F : B ratio dynamic changed with plant residues ($P < 0.01$). Bacterial copy number and F : B ratio fluctuation were influenced by incubation period ($P < 0.01$), bacterial copy number was gradually decreasing along with rice decomposition, thus leaded to an increase on

F : B ratio. Raw DNA of control treatment (Fig 5.5d) corresponded to the bacteria copy number of that, which appeared the similar decreasing movement. In addition to that, there were no particularly consistent dynamics among raw DNA, bacteria and fungi copy number (Fig 5.5b) observed.

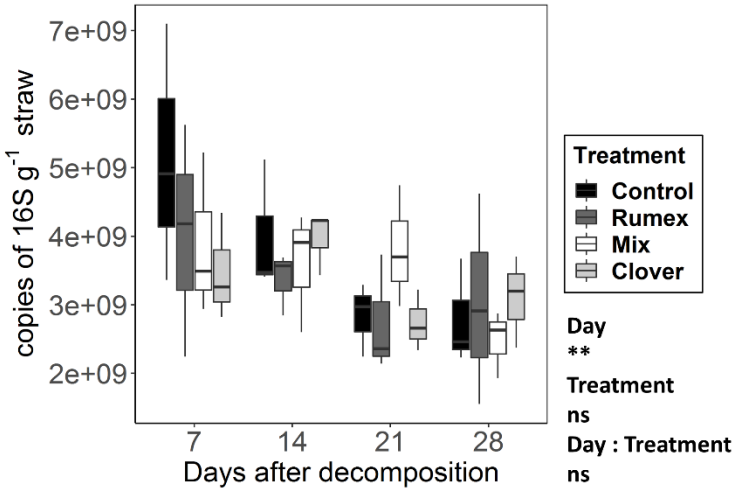

Fig 5.5a. The 16S bacterial abundance at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as ** p < 0.01; ns = not significant. Error bars represent standard deviations (n = 3).

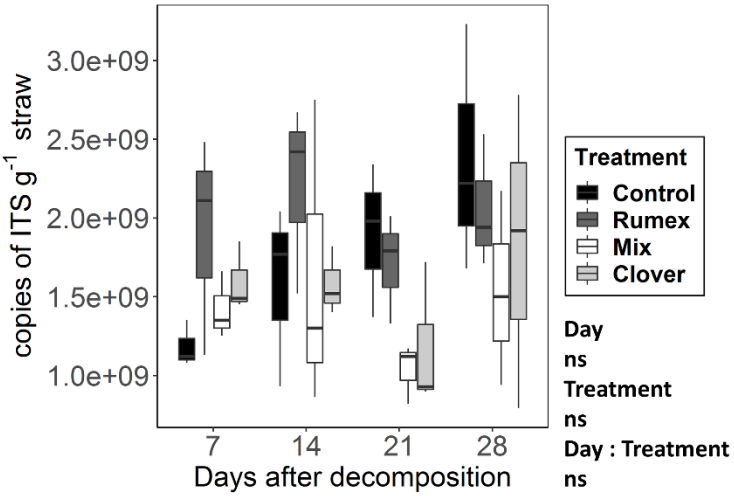

Fig 5.5b. The ITS fungi abundance at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as ns = not significant. Error bars represent standard deviations (n = 3).

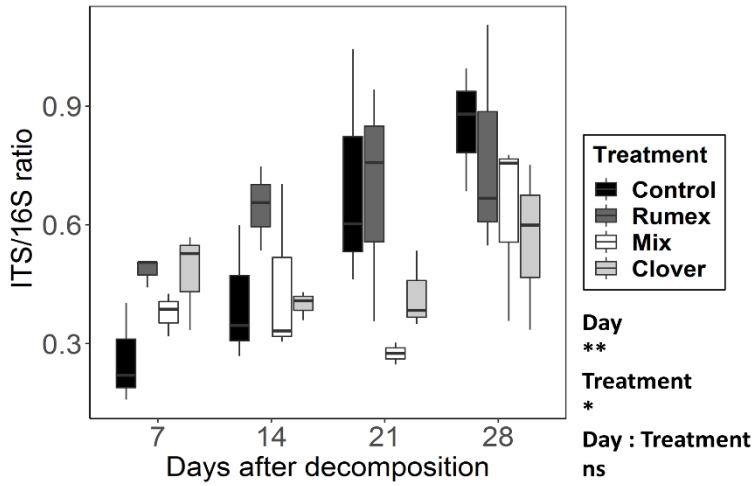

Fig 5.5c. The Fungi to bacteria ratio at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as * $p < 0.05$; ** $p < 0.01$; ns = not significant. Error bars represent standard deviations (n = 3).

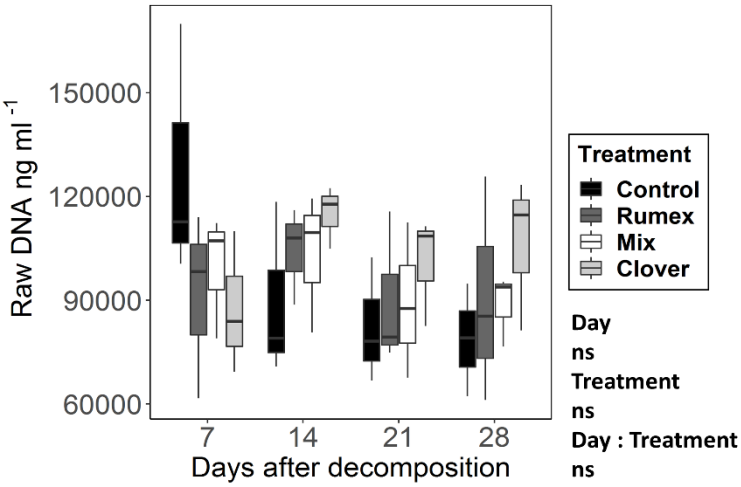

Fig 5.5d. The raw DNA concentration at 7 days, 14 days, 21 days and 28 days under control, *Rumex*, mix and clover treatments. Level of significance was determined by two-way ANOVA. Day : Treatment shows the interaction between the day and treatment. The significant level is shown as ns = not significant. Error bars represent standard deviations (n = 3).

5.4.5 Microbial biomass carbon (MBC)

The microbial biomass C in the soils at the end of the incubation period averaged around 2500 mg C kg^{-1} soil. There was no significant difference among the treatments (Fig 5.6).

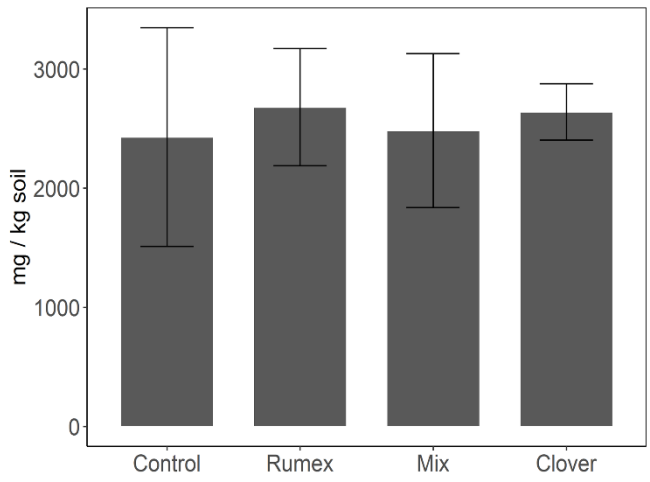

Fig 5.6. Microbial biomass carbon in soil (Control), Clover + soil, Rumex + soil and Mix + soil treatment after 28 days incubation. Level of significance was determined by one-way ANOVA (p=ns). Error bars represent standard deviations (n = 3).

5.4.6 Ammonium, Nitrate and pH

Ammonium concentration was significantly higher at clover + soil (7.22 ± 1.23 mg/L) followed by *Rumex* (5.31 ± 0.91 mg/L), mix (4.91 ± 0.44 mg/L) and control (4.22 ± 0.3 mg/L) (Fig. 5.7a). Nitrate concentration was significantly higher at clover (645 ± 179 mg/L) compared with other treatments. Control had the lowest nitrate concentration (3 ± 1 mg/L) (Fig 5.7b).

Soil pH ranged from 5.48 to 6.07 and differed significantly among treatments ($P < 0.001$). The lowest soil pH was observed in the soil with clover (5.48 ± 0.13). The control treatment (6.01 ±0.07) and the *Rumex* treatment (6.07± 0.03) showed similar and the highest pH. The soil pH of mix was (5.79 ± 0.01) (Fig 5.8).

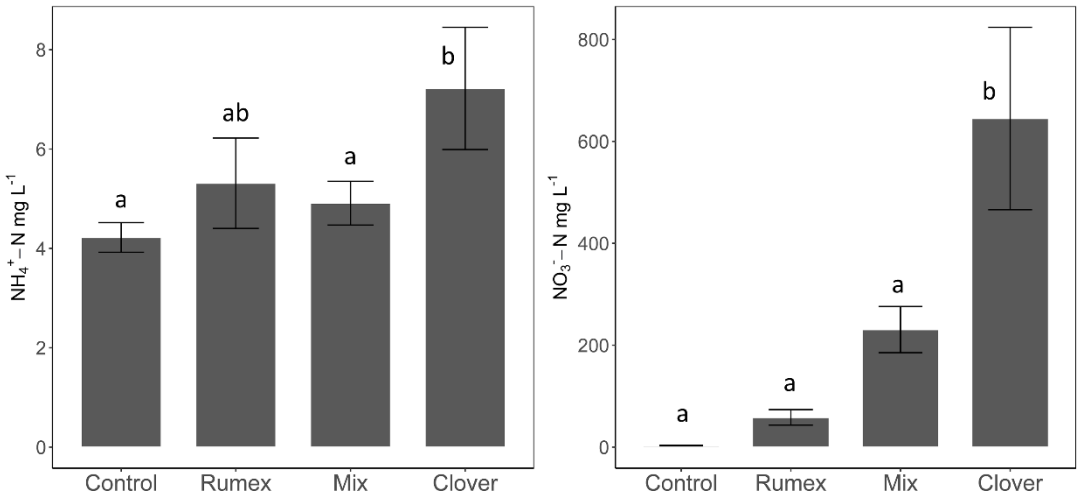

Figure 5.7. The NH_4^+-N (left) concentration in control, *Rumex*, Mix and Clover treatments after 28 days incubation. Level of significance was determined by one-way ANOVA ($p < 0.05$) (Fig 5.7a). The NO_3^--N (right) concentration in control, *Rumex*, Mix and Clover treatments after 28 days incubation. after 28 days incubation. Level of significance was determined by one-way ANOVA. Different lowercase letters indicate

significant differences between treatments (p < 0.001) (Fig 5.7b).Error bars show standard deviation (n = 3).

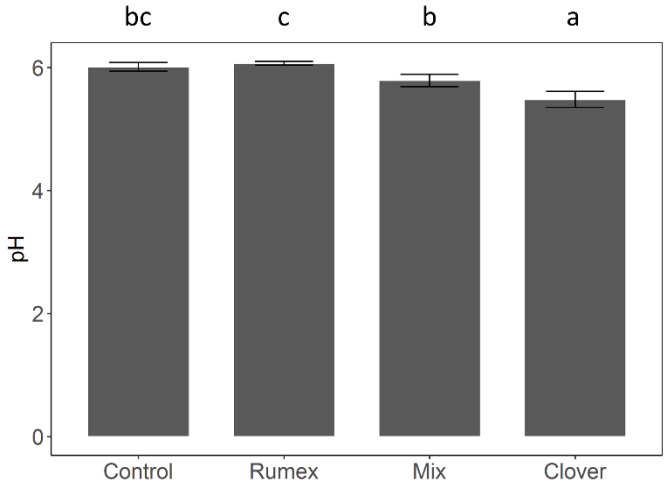

Fig 5.8. pH of control, *Rumex*, Mix and Clover treatments before incubation. Level of significance was determined by one-way ANOVA. Different lowercase letters indicate significant differences between treatments (p < 0.001). Error bars represent standard deviations (n = 3).

5.5 Discussion

In this study, the amount of respired CO2-C was negatively correlated to the C/N ratio of the added residues. This suggests that the systems were N limited. Previous studies stated that additional nitrogen sources input were able to facilitate the cell backbone make up with carbon, rather than release carbon dioxide through microbes respire, on the other hand, additional nitrogen promote microbes growth (Fornara and Tilman, 2012; Frey et al.,2014; Kleber et al., 2015; Rui et al., 2016; Liu et al., 2018). Incorporated low C/N ratio plant residues promoted soil respiration, and the rice straw decomposition is influenced by C/N ratio as well as multiple

factors, for example, content of nitrogen and carbon, cellulose and lignin (Raiesi, 2006) and the biological activity.

However, the rice straw decomposition rate was not influenced by the treatments in the current study (Fig 5.1). Generally, the incorporated rice straw is able to serve carbon for soil microbial growth as well as increase the demand of nitrogen. Input of additional low C/N ratio plant residues in soil were supposed to lower the releasing rate of rice straw nitrogen. We assumed the nitrogen in Rumex and mix was not sufficient for microbial requirements at the first 7 days, thus the rice straw C/N ratio was increasing, was due to the assumption of nitrogen content in rice straw by microorganisms. Clover with greater water-soluble organic carbon which is readily decomposed to microbes (Hadas et al. 2004), accelerated microbial proliferation, further enhanced nitrogen requirement, thus the rice straw C/N ratio was decreasing. Thereby, the treatments except control showed a decreasing on rice straw C/N ratio trend, indicated the incorporated plant residues might have stimulated microbial proliferation.

Fungal abundance was promoted, in relation to bacterial abundance, in the Rumex treatment, especially at the earlier stages of the decomposition, compared to other treatments (Fig 5.5c). Contrastingly, previous papers suggested that in general, bacteria are predominant in the early rice straw decomposition stages because of the presence of readily decomposable C, while fungi dominate later stages due to the presence of recalcitrant C such as lignin remaining in the decaying rice straw (Wang et al., 2004; Boer et al., 2005). The F : B ratio of Rumex treatment was steadily rising to entire incubation period might be due to its composed of uneasy decomposed constituents and stem (Kasai et al., 1982; Hongo, 1988; Merfild, 2018) which was correspond with fungi biochemical characteristics. The control soil (rice straw only) did not show the relatively high F/B ratio in the early stage of the decomposition period. This might be because plant species shape microbes composition through root exudates as well as leaf (Bai et

al., 2015; Jacoby et al., 2017). Accordingly, different plant residue varieties might attract different microbial structure which control soil respiration, a further research aimed to figure out which plant residue benefit microbial decomposer is greatly necessary.

Soil microbial biomass carbon was similar among treatment after incubating 28 days (Fig. 5.6). A previous rice straw decomposition study also showed the same result during a short 28 days decomposition period, there were significant difference until 80 days decomposition (Guo et al., 2018). Thus, although further studies are needed, the addition of weed residues can influence soil microbial biomass compositions (e.g. F/B ratio) but not the size of the biomass. We need to further investigate whether the changes in the composition will influence the decomposition of rice straw in the latter stages.

5.6 Conclusion

We added low C/N ratio clover, Rumex, and mixture of clover and Rumex residues as extra nitrogen source to observe whether incorporated rice straw decomposition rate could be accelerated. As a result, our study found:

- Rumex treatment had relatively higher F : B ratio among four treatments; Clover treatment had largest ammonium and nitrate concentration;
- The accumulative C released from rice straw is associated with F : B ratio and soil respiration. Higher F : B ratio lead to lower soil respiration and higher accumulative C released from rice straw;
- Even though incorporated plant residues did not significantly improve fungal and bacterial abundance. However, the higher soil respiration appeared on treatments with plant

residues suggested that might only specific microbial taxa increasing instead of entire microbial community;

- The treatments except control showed a decreasing on rice straw C/N ratio trend, indicated the incorporated plant residues might have stimulated microbial proliferation.

Our study suggested input plant residues after harvesting potentially improve higher F : B ratio as well as increasing nitrogen nutrient. In order to acquire faster rice straw decomposition rate to release a great extent of carbon, nitrogen and mineral nutrient into soil, a long term incubation period and higher plant residue to rice straw ratio, more plant residue varieties, as well as microbial composition are considerable to further confirm what factors most benefit rice straw decomposition rate.

Chapter 6

Synthesis and recommendations for further research

This chapter synthesises my research and identifies emergent opportunities for advancement by further research.

6.1 Overall summary and future work

6.1.1 Identifying the microbes communities in conventional and natural farming rice paddies in Japan and Taiwan

This study investigated the microbial community under conventional (C) and natural farming (NF) managed system respectively in Japan and Taiwan, as well as the delta 15N natural abundance in Japan. The microbial diversity was not significantly higher in NF rice paddy where had higher yields (5.6 ± 0.7 t ha−1) compared with C rice paddy (3.3 ± 0.7 t ha−1) in Japan. However, the microbial diversity was significantly higher in conventional farming rice paddy where produced more rice (6.5 t ha−1) compared to the natural farming rice paddy (1.5–2.4 t ha). Regarding the microbial community, our result was consistent with previous studies, indicated the higher yield rice paddies in were mainly predominant by class of *Acidobacteria* and *Planctomycetes*, *Deltaproteobacteria*. Although microbial diversity is also an indicator of soil microorganisms in a region, and it is accepted that microbial diversity plays an important role in agricultural production which provide a vast amount of ecological information in terms of the soil, but the relationships between the soil microbial diversity and the functioning and sustainability of agricultural ecosystems have not been fully understood, thus the microbial diversity could not explain the higher rice yield in the natural farming rice paddy in Japan. Also, in this study we did not sample soils in the different growing stage of rice as well as clearly separated rhizosphere and bulk soil. The interaction of microbes between plant roots and bulk soil potentially influences rice ecosystem functioning by promoting the circulation of materials (section 2.1.3).

We measured the δ^{15}N natural abundance at soil surface and rice root in order to look at where the N-Fixing “hot spot” in two differently managed rice paddies. As a result, the δ15N natural abundance was decreasing from water inlet to outlet, and was lower in summer (July) compared with spring (June). Apart from measuring the δ^{15}N natural abundance, surveys of the nifH gene

which encodes the iron protein subunit of nitrogenase are commonly used to characterize diazotroph populations and assess the potential for N_2-fixation, studies also have indicated that changes in the abundance and composition of diazotroph communities are related to variations in the N_2-fixation rates in various soils (Raymond et al., 2004; Mirza et al., 2014). Several environmental factors have been suggested to influence the rates of N_2-fixation process, these factors including soil temperature, moisture, oxygen, carbon quality and quantity, nitrogen availability and P, Mo and Fe. Thus, for the future experiments, it is necessary to include the factors mentioned above and make an effort to figure out which are the most specific keys to control rice yields, thus farmers who perform natural farming system are able to shorten their time pursuing a healthy soil ecosystem for rice growth.

6.1.2 Inter–tillage during natural farming rice paddy production negatively impacted the microbial abundances in soils but not on diversities

In chapter 3 we compared the microbial community between Japan and Taiwan, conventional and natural farming rice paddies, higher and lower yields. In chapter 4, we compared the microbial community between five times inter-tillage (5T) and no-tillage (NT) under natural farming treatment rice paddy. Soils were sampled at rice proximity, soil surface and 10 cm depth in a natural farming rice paddy, during the early to late vegetative phase (June to July), in Hokkaido, Japan. We observed there was no clear differences between 5T and NT, regarding the bacterial community structures and diversity indices. Instead, the sampling timings markedly impacted the bacterial community structures for the rice proximity and soil surface, showing increasing diversity indices at the late vegetative stage, compared to the early vegetative stage, suggesting the interaction between the crop growth and bacterial communities.

Different from our previous study, we did not observe the significant difference between the rice yield from NT (2.3 ± 0.7 t ha−1) and 5T plots (2.7 ± 0.9 t ha−1). A potential reason is due to the paddy including 5T and NT had been ploughed before growing rice, thus we did not observe the different diversity during the soil sampling period. In addition, weeding through inter-tillage did not improve rice yield. On the other hand, no-tillage seems feasible to be performed.

However, the microbial community dynamic was just be investigated within a short course, not across over the entire rice growing stage, beside, the interaction between soil environmental factors and microorganism was not considered in this study. For instance, studies has suggested the variations of enzymatic activities in the soils were significantly explained by total nitrogen, total potassium, and moisture, and the enzymatic variability in the low-yield soil was related to potassium, available nitrogen, pH, and total carbon; and that in the high-yield soil was partially associated with potassium and moisture (Luo et al., 2016). Therefore, the future need to contain

more comprehensive microbial, chemical and physical properties, as well as considering the complete rice growth stage. This might be the only way to find how tillage actually influences microbial community fluctuation compare to NT.

6.1.3 Weeds incorporation affected on microbial abundance but not on straw decomposition

In a natural farming rice paddy, where relies on organic residue incorporation, rice straw plays an essential role. Rice straw incorporation has the potential to improve soil quality and maintain sustainable soil productivity by increasing soil organic carbon sequestration and nutrients deposition (section 2.2.4). We found a quite amount of undecomposed rice straw while

sampling soils for the chapter 4 experiment. We hypothesis that incorporated extra nitrogen might be able to stimulate rice straw decomposition rate. The dominated species *Trifolium Pretense* (clover) and *Rumex obtusifolius* (*Rumex*) as extra nitrogen for this incubation experiment. As a result, the rice straw decomposition rate was not influenced by the treatments, but we observed fungal abundance was promoted, in relation to bacterial abundance, in the *Rumex* treatment, especially at the earlier stages of the decomposition, compared to other treatments.

In order to acquire faster rice straw decomposition rate to release a great extent of carbon, nitrogen and mineral nutrient into soil, a long term incubation period and higher plant residue to rice straw ratio, more plant residue varieties, as well as microbial composition are considerable to further confirm what factors most benefit rice straw decomposition rate. In this study we did not investigate the attribution of microorganisms which are in charge of residue decomposition. The related microbial group will be measured in the future experiments.

6.2 General comments

- In Chapter 3, we did not separate the rice rhizosphere and bulk soil, and the sampling times was only once in Taiwan. The sampling amount was not enough for explaining the realistic condition. Also, soil nifH gene analysis was not investigated in Chapter 3 and Chapter 4. The nifH gene abundance is one of an essential key factors to confirm whether a natural farming rice paddy be able to achieve autarky.

- In Chapter 5, the incubation period was one month. Multiple previous incubation studies has been conducted at least four months in correspondence to the rice growth course. In addition to incubation course, low temperature incubation experiment is needed to be

develop. In Hokkaido, Japan, rice straw incorporation followed by winter in about two months later. How to improve the straw decomposition rate under low temperature will be a considerable issue in a natural farming paddy system especially in Hokkaido.

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