Application of biogas digestate with rice straw mitigates nitrate leaching potential and suppresses root-knot nematode (*Meloidogyne incognita*)

Yuexi Wang

ID NO. 16701113

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Content

Chapter 1 General introduction

1.1 Background of biogas digestate

With the concern of climate changes caused by the extensive use of fossil fuels that give rise to large amount of greenhouse gas emissions, and leaching of nutrients and farm and industry wastes to natural environment. Reduction of greenhouse gases emissions is an intractable and urgent task to mitigate climate changes (Liu et al., 2019). Renewable energy is considered to be a of most effective way to minimize CO_2 emissions, and the sources of clean, inexhaustible and increasingly competitive energy, because some of it such as wind and solar are replenished and will never run out. While many renewable energy sources such as, wind, solar power, and biomass depend on inconstant natural climate conditions, biogas is produced under rather stable conditions. Because it is produced through the degradation (anaerobic digestion, AD) of organic materials, such as manures, crop residues, food waste (Moller & Muller, 2012; Velazquez et al., 2015). AD is designed and controlled by different temperature and water content (Ward et al., 2008), it is not only an important solution to the reuse or recycling of organic waste, but also its product biogas has an advantage better than other renewable energy sources, which can be stored, meaning it accustomed variable model with regards to storage, transport, spreading (Logan and Visvanathan , 2019). The residual by-product of AD, which is called biogas digestate, is usually spread as a fertilizer since its application to soil improves soil properties (Petersen et al., 2003), provides nutrients (N, P, K) (Nkoa, 2014; Burnett et al., 2016), also can suppress soil-borne pathogens, plant parasitic nematodes, or their disease (Jothi et al., 2003; Goberna

et al., 2011; Cao et al., 2016).

1.1.1 Anaerobic digestion

AD is a multiple bio-degradation process, it can be applied to a wide range of organic wastes including industrial and municipal waste waters, agricultural, municipal, food industry wastes, in which some components of organic wastes, and plant residues will be decomposed into biogas, mainly consisting of methane and carbon dioxide, in the absence of oxygen. It involves with four principal metabolic reactions: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Park et al., 2005; Liu et al., 2008; Charles et al., 2009).

This process has some advantages over other forms of waste treatments, such as less biomass sludge is produced in comparison to aerobic treatment technologies (Ward et al., 2008); Successful in treating wet wastes of less than 40% dry matter (Mata-Alvarez, 2002); a high rate of pathogens destructions can be achieved (Sahlstrom, 2003). It is considered a viable technology in the competent treatment of organic waste and the simultaneous production of a renewable energy (De Baere, 2006; Jingura, R.M. and Matengaifa, R., 2009.).

On the other hand, the anaerobic process has some disadvantages such as long retention times and low removal efficiencies of organic compounds (Park et al., 2005). Therefore, the keys in the anaerobic digester design are to maintain a constantly high organic loading rate and to obtain a short retention time and the maximum volume of biogas (Ward et al., 2008).

1.1.2 Types of anaerobic digestion system

Based on operating temperatures, anaerobic reactors are mainly divided into two types: mesophilic (30 to 40°C) fermentation and at moderate thermophilic (50 to 60°C) fermentation, depending on the optimal temperature ranges for methanogens (Van Lier, 1996; Kuo and Cheng, 2007). In addition, psychrophilic (< 20°C) anaerobic digestion is also known as an easy-to-use process (Saley and Westerman, 1994; Rajagopal et al., 2017).

Depending on the water content in the digester, bio-digesters are categorized into wet and dry fermentation. Wet fermentation refers to total solid contents less than 10-16%, while dry fermentation refers to digester fed with 22-40% (Mata-Alvarez, 2002; Ward et al., 2008). Dry bioreactors are mainly filled with municipal solid waste or agricultural crops wastes, and wet bioreactors are used for different types of manure (Ward et al., 2008).

1.1.3 Materials of anaerobic digestion

1. Agriculture waste

Agricultural waste such as animal waste, crop residue etc., usually has higher cellulose and hemicellulose contents and lower lignin contents, is useful for more bio-energy production. The co-digestion of agriculture waste with crop residue produces more biogas with high methane percentage than manure or biogas plant alone (Al Seadi and Lukehurst, 2012; Ebner et al., 2016). Muscolo et al. (2017) observed that the by-product (biogas digestate) from anaerobic co-digestion of agricultural wastes applied into the soil enhanced soil organic matter (SOM), microbial biomass carbon (MBC).

Animal waste comes from livestock and poultry, wastewater, feedlot runoff, silage juices, bedding, and feed (Chen et al., 2008). These wastes result in environmental and human health risks such as contaminants in drinking water sources, non-point source pollution, transmission of disease-causing bacteria and parasites associated with food. Animal waste contains high concentrations of nitrogen (N) and phosphorus (P).

Crop residue stands for a part of agriculture waste and it is produced from stalks, straws, leaves of a variety of crops, which are used for energy generation (Kalra and Panwar, 1986). The primary crop residues are categorized into cereal crop residue, leguminous crop residue, root crop residue, and oil seeds residue (Zhang et al., 2017). Celluslose, hemicellulose, and lignin are the main components of crop residues.

Fruit and vegetable wastes are generated after processing, packing, distribution. They tend to have lower total solids and higher volatile solids, and are easily degraded in an anaerobic digester (Ward et al., 2008).

2. Municipal solid waste

Municipal waste is defined as waste collected by or on behalf of municipalities (Otten, 2001). The bulk of the waste originates from households, commerce and trade, office buildings, institutions and small businesses, but excludes industrial, hazardous, and construction wastes (Rajkumar et al., 2010; Jha et al., 2011). Municipal solid waste generally includes degradable (paper, textiles, food waste, straw and yard waste), partially degradable (wood, disposable napkins and sludge) and non-degradable materials (leather, plastics, rubbers, metals, glass, ash from fuel burning like coal, briquettes or woods, dust and electronic waste) (Heart, 2009; Jha et al., 2007;

Tchobanoglous et al., 1993). The municipal waste is an easily-degraded material which can provide more biogas through anaerobic decomposition (Ward et al., 2008).

3. Industry waste

A Food industry wastes

The food industries that could benefit from anaerobic treatment include fruit and vegetable canning, edible oil refining, dairy production, seafood processing, meat processing, starch and sugar production, brewing, and fermentation. Wastes from food processing are high in organic matter and are therefore ideal for anaerobic digestion. Seafood processing wastewaters contain high concentrations of different cations and anions, mainly Na^+, Cl^-, and SO_4^{2-} (Feijoo et al., 1995). Meat processing wastes are substantially different from other food industry wastes. They are very strong wastes containing grease, blood, faeces, and recalcitrant organic matter such as straw and hair. During anaerobic digestion, protein and lipids degradation leads to the accumulation of ammonia, which are important inhibitors of the anaerobic microorganisms (Salminen and Rintala, 1999).

B Paper and pulp industry wastes

The pulp and paper industry has several high strength waste streams that are of concern from an environmental standpoint. Since the pulp produced corresponds to only 40–45% of the original weight of the wood, the effluents exhibit high chemical oxygen demand (COD) concentrations (Ali and Sreekrishnan, 2001), which together with effluent's warm temperature (the waste is typically around 35 °C), makes anaerobic digestion a favorable waste treatment technique.

C Textile industrial wastes

The main sources of wastewater generated by the textile industry originate from the washing (or scouring) and bleaching of natural fibers and from the dyeing and finishing steps. Given the great variety of fibers, dyes, process aids, and finishing products in use, these processes generate wastewaters of great chemical complexity (Vandevivere et al., 1998).

D Petrochemical refineries wastes

Anaerobic digestion could also be of use in petrochemical refineries. It has been found that after prolonged acclimation, aldehydes, acids, alcohols, and esters could be used for methane production (Chou et al., 1978). Chou et al. (1978) concluded that anaerobic digestion of petrochemical wastes would not only result in a saving of energy over aerobic processes but would also produce methane on a scale for use as a fuel.

1.2 General effects on the soil biota

1.2.1 Effects of biogas digestate on soil nitrifiers

Soil nitrification, the first and rate-limiting step is the oxidation of ammonia to nitrate, which was performed by ammonia oxidising bacteria (AOB), results in soil acidification and groundwater pollution by N losses via leaching of nitrate-based fertilisers, with associated atmospheric pollution by nitrous oxide (N_2O) emission (Stephen et al., 1996; Prosser & Nicol, 2012; Bissett et al., 2014). Biogas digestate as an organic fertilizer contain higher ammonia (NH_4^+), more mineral nitrogen (N), the application of BD may lead to negative effects on AOB groups. Nyberg et al. (2004) investigate whether the different kinds of wastes after anaerobic digestion contain

substances with the negative effect on ammonia oxidizing bacteria activity. The results showed that the substances inhibit potential ammonia oxidation and AOB activity after application of anaerobia digestion residue in agriculture soil. The application of 80 kg N ha^{-1} digestate into the soil enhanced nitrification rate and modify the soil AOB community in compare with control (no fertilizer) (Gómez-Brandón et al., 2016).

1.2.2 Effects of biogas digestate on soil microbial community structure

The application of biogas digestate (BD) may effectively affect the structure and diversity of soil microbial community, resulting in the improvement of soil quality (Sullivan et al., 2006). Biogas digestate contains different kinds of nutrients, particular higher in nitrogen, soil amended with digestate will result in C/N ratio decrease (Cheng et al., 2017) and soil pH increase (Cheng et al., 2018) that will further lead to soil microbial community structure changes. Several studies have already demonstrated that the effect of digestate application on soil microbial community structure changes in terms of soil chemical properties' changes, such as Hupfauf et al., (2016) revealed the relationship between phosphate solubilising bacteria community and P uptake after digestate amendment, and also nitrogen dynamics with soil nitrifiers changes (Sawada and Toyota, 2014). Due to the fact that changes in microbial communities can occur more quickly than changes in other soil characteristics, such as nitrifiers (Gómez-Brandón et al., 2016), phosphate solubilising bacteria (Hupfauf et al., 2016) etc. the study of microbial parameters is deemed a sensitive indicator when evaluating the soil disturbance and impact assessment after application of (organic) fertilizers (Odlare et al., 2008; Alburquerque et al., 2012; Coban et al., 2015).

Biogas digestate contains higher ammonia (NH_4^+) than undigested manures, more mineral nitrogen (N), less organic carbon (C), phosphorus, potassium and other bioactive substances: such as phytohormones (e.g. gibberellins, indoleacetic acid), nucleic acids, monosaccharides, free amino acids, vitamins and fulvic acid, etc., which provide efficient nutrients for plant growth and improve the tolerance to biotic and abiotic stress (Liu et al., 2009; Yu et al., 2009). Microorganisms as biochemical media can transform chemical components between soil and plants, being the source and sink of nutrients, or in physical properties of soils, such as aggregation, which play a leading role in soil (Anderson, 2003; Anderson and Domsch, 1980). Soil microbial biomass can be a living metabolizing unit, the application of BD into the soil, which was an indicator to reflect any changes of soil health (Beare et al., 1995; Elliott 1997; Pankhurst et al., 1997).

In an 8-week pot experiment on loamy sand, microbial basal respiration and metabolic quotient were higher with the digestates than that of untreated slurry or the mineral treatments, community level physiological profiles with MicroResp showed particularly strong effects of digesates treatment (Hupfauf et al., 2016). Juárez et al., (2013) report that amendment with digestate (120 kgN ha^{-1}) enhanced soil total C, NH_4^+, and total P, resulted in significantly effect on soil microbial community compare with manure treatment. Johansen et al. (2013) found a pronounced change in microbial diversity measures compared to the controls (no fertilizer) after application of anaerobically digested grass-clover, in compared with other anaerobically digested materials, grass-clover devoted to more readily degradable organic C than the other

materials. Application of digestate had no obvious effect on barley yield and earthworm population, but increase a aggregate stability and reduce a the risk of N losses (Frøseth et al., 2014). Caracciolo et al., (2015) found that the application of anaerobic digestate at rates of 30 and 60 t ha^{-1}, respectively, even from the low concentration AD significantly increased the diversity of the microbial community and also improved the soil organic carbon and nitrogen contents of a degraded soil. In contrast, biogas slurries have negative effects on the microbial colonization of root, and also have negative effects on saprotrophic fungi (Wentzel and Joergensen, 2016). A 4-year study showed that application of organic municipal waste based digestate increased much more soil microbial biomass (SMB) C in compare with control (unfertilized), and had beneficial short-term effects on microbial community (Poulsen et al., 2013).

Chen et al. (2012) found a certain shift in the microbial community to slower-growing microorganisms by amended with biogas digestate to soil. This is also supported by Sapp et al. (2015) who performed an greenhouse experiment where inorganic fertiliser or digestate from sewage sludge were applied into soil, the results showed that the addition of digestate obviously influenced the bacterial community structure directly, the plant growth was also increased by the digestate addition thereof had effects on bacterial community structure and diversity indirectly. Walsh et al. (2012) observed that the application of liquid digestate result in a higher growth of bacterial compare to mineral fertilizer and stimulated the bacterial decomposer community. The application rate of 100 Mg ha^{-1} digestate (sugar beet pulp 50%, fruit marc 42%, and maize silage 8%) in metal polluted soil showed that microbial activity and physiological

diversity were significantly enhanced, bacterial and fungal populations were also increase (Garcia-Sanchez et al., 2015).

1.2.3 Effects of biogas digestate on soil borne-plant pathogen

Soil borne plant pathogens are generally categorized into virus, bacterium, fungus or nematode, which can exist in the soil for long period even without a host (Huber et al., 1970; Dixon & Tilston, 2010). Amendment with digestate into the soil may affect the structure and diversity of soil microbial community, the diversity of soil microbial communities can be the key to the capacity of soils to suppress soil-borne plant pathogen (Elsas et al., 2004; Goberna et al., 2011).

A pot and field experiments were conducted to investigate the effects of biogas slurry application on Fusarium wilt disease suppression, the results showed that amendment with biogas slurry significantly suppressed Fusarium wilt both of field (43.1%) and pot (95.9%) experiment (Cao et al., 2016). Goberna et al., (2011) reported that the addition of digestate effectively reduced the number of all pathogens survived in soils compared to those amended with manure; anaerobic digestion of cattle manure completely destructed *E. coli* and *Salmonella* sp. and made a significant reduction of *Listeria* sp. from 10^5 ml^{-1} to 10^4 ml^{-1}. *E. coli* and *Salmonella* were both completely eliminated after addition of digestate one month later (unless the manure was applied to sterilized soil). *Listeria* numbers in soil were similarly significantly reduced to control level within three months incubation period. The different methods of manure application as well as the microbial competition between native soil and manure also can be an pronounced indicator to control the survival of potential pathogens in

amended soils (Unc and Goss, 2004; Hodgson et al., 2016).

Tao et al. (2014) conducted a lab-scale study to investigate the effect of digestate on in vitro mycelial growth of seven phytopathogenic fungi: *Fusarium oxysporum, Sclerotinia sclerotiorum, Rhizotonia cerealis, Bipolaris sorokinianum, Rhizoctonia solani, Exserohilum turcicum,* and *Bipolaris maydis*, the results showed that the absolute growth rate of seven fungi was significantly suppressed except for *Exserohilum turcicum* compared to the controls (water applied), the colony sizes of *Fusarium oxysporum, Sclerotinia sclerotiorum, Rhizotonia cerealis, Bipolaris sorokinianum, and Rhizoctonia solani* were significantly smaller than controls (water applied). The same result was also reported by Bustamante et al., (2012) who stated that *Fusarium oxysporum* f. sp. *melonis* (FOM) growth was significantly suppressed by addition of digestate.

Pampillon-Gonzalez et al. (2017) found that there were no *Salmonella* spp. or eggs of helminths were detected in the anaerobic storage of the pig slurry, which efficiently killed most of the pathogens. Westphal et al. (2016) reported that amendment with the digestate significantly reduced the densities of *Heterodera schachtii* in nematicide-treated plots, and digestate had a highly significant effect on the fungal community composition in the rhizosphere of *mangold* experiment. But several studies also reported that the organic amendment application has no obvious suppressive effects on soil-borne pathogens (Goberna et al., 2011) and nematodes (Nkoa, 2014).

1.3 Effects of biogas digestate on soil nitrogen cycle

Nitrogen is an essential nutrient in soil micro-organism environment and plant-

based systems, soil N cycle may come after several pathways including: biological N fixation (BNF), litter decomposition, N mineralization, immobilisation, nitrification, denitrification, as well as N leaching and ammonia volatilization, additional N input such as manure, urea, compost, compound fertilizer, biogas digestate etc. application will direct or indirect effect soil N cycle (Chapin, et al., 2011; Manning, 2012). Digestates decomposition in soil were compared to those on undigested manures, the liquid (LS) and solid fraction (SS) of a biogas slurry was shown to either promote or suppress N mineralization and N apparent recovery fraction. However, the composted solid fraction (CSS) mitigated N immobilization (Grigatti et al., 2011). Likewise, a 181 days incubation study indicated that digestate cattle slurry-maize mix and the liquid induced net N mineralization (≈30% of manure organic N) due to a low C to organic N ratio,. However, undigested cattle slurry and the solid fraction induced net N immobilization (9–16% of manure organic N) due to high C to organic N ratio, high cellulose and volatile fatty acids content (Cavalli et al., 2017). The dissolved organic C, and the corresponding organic C: total N ratio of dissolved substances can be considered the most reliable indicators in describing digestate biodegradability (Alburquerque et al., 2012).

Ammonium (NH_4^+) in biogas digestate is readily nitrified into very mobile soil nitrate (NO_3^-) (Möller and Müller 2012; Nkoa 2014) which will promote nitrate (N) leaching. Sawada and Toyota (2015) reported that biogas digestate applications (300 kg NH_4-N ha^{-1}) led to rapid increase in nitrate contents in soil. Cheng et al. (2018) conducted a plot experiment with biogas slurry irrigation in purple soil at nitrogen

application rates of 0, 48, 144, 240, 336, and 480 kg NH_4-N ha^{-1}, as a result, the last two rates have triggered N leaching being detrimental to groundwater safety. Several studies had compared with the nitrate leaching risk between biogas digestate and undigestate manure application. Goberna et al. (2011) found that amending soils with digestate enhanced nitrate losses during the first 30 d of a 100-day incubation period, the biogas digestate resulted in three times as much NO_3^- leaching as the manure led to. Fertilizing with the anaerobically digested materials increased the soil concentration of NO_3^- approx. 30–40% compared to undigested materials (Johansen et al., 2013). Gomez-Brandon et al. (2016) observed that digestate led to a 49% higher soil nitrification rate than manure in the 60 days incubation periods.

1.4 Objective of the study

The main objective of the present study was to explore the effect of wet and dry digestate mix with rice straw application on root-knot nematode (*Meloidogyne incognita*) suppressiveness and the mitigation of nitrate leaching potential. In the chapter 2, I conduct two main experiments to estimate the effect of wet and dry digestate application on the population of soybean cyst nematode (*Heterodera glycines*) and potato rot nematode (*Ditylenchus destructor*). Chapter 3 mainly focus on the effect of wet and dry digestate mixed with rice straw on nitrate leaching potential and the dynamics of soil inorganic nitrogen and extractable total carbon and nitrogen. Considering the results of chapter 3 and the previous study (Min et al., 2011) on root knot nematode, I chose to mainly study on root knot nematode suppressive effect after digestate amendment and the relationship between root knot nematode and liable

carbon. I hypothesize the mechanism of root-knot nematode suppressiveness was related to soil liable carbon dynamics. In chapter 4, I mainly discuss the future direction of rice straw and biogas digestate management and life-cycle environment. Depending on chapter 2 results discussed the effect of different practice of the conventional tillage (incorporation rice straw in soil) and conservation tillage (surface retention of rice straw) on soil carbon dynamics. Moreover, considering the current global option of wet biogas digestate management and solid content of dry digestate, I discussed the available practice of both types of digestate and its economic value for better utilize for the farmers in the future.

Chapter 2 Effect of biogas digestate mixed with rice straw on nitrate leaching potential

2.1 Abstract

The objectives of this study were to mitigate nitrate leaching risk in the application of biogas digestate to soil by co-application of rice straw. Because of the higher carbon content of rice straw that will result in soil nitrogen deficiency after amendment, therefore we hypothesis the application of biogas digestrate mixed with rice straw can mitigate nitrate leaching risk. This study consisted of the following seven treatments: i) control without any fertilizer (CONT), ii) chemical fertilizer (CF), iii) wet biogas digestate deriving from pig manure (WBD), iv, v) dry biogas digestate deriving from a mixture of pig manure and rice straw at an initial C/N ratio of 20 and 30 (DBD20 and DBD30), respectively, vi), vii) DBD20 mixed with a low and high amount of rice straw to adjust its C/N ratio to 16 (Mix1) and 30 (Mix2), respectively. The application rate of CF and digestates was adjusted to 200 mg N kg^{-1} soil based on their inorganic nitrogen contents. Nitrate contents readily increased in all the treatments with incubation, except for Mix2, and were consistently lower in Mix2 and Mix1 during most of the incubation periods. Wet and dry digestate applications resulted in increases in extractable total nitrogen (ETN) in both soils, and the amounts of ETN were almost similar to those of NO_3-N from 14 to 35 days of incubation. There were significant relationships between the nitrogen mineralization (*Nm*) or nitrate conversion (NC) and the differences in extractable organic carbon EOC from 0 day to 90 days soil. These results suggest that dry digestate mixed with rice straw might have a lower nitrate leaching potential.

2.2 Introduction

Biogas digestate is a highly valuable nutrient-rich and humus-rich fertilizer (Möller, 2015). In addition to the biofertilizer effect, the use of digestate can be an effective management practice as an organic amendment in agriculture for improving physical soil properties such as aggregate formation and moisture retention, sustaining soil organic matter concentrations, enhancing biological activities, and suppressing pathogenic organisms (Möller, 2015, Nkoa, 2014). However, when digestate is applied to soil, ammonium in the biogas digestate can be readily nitrified (Alburquerque et al., 2012). Previous studies have demonstrated that biogas digestate application led to a higher soil nitrification rate than manure (Cavalli et al., 2017, Gómez-Brandón et al., 2016) and compost (Viaene et al., 2017). Thus, the use of biogas digestate may increase nitrate leaching risks (Broz et al., 2017, Cheng et al., 2017, Cheng et al., 2018, Du et al., 2019, Forge et al., 2016), as reported in the previous study (Sawada and Toyota, 2015) where biogas digestate increased nitrate contents in soil through a stimulatory effect on nitrification.

Several management strategies to mitigate nitrate leaching have been proposed: (i) limiting N application rates, (ii) synchronizing N supply to plant demand, (iii) the adoption of cover crops, (iv) the use of nitrification inhibitors, and (v) the application of a C source such as wheat or rice straw (Di and Cameron, 2002). Of these, returning C-rich residues (e.g., straw) is an efficient tool to retain nitrate in the soil, due to the drastic increase in microbial immobilization of inorganic N, since microbes use labile C in straw as energy and carbon sources (Shindo and Nishio, 2005, Yang et al., 2018).

Thus, the combined application of straw with manure (Li et al., 2015, Omar et al., 2016) or mineral N fertilizer (Demiraj et al., 2018, Gai et al., 2019) reduces the superfluous accumulation of mineral N in soil and its losses (Pan et al., 2017). Therefore, I assumed that the combined use of rice straw and biogas digestate may be useful for sustainable crop production, since it reduces nitrate losses from agricultural soils and improves nitrogen utilization efficiency.

The objectives of this chapter were to evaluate the effect of the application of dry and biogas digestates with rice straw on the dynamics of inorganic nitrogen in soil, in particular nitrate nitrogen.

2.3 Materials and methods

2.3.1 Biogas digestates and rice straw

Two types of digestates, i.e., wet and dry biogas digestates, were used. The wet digestate was collected from a biogas plant in Aichi Prefecture, Japan, in which pig slurry was anaerobically digested at 35 °C with a hydraulic retention time of 15 to 20 days. Dry digestate was obtained from a dry thermophilic (55 °C) anaerobic digestion pilot plant that primarily used pig manure, and was supplemented with rice straw to adjust its C/N ratios to 20:1 or 30:1, with a sludge retention time of 40 days, in the Tokyo University of Agriculture and Technology, Institute of Engineering, Japan (Zhou et al., 2016). Both digestates were directly taken from the effluent of the digester and stored at 4 °C until use. Rice straw was collected from a paddy field, air-dried, cut into 2–3 cm lengths with scissors and ground into powder with a blender (Osaka Chemical Co., Ltd., Osaka, Japan). The chemical properties of the digestates and rice straw are

shown in Table 1 and each sample was analyzed with three replicates. Ammonium-N content was measured using the indo-phenol blue method (Bolleter et al., 1961). Extraction was performed by: (i) mixing 5 mL of wet digestate or 5 g of dry digestate and rice straw with 25 mL of 2 M KCl, (ii) shaking for 1 h at 120 rpm, and (iii) filtering through No. 5C filter paper (ADVANTEC Toyo Kaisha, Ltd., Tokyo, Japan). The water soluble total C (WSC) and N of the digestates were measured with a TOC-$V_{CSH/CSN}$ (Shimadzu, Kyoto, Japan) using the extracts. Carbon and N contents of the solid parts after extraction were measured with a CN coder (MT-700, YANACO New Science, Kyoto, Japan). Total C and N of digestate were then estimated from the sum of C and N in the water soluble and solid fractions. pH was determined in a 1:2.5 water-soluble extract.

Table 2.1 Chemical properties of digestates used in the present study.

	Water Content (%)	**pH (H_2O)**	**Total C (g kg^{-1} or L^{-1})**	**Total N (g kg^{-1} or L^{-1})**	**C/N Ratio**	**WSC*[1] (g C kg^{-1} or L^{-1})**	**WSN *[2] (g N kg^{-1} or L^{-1})**	**NH_4-N (g N kg^{-1} or L^{-1})**
WBD	97	6.2	12	5.0	2.4	2.81	3.88	4.2
DBD20	81	8.8	53	4.3	12.3	7.66	2.77	2.7
DBD30	80	8.7	56	3.4	16.5	5.06	1.78	1.6
Rice straw	4.0	6.2	353	5.5	64.2	ND *[3]	ND *[3]	0.1

WBD: wet biogas digestate, DBD20: dry biogas digestate adjusted to its original C/N ratio of 20 and then fermented (C/N ratio = 12), DBD30: dry biogas digestate adjusted to its original C/N ratio of 30 and then fermented (C/N ratio = 16). Data expressed on a fresh weight basis, data for WBD are expressed on a g L^{-1}. *[1] WSC: water soluble C, *[2] WSN: water soluble N, *[3] ND: not determined.

2.3.2 Soils

Two soils (an Andosol and a Fluvisol), which are typical in Japanese cropland and paddy fields, respectively, were used. Kikugawa soil was a culture soil (an Andosol

amended with compost) naturally infested with the root-knot nematode *Meloidogyne incognita* and collected from a tomato farm in Kikugawa city, Shizuoka Prefecture, Japan. Soil was taken from the plow layer (ca. 20 cm). Fuchu soil (a gray lowland soil, Fluvisol) was also collected from the plow layer (ca. 10 cm layer) of an upland field in the Field Museum Hommachi (FM Hommachi), Field Science center, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan. Freshly collected soil samples were sieved to 5 mm and stored at field moisture (fresh soil moisture content was 28% and 14% in Kikugawa and Fuchu soil, respectively) at 5 °C until use. Subsamples of the soils were air-dried and then analyzed for the physicochemical properties. The main characteristics for Kikugawa soil were: 73.2 g C kg^{-1} soil of total C, 7.0 g N kg^{-1} soil of total N, pH (H_2O) 7.0, maximum water holding capacity (MWHC) 1.29 g g^{-1}, and texture; sandy loam (16% clay, 18% silt and 66% sand). The properties for Fuchu soil were: 35.0 g C kg^{-1} soil of total C, 3.6 g N kg^{-1} soil of total N, pH (H_2O) 5.7, MWHC 0.81 g g^{-1} and texture; sandy clay loam (23% clay, 27% silt and 50% sand) (Sunaga et al., 2009).

2.3.3 Experimental Setup

The following seven treatments were prepared: (i) control (no addition of biogas digestate and chemical fertilizer, CONT), (ii) compound chemical fertilizer (N:P:K = 8:8:8, Asahi Industries, Tokyo, Japan) (CF), (iii) wet biogas digestate (WBD), (iv) dry biogas digestate adjusted to its original C/N ratio of 20 and then dry fermented (C/N ratio = 12) (DBD20), (v) dry biogas digestate adjusted to its original C/N ratio of 30 and then dry fermented (C/N ratio = 16) (DBD30), (vi) DBD20 mixed with a low

amount of rice straw to adjust the C/N ratio to 16 (DBD20:rice straw = 1:0.06) (Mix1), (vii) DBD20 mixed with a higher amount of rice straw to adjust the C/N ratio to 30 (DBD20:rice straw = 1:0.4) (Mix2). Their application rates were adjusted to 200 mg ammonium (NH_4^+-N) kg^{-1} dry soil (equivalent to ~300 kg NH_4-N ha^{-1}) except for CONT, since this rate is commonly used for tomato cultivation. The N contained in rice straw was not considered in this chapter, because the amounts (0.003 and 0.019 mg N kg^{-1} dry soil in Mix1 and Mix2, respectively) were low compared with the N contained in the digestate. Since N is the main yield-limiting factor, the application rate was determined based on the amounts of the NH_4^+-N fraction in the digestates (Möller and Müller, 2012). The actual added amounts of WBD, DBD20, DBD30, Mix1 and Mix2 were 48, 74, 125, 74 and 74 mg g^{-1} dry soil, respectively. In Mix1 and Mix2, rice straw was added at rates of 4.4 and 29.7 g kg^{-1} dry soil, respectively.

2.3.3.1 Experiment 1

A Application of biogas digestate on tomato cultivation

a. Tomato germination experiment

Tomato seeds (Solanum lycopersicum var. Fukuju) were pre-germinated in a Petri dish for 4 days at temperature conditions (25°C) in a biotron (LPH 200, NK System) (12 hr day and 12 hr night conditions). Then, three germinated tomato seedlings were planted in a vinyl pot (9 cm in diameter, 7.5 cm in height) containing 80 g of Akadama soil (no infested soil, soil texture: clay loam (clay 39%, silt 33%, and sand 28%, pH (H_2O): 6.7) and grown for 30 days.

b. Application of biogas digestate on tomato cultivation experiment

This experiment was divided into two levels of nematode density (lower and higher density). All treatments set up were followed above from i to iv with five replicates. Their application rates were adjusted to 300 mg ammonium (NH_4^+-N) kg^{-1} dry soil (equivalent to ~450 kg NH_4-N ha^{-1}) except for CONT, since this rate is maximum used for tomato cultivation. The actual added amounts of WBD, DBD20 and DBD30 were 100, 168 and 100 mg g^{-1} dry soil (equivalent to 150, 252 and 150 Mg ha^{-1}), respectively. Each pot of lower level and higher lever density experiment were filled with 40 g and 200 g infested soil, respectively. Then, three one-month old tomato plants with soil was transplanted into a vinyl pot (13 cm in diameter, 25 cm in height) filled with enough culture soil to 850 g. After 6 months cultivation period, investigate plant parameters, disease degree and inorganic matter content.

2.3.3.2 Experiment 2

A Application of biogas digestate with rice straw on nitrate leaching potential during 35 day incubation periods

The experiment set up was same as 2.3.3.

a. Inorganic matter and pH measurement

This experiment was set up to evaluate periodic changes in inorganic nitrogen contents in soil. Soil samples supplemented with (i) to (vii) described above were mixed thoroughly with a spatula and each 5 g (60 °C oven dry basis) was dispensed into a 50 mL glass vial. The vials were covered with aluminum foil and incubated for 0, 7, 14 and 35 days at 27 °C to analyze ammonium nitrogen and nitrate nitrogen. Average temperatures in our summer period ranged from 25 to 30 °C, and thus, our incubator

was set up at 25 to 27 °C. Therefore, a total of 126 vials (3 replicates × 7 treatments × 6 sampling dates) were prepared for Kikugawa soils. The moisture levels of soil were maintained at 60% MWHC during the incubation period by adjusting with distilled water every week. Ammonium and nitrate (NO_3^--N) in the soils were analyzed using these vials, which were destructively collected, by extracting from 5 g (60 °C oven dry basis) soil with 25 mL (1:5 w/v) of 0.5 M K_2SO_4 solution. Extraction was performed by 1 h shaking at 120 rpm and by filtering through No. 5C filter paper (ADVANTEC Toyo Kaisha, Ltd.). Concentrations of NH_4^+ and NO_3^- in extracts were analyzed colorimetrically using the methods of Kandeler and Gerber (1988) and Cataldo et al. (1975), respectively. For measuring soil pH (H_2O), the experiment was separately prepared as described above. Five g (60 °C dry basis) of soil in each vial was mixed with 25 mL distilled water and shaken for 1 h and then pH was measured with an electrode (Metrohm AG).

B Application of biogas digestate with rice straw on nitrate leaching potential during 90 day incubation periods

This experiment was different from A and separate prepared before for investigating longer incubation periods. The experiment was conducted with two types of soil, one was Kikugawa nematode infested soil, and another one was Fuchu soil. This experiment was set up to evaluate periodic changes in inorganic nitrogen contents in soil. Soil samples supplemented with (i) to (vii) described above were mixed thoroughly with a spatula and each 5 g (60 °C oven dry basis) was dispensed into a 50 mL glass vial. The vials were covered with aluminum foil and incubated for 0, 7, 14, 35, 60, 90 days at

27 °C to analyze N immobilization and subsequent N mineralization rates Average temperatures in our summer period ranged from 25 to 30 °C, and thus, our incubator was set up at 25 to 27 °C. Therefore, a total of 252 vials (3 replicates × 7 treatments × 6 sampling dates × 2 types of soil) were prepared for the Fuchu and Kikugawa soils. The moisture levels of soil were maintained at 60% MWHC during the incubation period by adjusting with distilled water every week. Ammonium, nitrate (NO_3^--N), and extractable organic C (EOC) in the soils were analyzed using these vials, which were destructively collected, by extracting from 5 g (60 °C oven dry basis) soil with 25 mL (1:5 w/v) of 0.5 M K_2SO_4 solution. Extraction was performed by 1 h shaking at 120 rpm and by filtering through No. 5C filter paper (ADVANTEC Toyo Kaisha, Ltd.). Concentrations of NH_4^+ and NO_3^- in extracts were analyzed colorimetrically using the methods of Kandeler and Gerber (1988) and Cataldo et al. (1975), respectively. EOC was measured with a TOC-$V_{CSH/CSN}$. For measuring soil pH (H_2O), the experiment was separately prepared as described above. Five g (60 °C dry basis) of soil in each vial was mixed with 25 mL distilled water and shaken for 1 h and then pH was measured with an electrode (Metrohm AG). EOC and pH (H_2O) were only measured at day 0 of incubation as regulation factors for nitrification and nematode population.

Net N-mineralization (N_m) and net nitrate conversion (NC) were determined as the percentage of the added-N from the digestate that had been converted into inorganic N and nitrate, respectively, according to Alburquerque et al. (2012) as:

$$N_m(\%) = 100 \times \frac{\left[(\text{inorg-N}_{90\text{d}} - \text{inorg-N}_{0\text{d}})_{\text{soil+digestate}} - (\text{inorg-N}_{90\text{d}} - \text{inorg-N}_{0\text{d}})_{\text{soil}}\right]}{(\text{added total N})} \quad (1)$$

$$\mathrm{NC}(\%) = 100 \times \frac{\left[(\mathrm{NO_3\text{-}N}_{90d} - \mathrm{NO_3\text{-}N}_{0\mathrm{d}})_{\mathrm{soil+digestate}} - (\mathrm{NO_3\text{-}N}_{90d} - \mathrm{NO_3\text{-}N}_{0\mathrm{d}})_{\mathrm{soil}}\right]}{(\text{added total N})} \quad (2)$$

2.3.4 Statistical analysis

All results for inorganic nitrogen, EOC content, pH were obtained in triplicate and expressed as means and standard deviations. The effects of all fertilizer treatments and incubation time, as well as their interactions on NO_3-N and NH_4-N and nematode numbers, were tested with a two-way ANOVA followed by a Tukey HSD mean comparison ($p < 0.05$) using the software SPSS version 22.

2.4 Results

2.4.1 Experiment 1

2.4.1.1 Application of biogas digestate on tomato cultivation

The lower and higher level density experiment, both of them showed that parameters of SPAD, dry shoot weight and dry root weight were much higher than that of CONT treatment after 6 months cultivation period (Figure 2.1 & 2.4). Gall formation was not observed in digestate treatment and much lower than that of CONT in lower level density experiment (Figure 2.3). In higher level density experiment, gall formation was not observed in most dry digestate treatment and much lower than that of CONT, gall formation of wet digestate was also lower than that of CONT (Figure 2.5). The application of dry biogas digestate can effectively suppress gall formation and help plants grow well, especially when the soil was not highly infested with root knot nematodes.

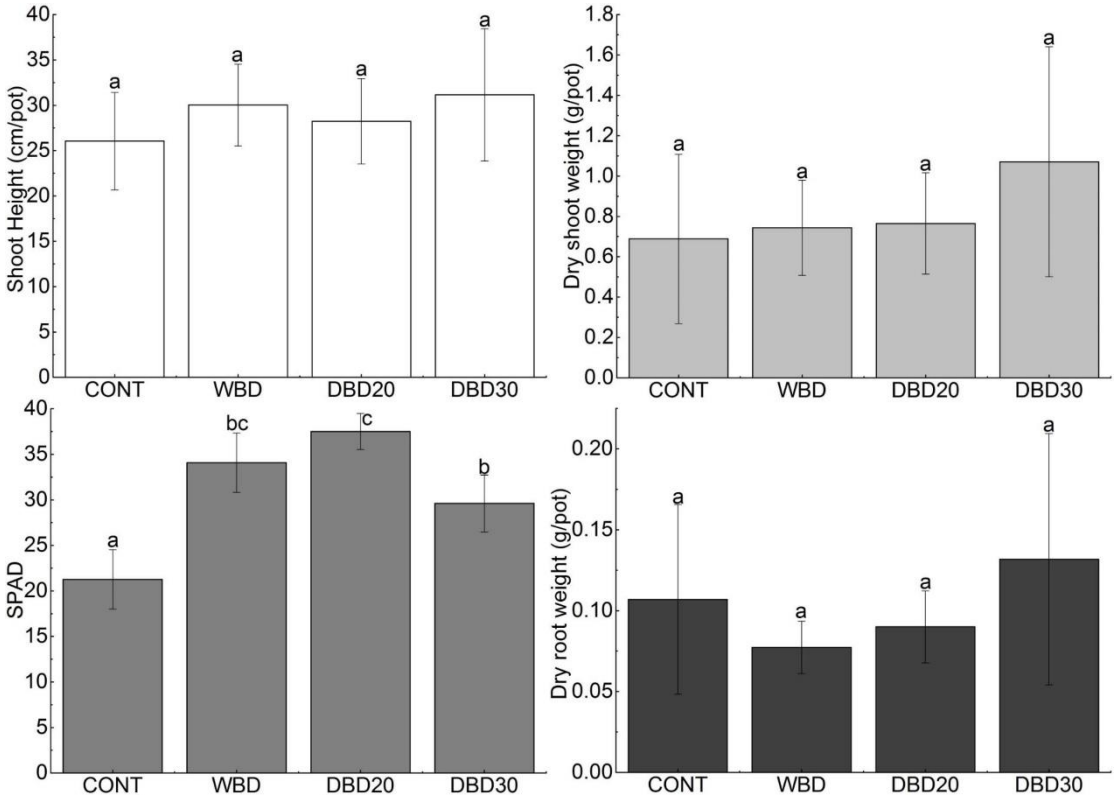


Figure 2.1 Effect of wet and dry biogas digestate application on tomato cultivation in lower density root knot nematode infested soil during 6 months periods. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30. The values are the means ± SD. Bars represent standard deviations (n = 3).

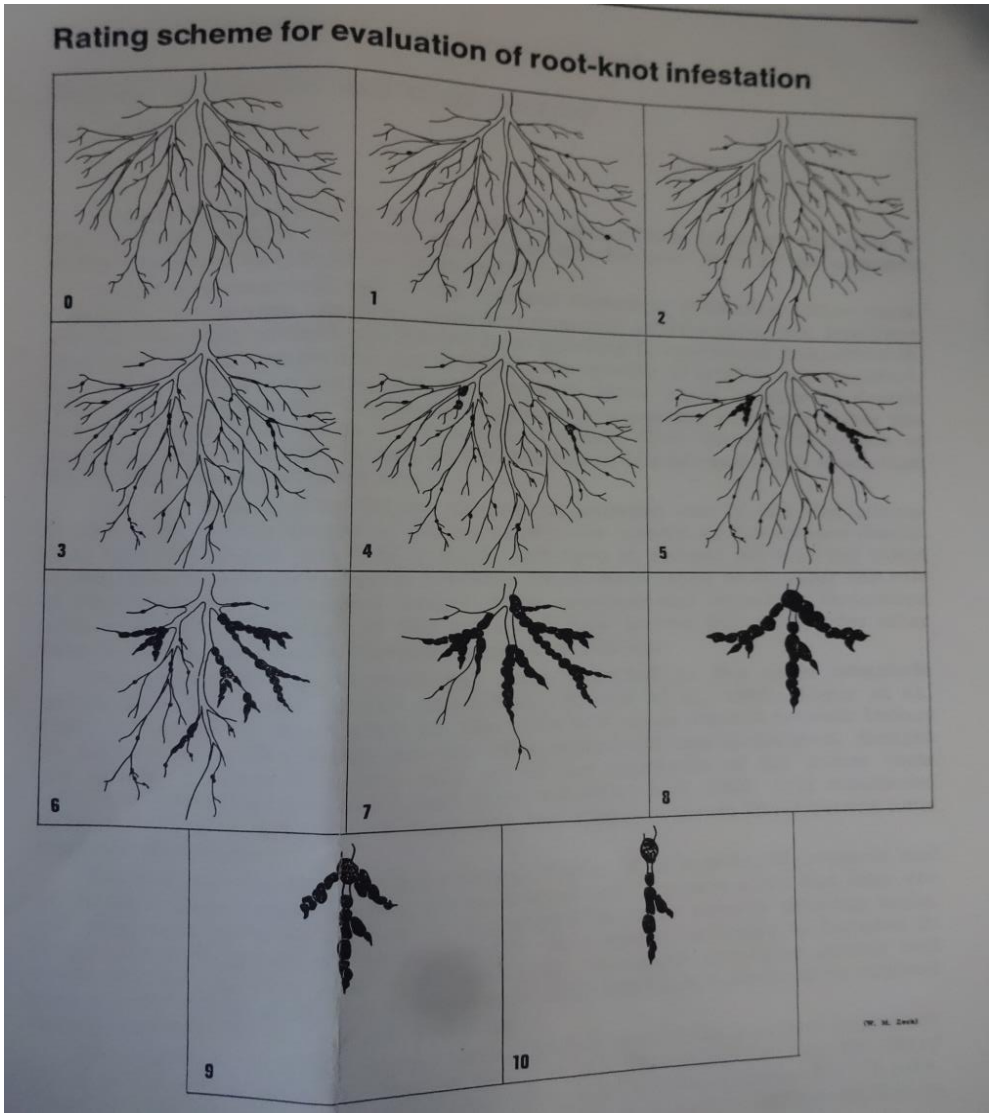


Fig. 2.2 Rating scheme for evaluation of root-knot nematode infestation according to (Zeck, 1971). Gall index was evaluated on a 0 to 10 scale, 0 = no galls, 1 = very few small galls, 2 = numerous small galls, 3 = numerous small galls of which some are grown together, 4 = numerous small and some big galls, 5 = 25% of roots severely galled, 6 = 50% of roots severely galled, 7 = 75% of roots severely galled, 8 = no healthy roots but plant is still green, 9 = roots rotting and plant dying, 10 = plant and roots dead. Values are means (n = 3) ± standard deviation. Different letters indicate significant difference ($p < 0.05$).

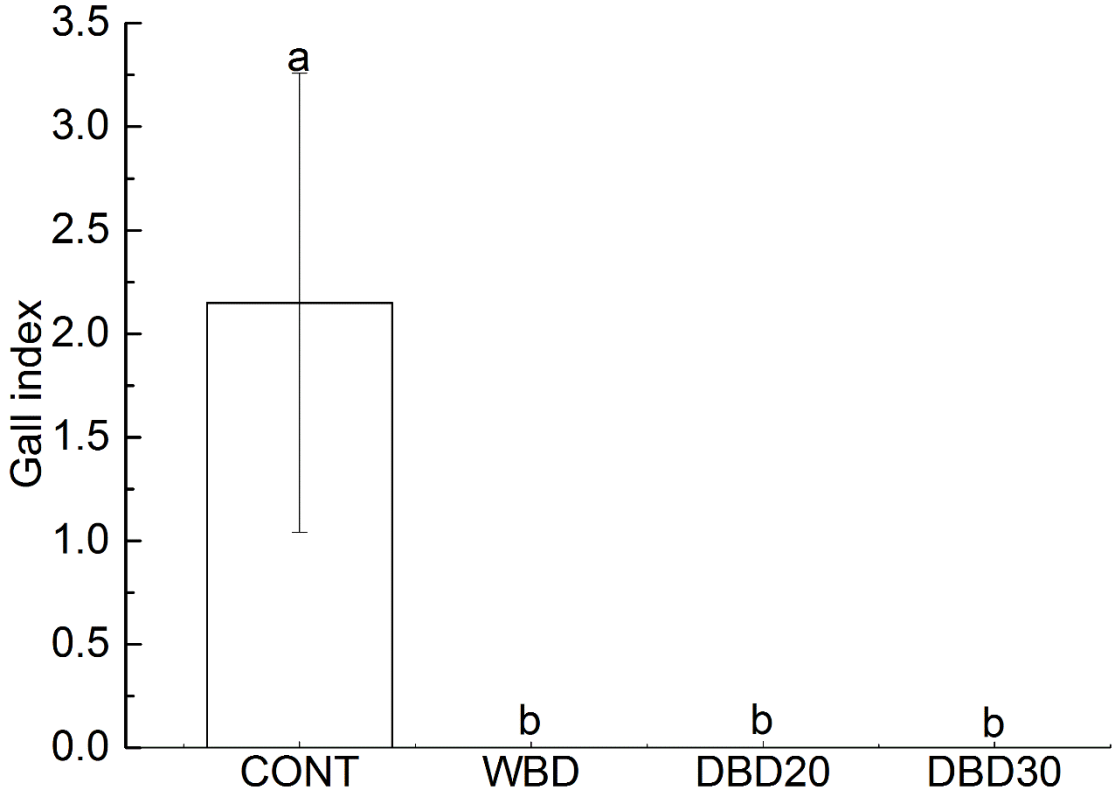


Figure 2.3 Root gall index after wet and dry biogas digestate application in lower density root knot nematode infested soil. The values are the means ± SD. Bars represent standard deviations (n = 3).

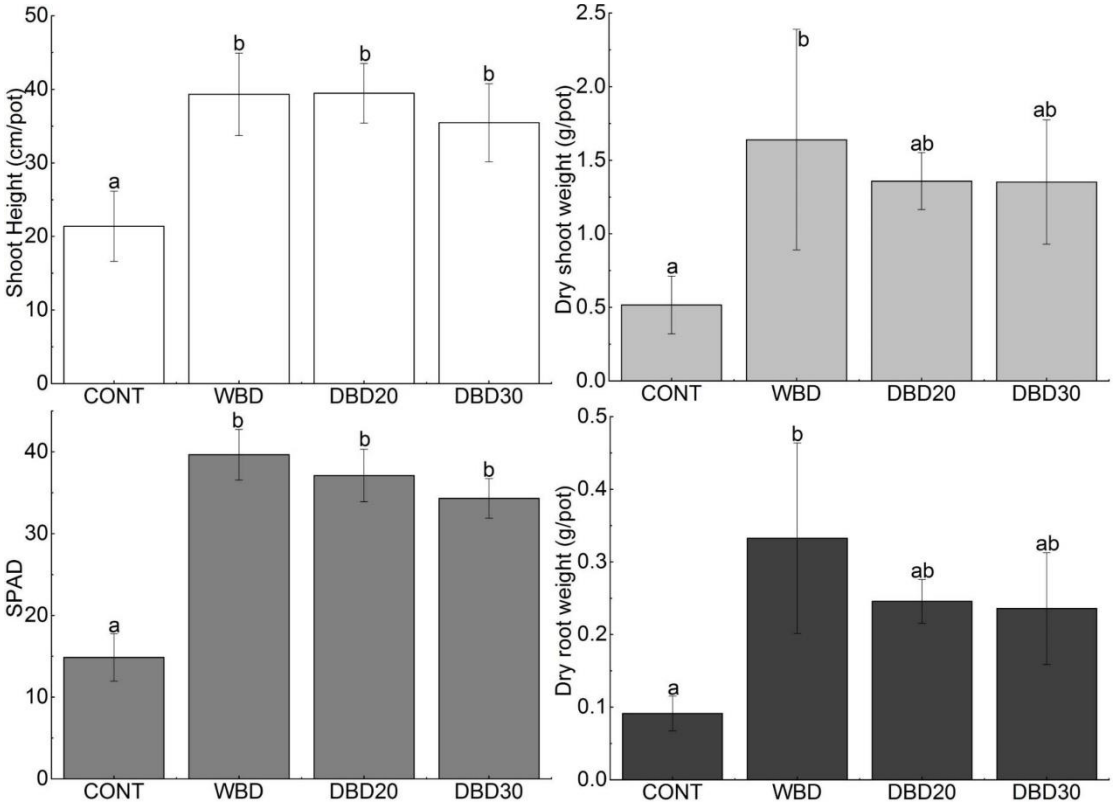


Figure 2.4 Effect of wet and dry biogas digestate application on tomato cultivation in higher density root knot nematode infested soil. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30. The values are the means ± SD. Bars represent standard deviations (n = 3).

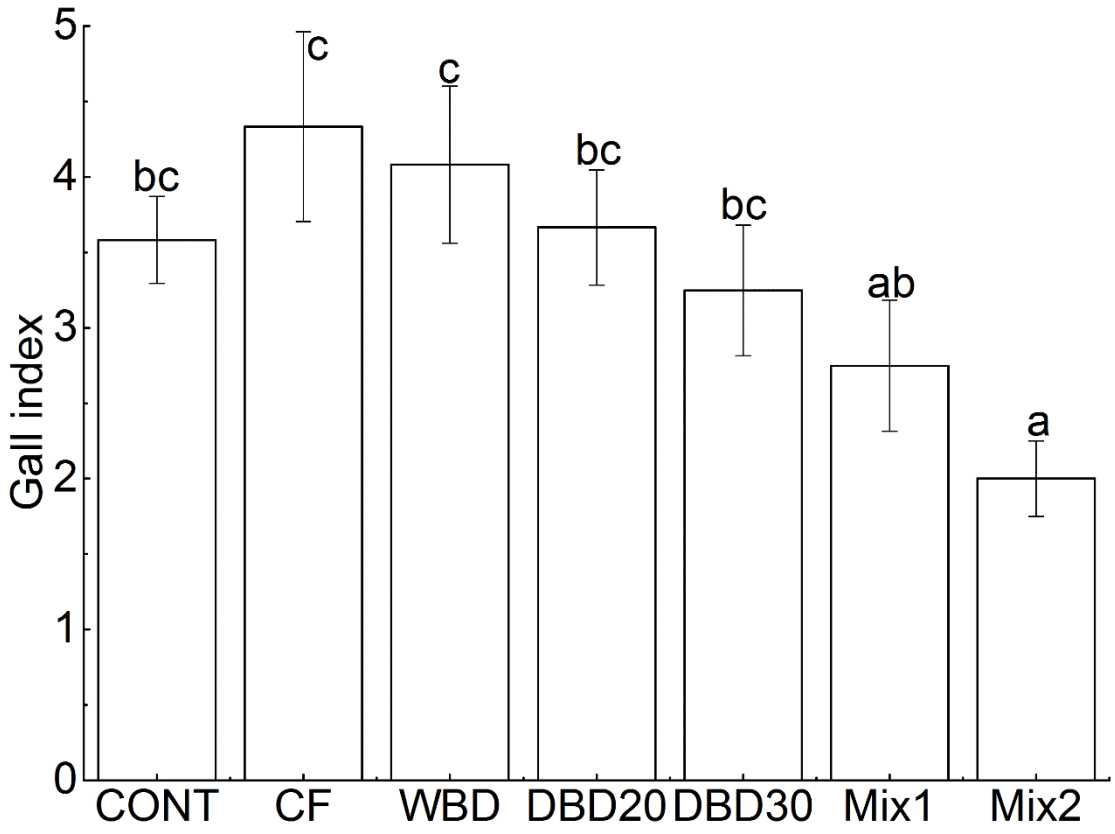


Figure 2.5 Root gall index after wet and dry biogas digestate application in higher density root knot nematode infested soil. The values are the means ± SD. Bars represent standard deviations (n = 3).

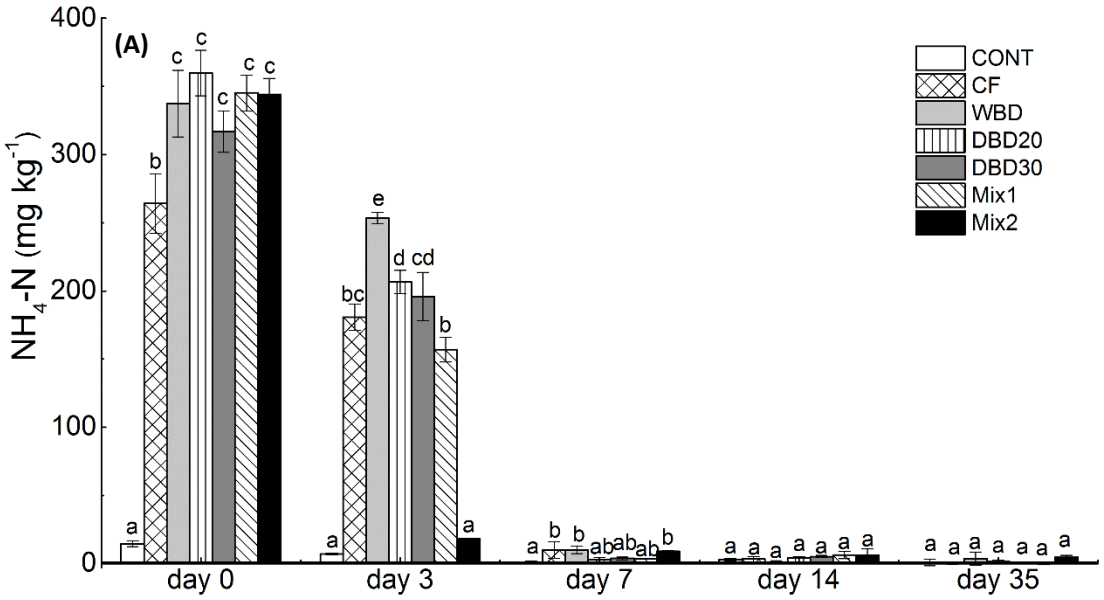
2.4.2 Experiment 2

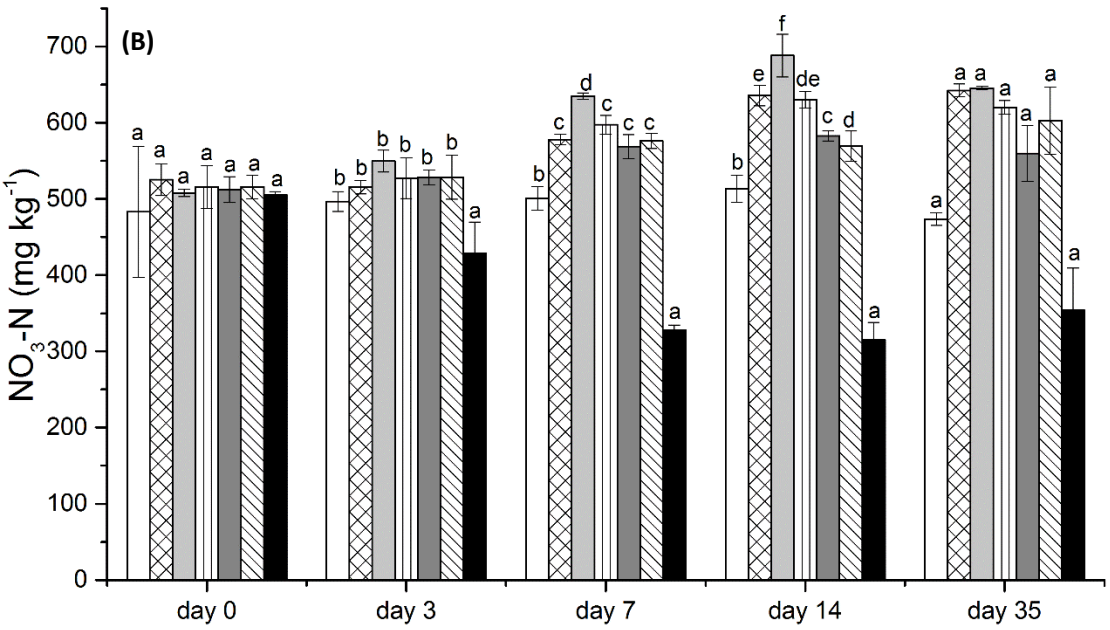
2.4.2.1 Application of biogas digestate with rice straw on soil inorganic matter and pH during 35 incubation periods

At 3 days of incubation, all fertilizer treatments were still higher than 150 mg NH_4–N kg^{-1} and showed significant difference (P<0.05) with CONT except Mix2 treatment, there was no significant difference (P<0.05) between CONT and Mix2 (Figure 2.6). At 7 days of incubation, the NH_4–N concentration decreased to less than 10 mg NH_4–N kg^{-1} soil in most of the treatments (Figure 2.6). At 35 days of incubation, the NH_4–N concentration was markedly low in all treatments (1 to 2 mg N kg^{-1} soil).

In CONT, NO_3-N concentration of 0 day was still remain the same after 35 days incubation period (Figure 2.6). NO_3-N concentration of all fertilizer treatment were increase by 50-180 mg N kg^{-1} soil expect for Mix2 treatment (Figure 2.6). NO_3-N concentration of Mix2 treatment constantly decreased from 0 day (506 mg N kg^{-1} soil) to 14 day (315 mg N kg^{-1} soil) incubation period, and increase to 354 mg N kg^{-1} soil on 35 day incubation period.

The pH (H_2O) values were not markedly different among the treatments and incubation periods during 14 days incubation period (Figure 2.6). pH of all fertilizer treatment were increase on 0 day. And on 35 days incubation period, pH of all fertilizer treatment were showed significant difference with CONT expect Mix2 treatment.


(A)
CONT
CF
WBD
DBD20
DBD30
Mix1
Mix2
NH4-N (mg kg-1)
400
300
200
100
0
day 0
day 3
day 7
day 14
day 35


(B)
NO3-N (mg kg-1)
700
600
500
400
300
200
100
0
day 0
day 3
day 7
day 14
day 35

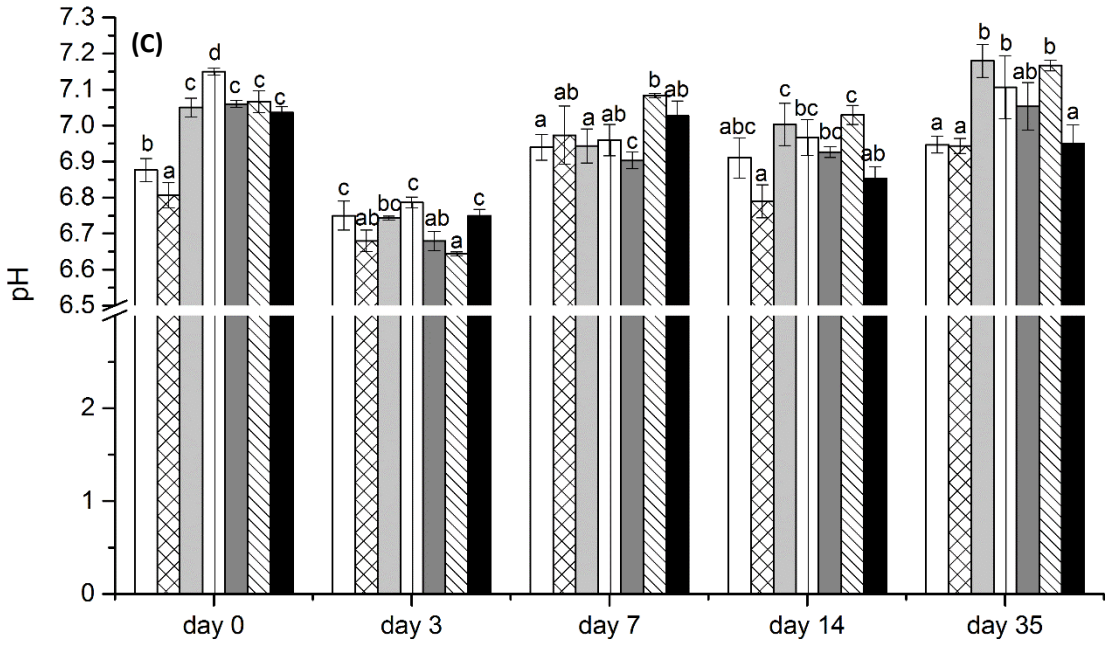


Figure 2.6 K_2SO_4–extractable NH_4-N (A), NO_3-N (B) and pH (C) of Kikugawa soil after 0, 3, 7, 14 and 35 days of incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Different letters within the same incubation times indicate significant differences at $p < 0.05$.

2.4.2.2 Effect of fertilization on the dynamics of inorganic N during 90 days incubation periods

Ammonium concentrations of Kikugawa and Fuchu soil at day 0 were 29 and 44 mg NH_4–N kg^{-1} soil and increased to 159–244 and 216–267 in the amended treatments, respectively, which nearly corresponded to the application rate (200 mg NH_4–N kg^{-1} soil). With incubation, NH_4–N concentration markedly decreased to less than 20 mg NH_4–N kg^{-1} soil at day 7 of incubation in all of the treatments in Kikugawa soil, indicating the ready occurrence of nitrification (Figure 2.7). In Fuchu soil, it was still higher than 20 mg NH_4–N kg^{-1} soil in CF, WBD, DBD20, and DBD30 at day 7, while it was less than 10 mg NH_4–N kg^{-1} soil in Mix1 and Mix2 (Figure 2.7). After day 35, the NH_4–N concentration was consistently lower than 10 mg NH_4–N kg^{-1} soil in both soils. Throughout day 35 to day 90 of incubation period, there were no significant ($p < 0.05$) differences between CONT and the other amended treatments in Fuchu soil, and also no significant ($p < 0.05$) differences between CONT and CF, DBD20, DBD30 and Mix1 treatments in Kikugawa soil.

Nitrate concentration in CONT increased by 466 and 141 mg N kg^{-1} soil at day 90, in Kikugawa soil and Fuchu soil, respectively (Figure 2.8). This result indicated that N_m was higher in Kikugawa soil than in Fuchu soil. At day 7 of incubation, NO_3-N was significantly ($p < 0.05$) higher in WBD, DBD20 and Mix1 treatment, followed by CF and DBD30 treatment in Fuchu soil, indicating that the net nitrification rate was highest in the WBD, DBD20 and Mix1 treatments. Nitrate concentration was significantly ($p < 0.05$) lower in the Mix2 treatment in both soils than in other treatments throughout the 90 days. The final NO_3-N concentration in Mix2 was at the same level as the control in

both soils, indicating that most of the N added to Mix2 was microbially immobilized even at day 90. In comparing the wet and dry digestate, NO_3-N concentration was significantly ($p < 0.05$) lower in DBD30 than in WBD in Fuchu soil from day 14 to day 90, and similar tendencies were observed in Kikugawa soil.

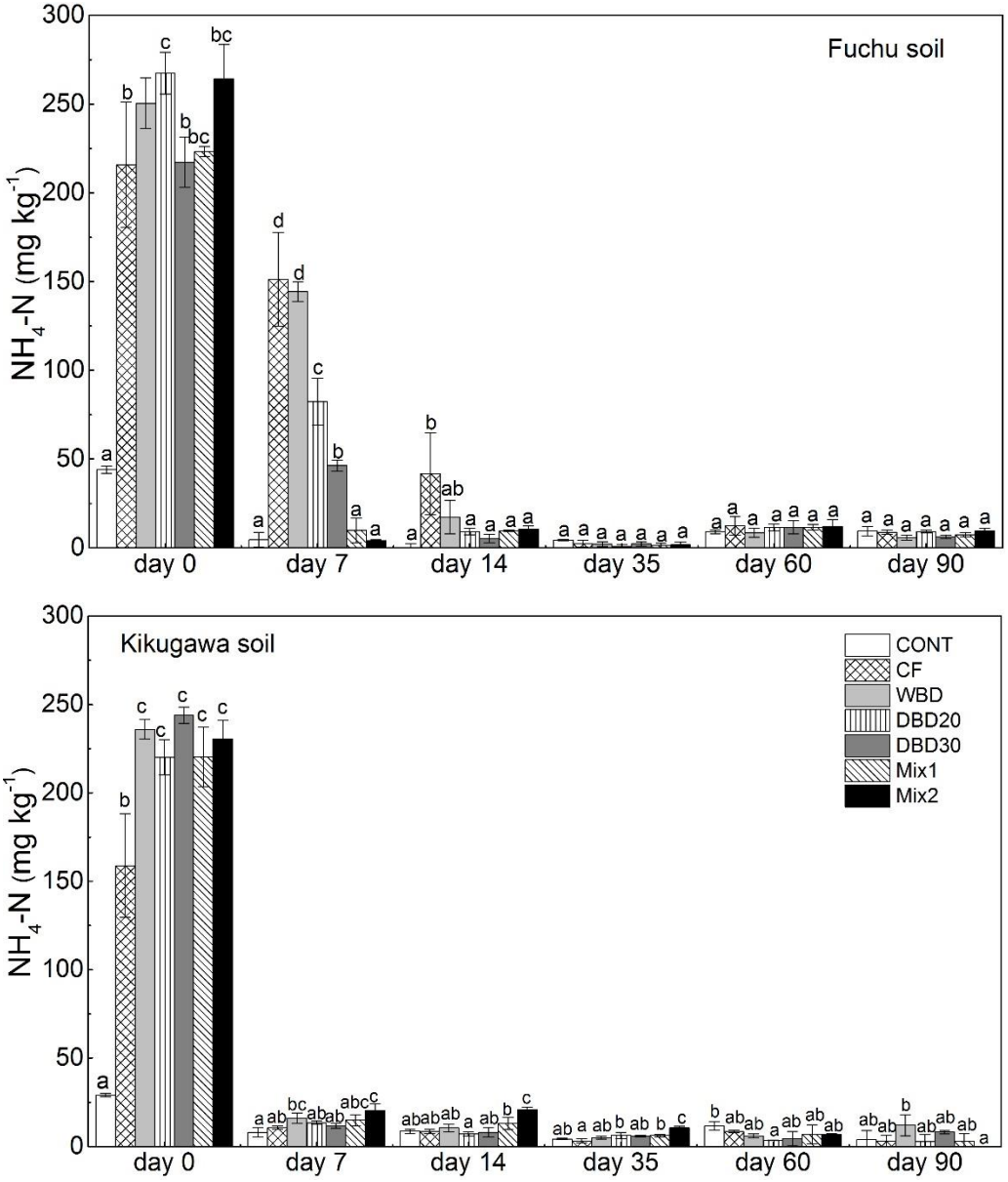


Figure 2.7 K_2SO_4–extractable NH_4-N in Kikugawa and Fuchu soils after 0, 7, 14, 35, 60 and 90 days of incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Different letters within the same incubation times indicate significant differences at $p < 0.05$.

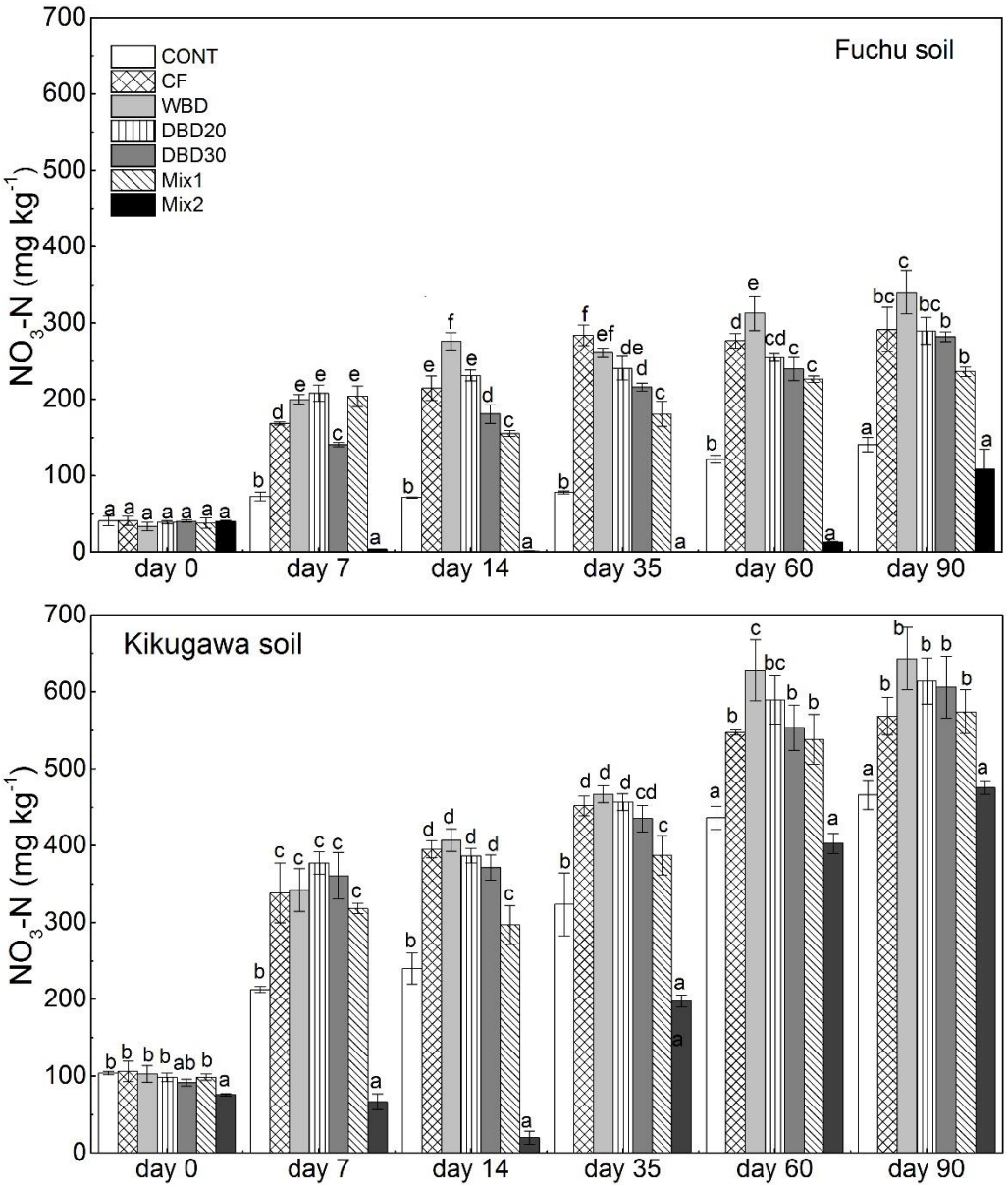


Figure 2.8 K_2SO_4–extractable NO_3-N in Kikugawa and Fuchu soils after 0, 7, 14, 35, 60 and 90 days of incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Different letters within the same incubation times indicate significant differences at $p < 0.05$.

N_m was the lowest in Mix2 (−34% and −53%) in Kikugawa and Fuchu soil, and lower in DBD30 (−15% and −8%) and Mix1 (−23% and −24%) than in WBD (−14% and −1%) in Kikugawa and Fuchu soil, respectively. While N_m was −12% in DBD20 in Kikugawa soil, it decreased to −23% in Mix1 to which 4 mg rice straw g^{-1} of soil was added to DBD20.

NC (8% and −7%) was the lowest in Mix2 in Kikugawa and Fuchu soils, respectively. NC was lower in Mix1 (33% and 29%) than in DBD20 (48% and 47%) in Kikugawa and Fuchu soil, respectively.

2.4.2.3 Factors affecting the nitrate leaching potential

The pH (H_2O) values were significantly ($p < 0.05$) higher in soils treated with wet and dry digestate than in CONT and CF at day 0 for both soils, except for Mix2 for Kikugawa soil (Table 2.2). There was no significant relationship between initial soil pH and NO_3-N at day 7, indicating that soil pH did not affect initial nitrification rates.

Extractable organic C (EOC) content at day 0 in both soils was significantly ($p < 0.05$) higher in treatments with dry digestate than in CONT and CF, except for DBD20 in Fuchu soil (Table 2.2). EOC content at day 0 was highest in Mix2 among all treatments in both soils.

There were significant relationships between the N_m ($R^2 = 0.593$, $p < 0.05$ in Kikugawa soil and $R^2 = 0.678$, $p < 0.05$ in Fuchu soil, Figure 2.9 A) or NC ($R^2 = 0.750$, $p < 0.05$ in Kikugawa soil and $R^2 = 0.762$, $p < 0.05$ in Fuchu soil, Figure 2.9 B) and EOC at day 0. There was a significant positive relationship between the N_m and NC ($R^2 = 0.632$, $p < 0.05$ in Kikugawa soil and $R^2 = 0.683$, $p < 0.05$ in Fuchu soil, Figure 2.9 C).

Table 2.2 Changes of soil pH and K_2SO_4–extractable organic C (EOC) content in Kikugawa and Fuchu soils after different fertilizer amendments on day 0.

Treatment	Kikugawa Soil		Fuchu Soil	
	pH (H_2O)	EOC (mg kg^{-1})	pH (H_2O)	EOC (mg kg^{-1})
CONT	6.6 ± 0.1ab	507 ± 18a	5.8 ± 0.0b	139 ± 11a
CF	6.5 ± 0.0a	502 ± 24a	5.6 ± 0.1a	199 ± 36ab
WBD	6.8 ± 0.0c	537 ± 9ab	6.5 ± 0.1e	182 ± 13a
DBD20	6.9 ± 0.1d	609 ± 27b	6.6 ± 0.0f	254 ± 10bc
DBD30	6.9 ± 0.0d	766 ± 24c	6.8 ± 0.0g	346 ± 14d
Mix1	6.9 ± 0.0d	704 ± 38c	6.3 ± 0.1d	267 ± 3c
Mix2	6.6 ± 0.0b	927 ± 59d	6.2 ± 0.1c	604 ± 39e

Values are means (n = 3) ± standard deviations. Different letters indicate significant difference ($p < 0.05$).

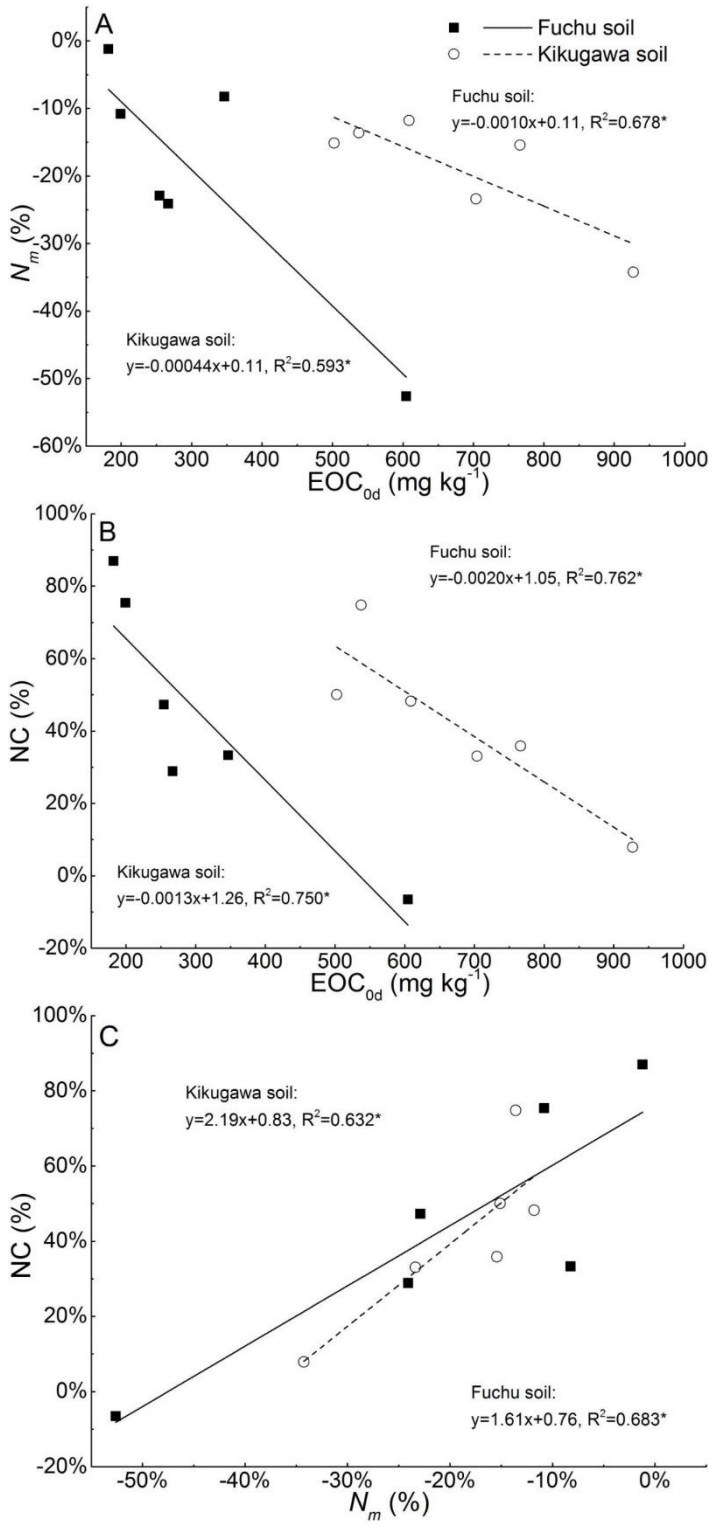


Figure 2.9 The relationships between N_m (A) or NC (B) and the differences in EOC at day 0, and between N_m and NC (C) in Fuchu soil and Kikugawa soil. N_m: net N-mineralization, NC: net nitrate conversion (%), EOC: extractable organic carbon. * $p <$ 0.05.

2.4.2.4 Effect of fertilization on extractable organic C (EOC) and total N (ETN)

Extractable organic C (EOC) at 0 day in both soils was significantly higher in treatments with dry digstate than in CONT, CF and WBD. Due to the higher rice straw amendment, EOC at 0 day were two times higher in Mix2 in both soils than in the other treatments (Figure 3.0). EOC rapidly decreased following the application of both digestate (Figure 3.0). In Mix2, EOC in both soils markedly decreased at 7 days of incubation, and remained unchanged from 14 days to 35 days of incubation but it was still the highest than in the other treatments (Figure 3.0).

Wet and dry digestate applications resulted in increases in extractable total nitrogen (ETN) in both soils, and the amounts of ETN were almost similar to those of NO_3-N from 14 to 35 days of incubation (Figure 3.1), indicating that ETN mainly consists of NO_3-N and that the contribution of NH_4-N and labile organic N to ETN was negligible after 14 days of incubation.

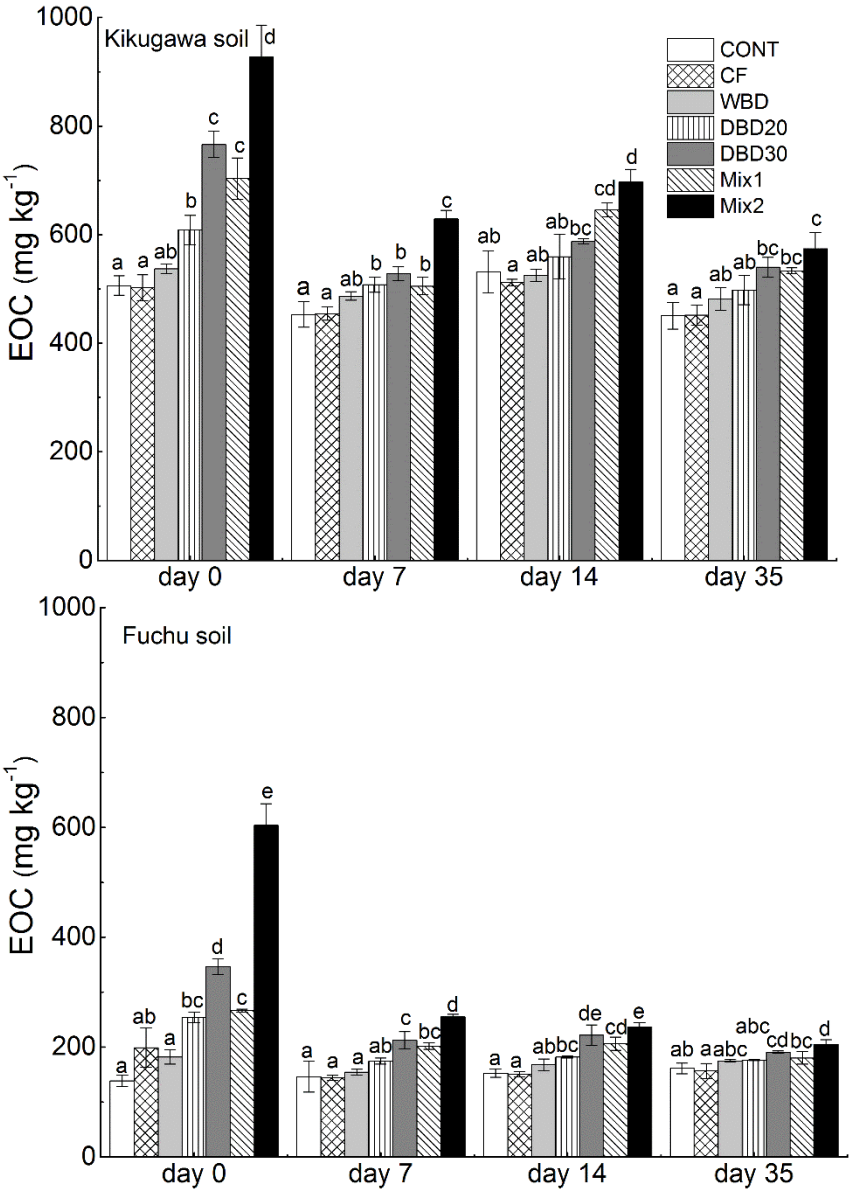


Figure 3.0 K_2SO_4–extractable organic C (EOC) in Kikugawa and Fuchu soils after 0, 7, 14, and 35 days of incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Different letters within the same incubation times indicate significant differences at $p < 0.05$

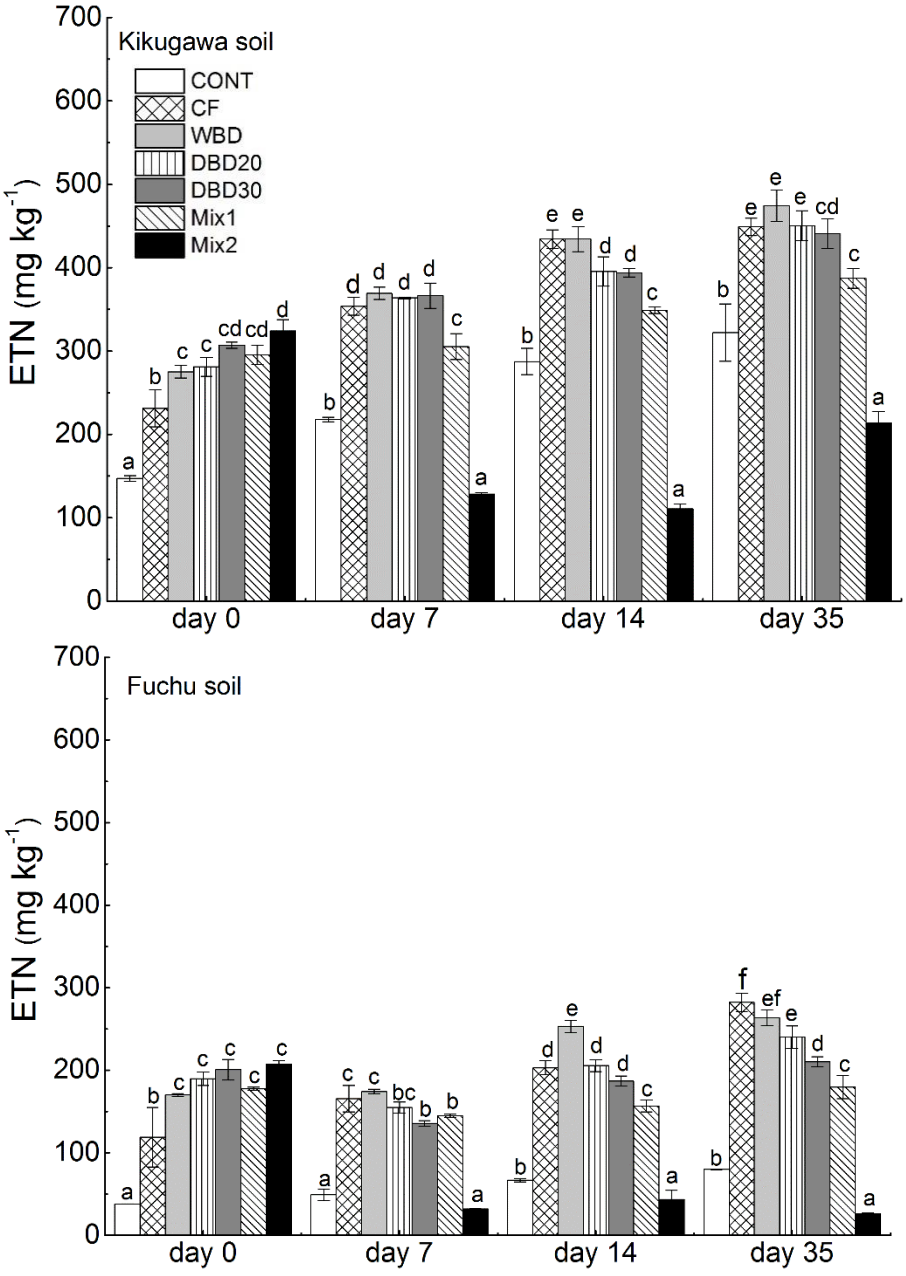


Figure 3.1 K_2SO_4–extractable total N (ETN) in Kikugawa and Fuchu soils after 0, 7, 14, and 35 days of incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Different letters within the same incubation times indicate significant differences at $p < 0.05$

2.5 Discussion

While net nitrification occurred in all the treatments in both soils (Figure 2.6 & 2.8), nitrate concentration was quite variable depending on the treatment; nitrate concentration was consistently lower in Mix2 and lower at day 14 and day 35 in Mix1 than in CF. Soil pH is usually a major factor affecting soil nitrification (Paul and Clark, 1989), but there was no relationship between the initial nitrification rates and initial soil pH values in this chapter, unlike the study by Sawada and Toyota (2015) in which biogas digestate application stimulated net nitrification by increasing soil pH. The reason that net nitrification was retarded in Mix1 and Mix2 was considered to be due to higher N immobilization rates than nitrification rates, because NC (%) decreased with higher carbon (Figure 2.9). This phenomenon is the occurrence of N starvation due to microbial immobilization of N and has been already reported by many studies (Cai et al., 2018; Ma et al., 2019; Reichel et al., 2018; Zhao et al., 2018). The size and C/N ratio of the easily degradable organic fraction of residues have critical roles in regulating N dynamics in soils (Galvez et al., 2012). According to Cheng et al. (Cheng et al., 2017), inputs of simple organic C more than 500 mg C kg^{-1} or complex organic C, such as plant residue, with C/N ratios of more than 18 induce net N immobilization. In my study, the C/N ratio in Mix2 was 30 and EOC, considered as a simple organic C, was 400 mg C kg^{-1} in Mix2, therefore, net N immobilization may occur in Mix2. This is also supported by Harmsen and Van Schreven (1955) and Alexander (1961), who concluded that the incorporation of crop residues with C/N ratios of 20–25 and 20–30, respectively, consistently produced net N-mineralization, whereas net immobilization

occurred in crop residues with C/N ratios higher than those values. The large negative values of N_m in Mix2 in both soils (−34% and −53% in Kikugawa and Fuchu soils, respectively) supported the negative net mineralization rates. Collectively, a part of the total N contained in the digestate samples and in the original soil may be immobilized by soil microbes growing using the labile C in rice straw. Indeed, Mix2 showed the highest EOC deriving from a large amount of rice straw (Table 2.2).

Alburquerque et al. (2012) reported digestate with a higher C/N ratio (18.5) did not induce net nitrate conversion (NC = −29%) while digestate with a lower C/N ratio (1.5) induced net nitrate conversion (NC = 84%) when they were mixed with soil and incubated. In our study, the C/N ratio (12) of DBD20 was increased to 16 in Mix1 by adding rice straw and NC decreased from 47% and 48% in DBD20 (Kikugawa and Fuchu soils) to 29% and 33% in Mix1, respectively. These results suggest that increasing the C/N ratio of biogas digestate by 4 stimulated N immobilization, and that the application of dry biogas digestate together with rice straw would be an appropriate strategy to mitigate the nitrate leaching potential.

In Kikugawa soil, the markedly low NO_3-N in Mix2 at day 14 of incubation increased from day 35 to day 90 of incubation, possibly due to the mineralization of the once immobilized N and soil organic N. In contrast, in Fuchu soil, NO_3-N started to increase only after day 60 of incubation, indicating that microbial immobilization consistently dominated the nitrogen cycling process for the first 60 days (Figure 2.8). The period of N retention and N supply processes differ among soils (Yadvinder et al., 2009). According to Zhao et al. (2018), N retention was much longer in a soil with

lower pH (5.3) than in a soil with neutral pH (7.6). This supports our results that N retention was much longer in Fuchu soil (pH = 5.7) than in Kikugawa soil (pH = 7.0). In addition, higher soil fertility in Kikugawa soil (total C: 73.2 g kg^{-1} soil) than in Fuchu soil (total C: 35 g C kg^{-1} soil) may have caused a higher mineralization rate and shorter N retention, that is, the earlier change from N immobilization to N mineralization. This hypothesis is supported by Pan et al. (2017) who, using three long-term different fertilized soils, reported that N mineralization starts earlier in a fertile soil after the occurrence of N immobilization.

Both types of digestates increased soil extractable C (EOC) at 0 day of incubation (Fig. 3.0), because they had water soluble C (Table 2.2). Mix2, in which a large amount of rice straw was added to soil, showed the highest EOC (Fig. 3.0). Since EOC represents the labile fraction of organic C (Galvez et al. 2012), the rapid decline observed in EOC in Mix2 at 7 days of incubation may have been due to its consumption by microbes, during which NO_3-N in soil was immobilized into the soil microbial biomass N.

Chapter 3 Effects of biogas digestate mixed with rice straw on root-knot nematode (*Meloidogyne incognita*)

3.1 Abstract

The objectives of this study was to evaluate its nematicidal activity on root-knot nematodes (RKN) *Meloidogyne incognita* in the application of biogas digestate to soil by co-application of rice straw. This study consisted of the following seven treatments, same with chapter 2. The application rate of CF and digestates was adjusted to 200 mg N kg^{-1} soil based on their inorganic nitrogen contents. Results of RKN densities showed that the application of dry biogas digestate, in particular Mix2, reduced the RKN density in soil, compared with CF. Furthermore, garden balsam was grown as a test plant for RKN using the soils after 90 days of incubation and the results showed that gall index was significantly lower in Mix2, Mix1, and DBD30 than in CF. There was a significant negative relationship between root-knot nematode population at day 90 and extractable organic carbon (EOC) at day 0. These results suggest that dry digestate mixed with rice straw might have a nematicidal property.

3.2 Introduction

Root-knot nematodes (RKN), *Meloidogyne* spp., are a major pest and cause significant losses in the yields or quality of crops (Kepenekci et al., 2018, Rudolph et al., 2015). Current management strategies are the use of chemical nematicides, organic amendments, resistant cultivars, soil solarization and biological control (Ntalli and Caboni, 2017, Xiang et al., 2018). Although chemical nematicides are frequently used, public demand for safer agricultural practices creates the need to discover alternative methods of root-knot nematode management. Previous studies have revealed that the application of biogas digestate reduced the severity of damage to tomato by root-knot nematodes (Jothi et al., 2003) and to sugar beet by *Heterodera schachtii* (Westphal et al., 2016). Several toxic compounds are involved in nematode suppressive properties, such as ammonia (Min, 2007, Renčo et al., 2010), fatty acids (Sayre et al., 1965), chitin, release of plant-specific toxins etc. (Mcsorley, 2011). The toxin contents of biogas digestates may vary due to the original materials, and thus, it can be difficult to generalize the performance and effects of digestates on nematodes. In addition, there are no papers available on the suppressive effects of dry biogas digestate on plant parasitic nematodes, although wet biogas digestate has been studied (Jothi et al., 2003, Min, 2007, Westphal et al., 2016). I assumed that dry digestate might have a suppressive effect on plant parasitic nematodes and the combined application of straw with digestate might enhance the suppressive effect through increasing labile C.

The objective of this chapter was to investigate the application of dry biogas digestate with rice straw can suppress root knot nematodes due to the increase in labile

C through mixing with rice straw.

3.3 Materials and Methods

3.3.1 Biogas Digestates and Rice Straw

Two types of digestates, i.e., wet and dry biogas digestates, were used. More details of rice straw and digestate were described same as chapter 2.

3.3.2 Soils

Kikugawa soil was a culture soil (an Andosol amended with compost) naturally infested with the root-knot nematode *Meloidogyne incognita* and collected from a tomato farm in Kikugawa city, Shizuoka Prefecture, Japan. Soil was taken from the plow layer (ca. 20 cm). Freshly collected soil samples were sieved to 5 mm and stored at field moisture (fresh soil moisture content was 28% a in Kikugawa) at 5 °C until use. Subsamples of the soils were air-dried and then analyzed for the physicochemical properties. The main characteristics for Kikugawa soil were: 73.2 g C kg^{-1} soil of total C, 7.0 g N kg^{-1} soil of total N, pH (H_2O) 7.0, maximum water holding capacity (MWHC) 1.29 g g^{-1}, and texture; sandy loam (16% clay, 18% silt and 66% sand).

3.3.3 Experimental Setup

The following seven treatments were prepared: (i) control (no addition of biogas digestate and chemical fertilizer, CONT), (ii) compound chemical fertilizer (N:P:K = 8:8:8, Asahi Industries, Tokyo, Japan) (CF), (iii) wet biogas digestate (WBD), (iv) dry biogas digestate adjusted to its original C/N ratio of 20 and then dry fermented (C/N ratio = 12) (DBD20), (v) dry biogas digestate adjusted to its original C/N ratio of 30 and then dry fermented (C/N ratio = 16) (DBD30), (vi) DBD20 mixed with a low

amount of rice straw to adjust the C/N ratio to 16 (DBD20:rice straw = 1:0.06) (Mix1), (vii) DBD20 mixed with a higher amount of rice straw to adjust the C/N ratio to 30 (DBD20:rice straw = 1:0.4) (Mix2). Their application rates were adjusted to 200 mg ammonium (NH_4^+-N) kg^{-1} dry soil (equivalent to ~300 kg NH_4-N ha^{-1}) except for CONT, since this rate is commonly used for tomato cultivation. The N contained in rice straw was not considered in this study, because the amounts (0.003 and 0.019 mg N kg^{-1} dry soil in Mix1 and Mix2, respectively) were low compared with the N contained in the digestate. Since N is the main yield-limiting factor, the application rate was determined based on the amounts of the NH_4^+-N fraction in the digestates (Möller and Müller, 2012). The actual added amounts of WBD, DBD20, DBD30, Mix1 and Mix2 were 48, 74, 125, 74 and 74 mg g^{-1} dry soil (equivalent to 72, 111, 187, 111, and 111 Mg ha^{-1}), respectively. In Mix1 and Mix2, rice straw was added at rates of 4.4 and 29.7 g kg^{-1} dry soil (equivalent to 6.6 and 44.6 Mg ha^{-1}), respectively.

3.3.3.1 Experiment 1

A. Application of biogas digestate with rice straw on root knot nematode

The effect of biogas digestate with rice straw on root knot nematode was conducted with two types of soil, one was nematode infested soil, and another one was Fuchu soil inoculated with eggs and J2. The following seven treatments were prepared: (i) control (no addition of biogas digestate and chemical fertilizer, CONT), (ii) compound chemical fertilizer (N:P:K = 8:8:8, Asahi Industries, Tokyo, Japan) (CF), (iii) wet biogas digestate (WBD), (iv) dry biogas digestate adjusted to its original C/N ratio of 20 and then dry fermented (C/N ratio = 12) (DBD20), (v) dry biogas digestate adjusted

to its original C/N ratio of 30 and then dry fermented (C/N ratio = 16) (DBD30), (vi) DBD20 mixed with a low amount of rice straw to adjust the C/N ratio to 16 (DBD20:rice straw = 1:0.06) (Mix1), (vii) DBD20 mixed with a higher amount of rice straw to adjust the C/N ratio to 30 (DBD20:rice straw = 1:0.4) (Mix2). Their application rates were adjusted to 200 mg ammonium (NH_4^+-N) kg^{-1} dry soil (equivalent to ~300 kg NH_4-N ha^{-1}) except for CONT, since this rate is commonly used for tomato cultivation. The actual added amounts of WBD, DBD20, DBD30, Mix1 and Mix2 were 48, 74, 125, 74 and 74 mg g^{-1} dry soil (equivalent to 72, 111, 187, 111, and 111 Mg ha^{-1}), respectively. In Mix1 and Mix2, rice straw was added at rates of 4.4 and 29.7 g kg^{-1} dry soil (equivalent to 6.6 and 44.6 Mg ha^{-1}), respectively.

100 g of soil (oven dry basis) was put in triplicate into 500 mL plastic bottles and treated with (i) to (vii), as described above. Each bottle of Fuchu soil was inoculated by pouring 2000 eggs in 10 mL of sterile distilled water and mixed thoroughly with a spatula. Eggs extraction method was followed below. The water content was adjusted to 60% of MWHC after addition of organic residues, including water contents in WBD, DBD20 and DBD30. A total of 42 (7 treatments × 3 replicates × 2 types soil) bottles were used. During the incubation period, the lid of each bottle was loosely closed to minimize water evaporation and to allow gas exchange. Every 7 days, the bottles were weighed, and moisture losses were replaced with deionized water to adjust to 60% of MWHC. Incubation was performed at 27 °C for 90 days. At 30, 60 and 90 days later, soils were mixed thoroughly with a spatula and then 10 g (oven dry basis at 60 °C) was taken from each bottle for DNA extraction (method as describes above).

a. Nematode inoculum production

To prepare the nematode inoculums, a root knot nematodes infested soil was collected from Kikugawa, Shizuoka prefecture, Japan. The infested soil was mixed with a commercially available sub-surface soil (Akadama) at a rate of 1:9, and the mixed soil was used to cultivate tomato plants. Pre-germinated tomato seeds (cv. Fukuju) prepared as above were cultivated in a vinyl pot (9 cm in diameter, 7.5 cm in height) containing 100 g of mixed soil in a biotron (LPH 200, NK System) (12 hr day and 12 hr night conditions) at temperature conditions (25°C). Watering was done daily using tap water. After one month of growth, the infected plants were gently removed from each pot and the entire root systems were carefully rinsed in tap water to remove the soil particles for collecting eggs and J2 of the nematode. The soil collected at this stage was mixed with the sub-surface soil at rate of 1:10, and the mixed soil was used to grow tomato for nematode production. For better growth of the tomato plants, compost was autoclaved at 121°C for 15 mins, and then mixed with the mixture soil at rate of 2%. These processes were continuously repeated every 2 or 3 months to keep the nematodes in plants.

Before extract root knot nematode from infected tomato roots, wipe the work area thoroughly with 75% ethanol to avoid contamination. Also wash in hot water a blender, two sieves of 106 and 20 µm aperture and scissors. The root were cut into 1 cm long pieces and shake with 1% NaOCl solution for 2 mins, depending on the gall formation and inoculation number, prepare enough NaOCl solution for shaking (CM-200, AS-ONE Co. Ltd, Osaka, Japan), each time NaOCl solution should cover roots in blender.

The method was modified from a protocol developed by Hussey and Barker (1973). Pouring the eggs and roots suspension in the following order of 106 and 20 µm aperture and wash well in tap water until all bleach smell is gone. Collected the debris and eggs in to a clean 10 ml plastic tube with minimum amount of water. Rinse well with tap water of 20 µm aperture and collect into same tube, don't touch the mesh part of the sieves as it may distort the pore size. Experiment 2

B. Application of biogas digestate with rice straw on root knot nematode and flower cultivation

I set up a parallel experiment to examine nematode suppressive effects. Kikugawa soil, which was naturally infested with the root-knot nematode, was used for this experiment. One hundred g of soil (oven dry basis) was put in triplicate into 500 mL plastic bottles and treated with (i) to (vii), as described above. The water content was adjusted to 60% of MWHC after addition of organic residues, including water contents in WBD, DBD20 and DBD30. A total of 21 (7 treatments × 3 replicates) bottles were used. During the incubation period, the lid of each bottle was loosely closed to minimize water evaporation and to allow gas exchange. Every 7 days, the bottles were weighed, and moisture losses were replaced with deionized water to adjust to 60% of MWHC. Incubation was performed at 27 °C for 90 days. At 30, 60 and 90 days later, soils were mixed thoroughly with a spatula and then 10 g (oven dry basis at 60 °C) was taken from each bottle for DNA extraction.

At the end of the incubation period, garden balsam seeds (*Impatiens balsamina*) were planted in the remaining soil (70 g, oven dry basis at 60 °C) to evaluate the gall

index caused by root-knot nematodes. The seeds were pregerminated in a Petri dish for 3 days at 25 °C in a Biotron (LPH 200, NK System) (12 h day and 12 h night conditions). Then, four germinated flower seedlings were grown in a vinyl pot (9 cm in diameter, 7.5 cm in height) containing 100 g of Kikugawa fresh soil with 60% of MWHC for 4 weeks at 25 °C in the Biotron. Then, the plants were uprooted and the gall index and dry matter weight were recorded. Gall formation on the plant roots was evaluated according to the levels (on a scale of 0–10) described by Zeck, (1971).

a. DNA extraction from soil

To extract soil DNA, soil samples was oven-dried at 60 °C for one night, and each of the oven-dried soil (10 g) was pulverized in triplicated with a ball mill (Retsch MM 400, Tokyo, Japan) for 2 min at 20 frequencies/second. DNA was extracted in duplicated from 0.5g soil according to the method of Sato et al. (2010).

The homogenize soil (0.5 g) was put into a 2 ml DNA extraction tube with 500µl of 20% skim milk, 0.75 g zirconia beads (0.1 mm diam.) and 0.25 g glass beads, 10 ul of soybean cyst nematode DNA (SCN-10^{-4} concentration) was also added and the tube was centrifuged (12,000 × g for 1 min, 25 °C). Then, 600 µl of lysis buffer (0.5% SDS, 100 mM Tris, 50 mM EDTA, pH 8.0) was added. The soil was bead-beaten 2 times at 5,000 rpm for 1 min each, followed by centrifugation (12,000 × g for 5 min, 25 °C). Then, 600 µl of the supernatant was transferred to a new 2 ml tube, and 377 µl of 5 M NaCl and 270 µl of 10% CTAB were added to the tube. After a10-min incubation at 60℃ and shaken 3 times at 3, 5 and 7 min. After incubation and cool down the tube to room temperature level, 500 µl of chloroform was added, and the tube was centrifuged at

15,000 × g for 15 min at 25 °C. 1.1 ml of supernatant was transferred to a new 2 ml tube, 500 µl of chloroform was added, and the tube was centrifuged at 15,000 × g for 15 min at 25 °C. The supernatant was then mixed with 600 µl of 20% PEG solution and centrifuged at 15,000 × g for 20 min at 4 °C to collect DNA as a pellet. The DNA pellet was washed with 70% ethanol and centrifuged at 15,000 × g for 5 min at 4 °C. Then the tube was dried using VC-15Sp (TAITEC Co. Ltd., Koshigaya, Japan) for 20 min. The DNA suspended in 100 µl of TE buffer.

b. Real-time PCR

qPCR was performed in a StepOne Real time PCR System (Applied Biosystems Japan Ltd, Tokyo, Japan) with a final volume of 10 µl containing 2 µl of 10 times diluted template DNA, 0.4 µl of 10 µM root-knot nematode primer (*Meloidogyne incognita*) RKNf [5′-GCTGGTGTCTAAGTGTTGCTGATAC-3′] -RKNr [5′-GAGCCTAGTGATCCACCGATAAG-3′] (Toyota et al., 2008) and 5µl of Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) under the manufacturer`s recommended conditions (95°C for 10 s, (95°C for 5 s and 60°C for 20 s, at increasing and decreasing rates of 0.2°C s^{-1}) for 45 cycles). A negative control was also included using distilled water instead of a template DNA. qPCR was done once per each DNA extract, since replicate samples showed almost identical values in qPCR. To know the DNA extraction efficiency of each soil, qPCR was performed as above procedure using 10 µM root-knot nematode primer.

3.3.4 Statistical analysis

All results for inorganic nitrogen, EOC content, pH and the number of root-knot

nematodes were obtained in triplicate and expressed as means and standard deviations. The effects of all fertilizer treatments and incubation time, as well as their interactions on NO_3-N and NH_4-N and nematode numbers, were tested with a two-way ANOVA followed by a Tukey HSD mean comparison ($p < 0.05$) using the software SPSS version 22.

3.4 Results

3.4.1 Experiment 1

3.4.1.1 Application of biogas digestate with rice straw on root knot nematode

While population of *M. incognita* decreased with time in the all digestate treatments and was significantly lower ($P<0.05$) in all digestate treatments at 30 days of incubation than in CONT (Figure 3.1). From 30 to 60 days of incubation, population of *M. incognita* was significantly ($P<0.05$) lower in dry digestate treatments than in that of CF. Population of *M. incognita* drastically decreased in DBD20, DBD30 and Mix2 from 30 to 90 days of incubation and at 90 days of incubation, it was significantly ($P<0.05$) lower in DBD30 and Mix2 than in the other treatments.

The inoculation experiment of Fuchu soil was not showed significant differences. (data not showed)

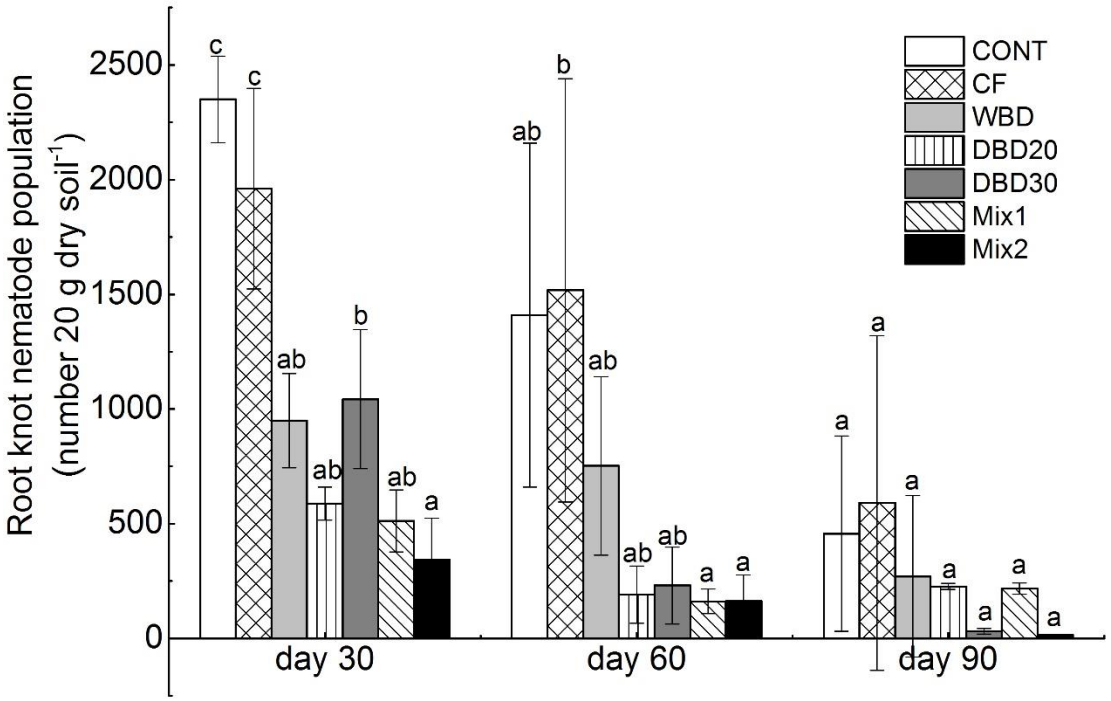


Figure 3.1 The number of root-knot nematodes in soil amended with different types of fertilizer during 90-day incubation period. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Letters indicate a significant difference among treatments ($p < 0.05$).

3.4.2 Experiment 2

3.4.2.1 Effect of fertilization on root-knot nematode

Populations of root-knot nematodes were significantly lower ($p < 0.05$) in Mix2 than in CF at day 30. At days 60 and 90, populations were significantly ($p < 0.05$) lower in DBD30, Mix1 and Mix2 than in CF. Populations significantly ($p < 0.05$) decreased in DBD30 and Mix2 from day 30 to day 90 (Figure 3.2). In comparing wet and dry digestate, populations were significantly ($p < 0.05$) lower in DBD30 than in WBD at days 60 and 90.

While plant growth was not significantly different among treatments, gall formation was significantly ($p < 0.05$) lower in Mix2 than in CF, WBD, DBD20 and DBD30, and it was significantly ($p < 0.05$) lower in Mix1 than in CF and WBD (Table 3.2). In comparing wet and dry digestate, gall formation tended to be lower by 10% to 20% in DBD30 and DBD20 than in WBD, although there were no significant differences.

There was a significant negative relationship between root-knot nematode population at day 90 and EOC at day 0 ($R^2 = 0.829$, $p < 0.01$, Figure 3.3)

Table 3.1 Gall index and dry matter production of garden flower (*Impatiens balsamina*) grown for one month in Kikugawa soil which was amended with different fertilizers and incubated for three months.

Treatment	Root Gall Index (0–10)	Dry Root Weight (g pot^{-1})	Dry Shoot Weight (g pot^{-1})
CONT	3.6 ± 0.3bc	0.17 ± 0.05	0.49 ± 0.04
CF	4.3 ± 0.6c	0.13 ± 0.08	0.43 ± 0.22
WBD	4.1 ± 0.5c	0.17 ± 0.06	0.51 ± 0.10
DBD20	3.7 ± 0.4bc	0.23 ± 0.01	0.62 ± 0.06
DBD30	3.3 ± 0.4bc	0.18 ± 0.05	0.55 ± 0.09
Mix1	2.8 ± 0.4ab	0.16 ± 0.04	0.53 ± 0.08
Mix2	2.0 ± 0.3a	0.17 ± 0.03	0.57 ± 0.06

Gall index was evaluated on a 0 to 10 scale, 0 = no galls, 1 = very few small galls, 2 = numerous small galls, 3 = numerous small galls of which some are grown together, 4 = numerous small and some big galls, 5 = 25% of roots severely galled, 6 = 50% of roots severely galled, 7 = 75% of roots severely galled, 8 = no healthy roots but plant is still green, 9 = roots rotting and plant dying, 10 = plant and roots dead. Values are means (n = 3) ± standard deviation. Different letters indicate significant difference ($p < 0.05$). (Zeck, 1971)

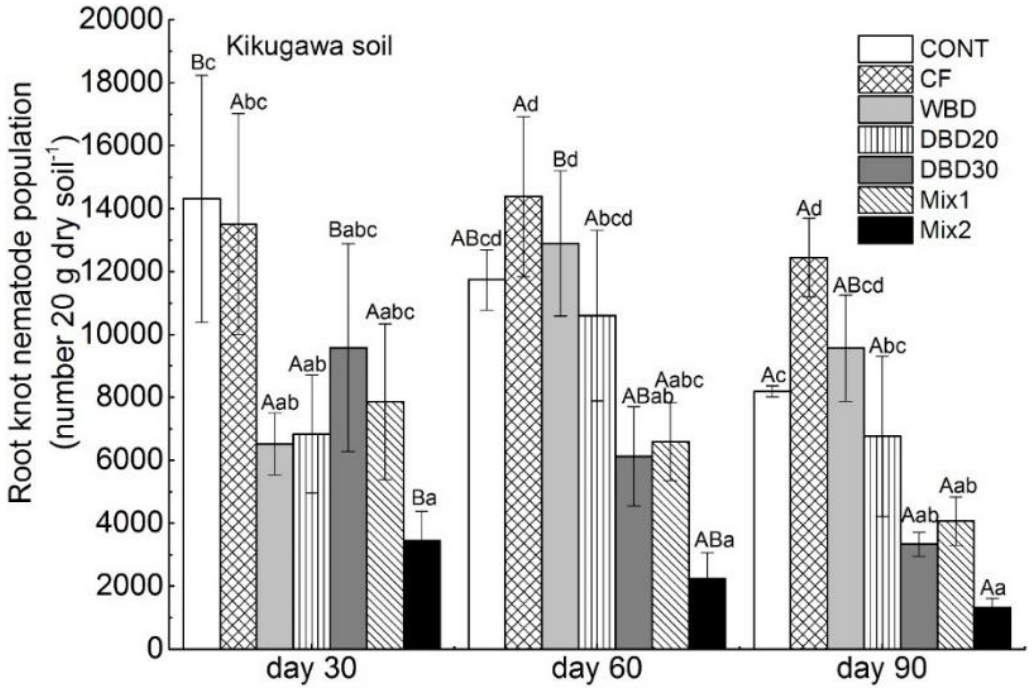


Figure 3.3 The number of root-knot nematodes in soil amended with different types of fertilizer during 90-day incubation period. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30, Mix1: DBD20 mixed with a low amount of rice straw to adjust its C/N ratio to 16, Mix2: DBD20 with a high amount of rice straw to adjust its C/N ratio to 30. Bars represent standard deviations (n = 3). Lowercase letters indicate a significant difference among treatments ($p < 0.05$); uppercase letters indicate significant differences among residue incorporation times for each treatment ($p < 0.05$).

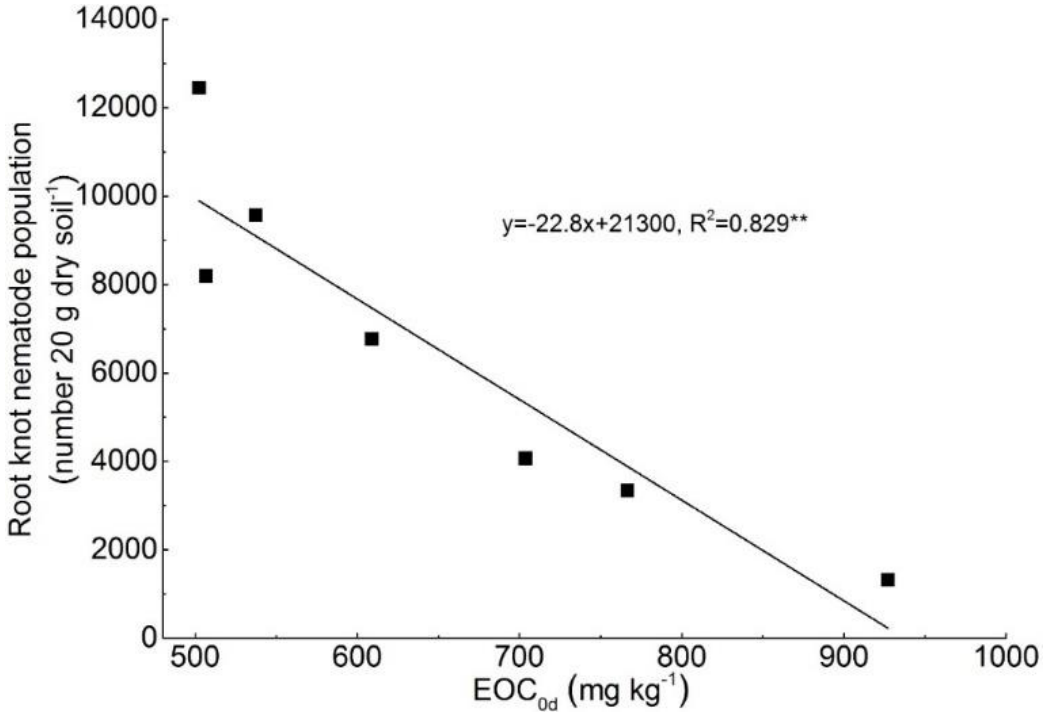


Figure 3.4 The relationship between root-knot nematode population at day 90 and EOC at day 0 in Kikugawa soil. ** $p < 0.01$.

3.5 Discussion

The populations of root-knot nematodes were drastically decreased in Mix2 and remained the lowest (Figure 3.1 & 3.3). These two times reproduced experiments showed similar suppressiveness, which means the application of dry digestate with rice straw on root-knot nematodes was reproducible and can successful controled nematodes from soil. This result was further supported by the significantly ($p < 0.05$) lower gall index in Mix2 than in CF (Table 3.1). The gall index was also significantly lower in Mix1 and DBD30 than in CF. The main differences among the treatments were C/N ratio and rice straw content. Mian and Rodriguez-Kabana (1982) reported that nematode suppression by organic amendment is directly related to N content or inversely related to the C/N ratio. Similarly, in a study by Agu (2008), plants of African yam bean treated with poultry and farmyard manures (C/N ratio of 4 to 12) showed a lower degree of disease caused by root-knot nematodes than those with other organic manures with C/N ratios higher than 30. In my study, all digestate amendment treatments were set up at the same level of 200 mg NH_4-N kg^{-1} dry soil, while their C/N ratios of all dry digestate treatments were more than 9.3, indicating no nematicidal effect based on the above references. However, Mix2 (C/N ratio of 30) showed the highest suppression compared with other treatments, which is contradictory to other studies in which populations of root-knot nematodes in soils decreased with the amendment of organic materials with a C/N ratio of less than 20 (Nico et al., 2004, Rodriguez-Kabana et al., 1987). In addition, Mix1 (DBD20 with rice straw added at a rate of 4.4 g kg^{-1}) also showed a lower gall formation of *M. incognita* than DBD20

(Table 3.1), indicating that rice straw could have a nematode suppressive property. Maareg et al. (2008) reported that gall formation by the root-knot nematode *Meloidogyne javanica* was markedly decreased (81.9%) by the addition of a higher amount (30 g kg^{-1}) of rice straw, which is the same amount as in Mix2, and that a minimum amount (10 g kg^{-1}) of rice straw also showed a 63.9% decrease in the gall formation. Recently, Zhao et al. (2019) reported that straw incorporation at 5 g kg^{-1} improved soil fertility, and thus increased wheat yield by 58%. Thus, rice straw amendment to soil may play an important role in the mitigation of the nitrate leaching risk, suppression of root-knot nematodes and soil fertility.

According to Jothi et al. (2003), a type of wet biogas digestate reduced the severity of damage to tomato as well as the population of *M. incognita*. In addition, Westphal et al. (2016) found that soil amendment with a wet digestate reduced host plant infection with *Heterodera shcachtii* and improved plant growth. However, in this study, populations of root-knot nematodes did not decrease in WBD and DBD20, compared with those in CONT and CF, from day 60 to day 90 (Figure 3.3). The exact reason why WBD20 and DBD20 did not decrease populations of root-knot nematodes was unclear, although the lower EOC in these two treatments could be involved (Figure 3.4). Several studies have already reported that not all types of organic amendments are beneficial in the suppression of root-knot nematodes (Bulluck et al., 2002, Korayem, 2003). In contrast, DBD30 significantly decreased populations of root-knot nematodes from day 60 to day 90 compared to WBD (Figure 3.3). DBD30 showed a significantly higher EOC at day 0 than WBD (Table 3.1). These results may suggest that a dry biogas

digestate, DBD30, is better than a wet biogas digestate, WBD, in terms of nitrate leaching risk and root-knot nematode management.

Bullock et al. (2002) indicated through a field study that non-fermented swine manure failed to decrease population of RKN on tomato but reduced gall indices. Organic amendment may have different effects on different soil microbial groups and gall index could be reduced by such a modified microbial group. For example, soil amendment with the nematode-antagonistic plant *Crotalaria juncea* enhanced nematode-trapping fungi as well as soil indigenous microbial populations (Wang and McSorley 2005). Organic amendment stimulates a broad range of (micro) organisms involved in the soil food web, many of which are potential predators or parasites of plant-parasitic nematodes (Oka 2010). Thus, nematode suppression might result from increased incidences and levels of nematode-antagonistic fungi following amendment application. Wang et al. (2001, 2002) also reported that the application of sunn hemp crop residues to soil decreased population levels of the plant-parasitic nematode *Rotylenchulus reniformis* and increased levels of nematode-trapping fungi in soil. In our study, the effect on nematode-antagonistic fungi was not tested, but such fungi could be responsible for nematode suppression in this study. However, the mechanism leading to this effective suppression observed in Mix2 needs to be further investigated.

3.6 Conclusion

In conclusion, addition of biogas digestate mixed with a higher amount of rice straw can effectively decrease nitrate leaching potential from soils by increasing the C/N ratio of amendment, which induced soil net N immobilization (= N starvation). Moreover, it also gave pronounced nematode suppression and thus could be used safely as a soil amendment in nematode management programs.

Chapter 4 Future direction

4.1 Rice straw management

Several management options available to farmers for the management of rice residues are burning (south Asia), incorporation, surface retention and mulching, livestock feeding and removing the straw (Mandal et al., 2004). The challenges are considered to be a poor feed for the animals due to high silica content (Drake et al., 2002), burning will lead to promoting loss of organic matter, killing of beneficial soil insects and microorganisms, and also causes air pollution (Muhammad et al., 2018). Removal is a loss of organic sources for soil health but is necessary to feed livestock and sustain mixed farming. Incorporation is a better option but it requires large amounts of energy and time; leads to temporary immobilization of nutrients, especially nitrogen; and the C:N ratio needs to be corrected by applying nitrogen at the time of incorporation (Pathak and Sarkar 1994; Talukder et al., 2008). Surface retention of residues help in increasing the organic carbon and total N in the top 5-15 cm of soil, while protecting the surface soil from erosion (Mandal et al., 2004). In my study, rice straw powder was thoroughly incorporated with soil, it successfully mitigated nitrate leaching potential from both types of soil, soil extractable carbon of both soil also increased and it might be one of the reason for responsible for suppressing the population of root-knot nematode from soil.

Because of importance of soil fertility improvement and sustainable crop system, soil incorporation and surface retention of rice straw practices were chosed to mainly discuss in this chapter, which would be a promising strategy on a global scale

(Dikgwatlhe et al. 2014).

The objective of this chapter included four parts, i) the beneficial effect of rice straw incorporation and surface retention on soil, ii) comparing with the difference between rice straw incorporation and surface retention, iii) the current options of biogas digestate management, iv) the available technology of wet and dry digestate application and its life-cycle environmental impact.

4.1.1 General conclusion of rice straw incorporation study

Considering the nitrate leaching potential of adding biogas digestate in soil, I hypothesize that amended with C – rich rice straw would help with mitigation of nitrate leaching risk in terms of N-deficiency (Manevski et al., 2016; Demiraj et al., 2018) and more liable C would also be related to the suppressiveness of root knot nematode population (Rodríguez-Kábana, 1986; Butler et al., 2011), therefore I set up two different amounts of rice straw mix with biogas digestate (Mix1 and Mix2) and incorporate with soil afterwards, the results showed that higher amount of rice straw (44.6 Mg ha^{-1}) mixed with biogas digestate effectively mitigate nitrate leaching potential in both type of soil by increasing the labile C contents of the amendment, which induces soil net N immobilization. Both of rice straw application rate showed effective suppressiveness of root knot nematode population compare with control treatment. For future and better in rice straw and digestate management, field study should be taken into account, rice and tomato cultivation, tilliage or no-tilliage practice, diary location and storage etc., all potential factors should be taken into account for better ecological and production system. This study was trying to give a guidance of

rice straw application rate for better management of rice straw.

4.1.2 Rice straw incorporation and the effect on soil organic carbon

The incorporation of rice straw to soil can not only improve soil physical properties, which can reduce soil erosion risks and improve soil moisture retention (Mandal et al., 2004), but can also reduce the loss of excess fertilizer N through immobilization and prolong nutrient availability (Powlson et al. 1985). Deep tillage for incorporation of crop residues has been shown to reduce soil bulk density (Kumar et al., 2004a) as well as penetration resistance of the plough layer (Walia et al., 1995). It also helps to decrease soil pH (Sidhu and Beri, 1989). The major disadvantage of incorporation of rice straw is the immobilization of inorganic N and its adverse effect due to N-deficiency (Mandal et al., 2004).

Soil organic carbon (SOC) is considered an important indicator of soil quality and agricultural sustainability because it improves soil aggregate stability and soil water retention, and provides a reservoir of soil nutrients (Liu et al., 2006). Based on different separation and extraction methods, active SOC can mainly be characterized as dissolved organic carbon (DOC), liable organic carbon (LOC), light fraction organic carbon (LFOC), and microbial biomass organic carbon (MBC) (Xu et al., 2011b). Wang et al., (2015) demonstrated that rice straw incorporation increased the concentrations of total SOC and active SOC fractions (including LFOC and DOC) significantly in paddy fields. After an 8-year long field trial, Zhu et al. (2010) reported that transferring the incorporation of rice straw from paddy fields to uplands resulted in a significant increase in SOC in the upland soils. Xu et al., (2011a) reported that, after 10 years rice

straw incorporation investigation, the straw incorporation enhanced SOC sequestration, total organic C concentration and C storage were significantly increased with straw incorporation rates. Water-soluble organic C (CWS) is composed of an array of molecules generally reflecting the composition of total SOC due to the soluble phase tending to be in equilibrium with the solid phase of SOC (Chantigny et al., 2002), and is regarded as an indicator of soil quality and functioning (Saviozzi et al., 2001). The 10-year rice straw incorporation experiment also showed that, CWS was significantly increased under the treatments of 50%S (50% of straw incorporation) and 100%S (100% of straw incorporation) (34% and 71%, respectively), compared with the 0%S (straw remove) treatment (Xu et al., 2011a).

4.1.2.1 Conventional tillage

Conventional tillage is a tillage system using cultivation as the major means of seedbed preparation and weed control. Conventional systems of tillage leave less than 30% of crop residues – and often none – on the soil surface after crop establishment. Conventional tillage is invariably deeper (20–35 cm) with inversion of the soil by mouldboard plough, disc plough or spading machine (Peigné et al., 2007). This form of tillage buries all superficial crop residues in the soil (Tisdale et al., 1985). In developing countries, tillage is broadly used for farmers to control weed in terms of poor access to herbicides thus incorporating residues in the process (Turmel et al., 2015).

4.1.3 Surface retention

The management of crop residues must be an integral part of future tillage practices for sustainable production systems. Direct drilling in surface mulched residues is a

practice that leaves straw residues from a previous crop on the soil surface without any form of incorporation. Surface retention of residues helps in protecting the fertile surface soil against wind and water erosion (Rasmussen and Collins, 1991). The large volume of residues remaining on the surface often leads to machinery failures, thus affecting sowing of seeds of the following crop. Farmers usually follow this method where no tillage or conservation tillage practices are prevalent.

Surface retention of some or all of the residues may be the best option in many situations. Residues decompose slowly on the surface, increasing the organic carbon and total N in the top 5-15 cm of soil, while protecting the surface soil from erosion (Rasmussen and Collins, 1991). Retention of residues on the surface increased soil NO_3^- concentration by 46%, N uptake by 29%, and yield by 37% compared to burning (Bacon, 1987, Bacon and Cooper, 1985b, Bacon and Cooper, 1985a).

4.1.3.1 Conservation tillage

Conservation tillage, by most definitions, includes crop production systems involving the management of surface residues (Lal, 1997). According to the Conservation Technology Information Center (CTIC, 1990) in West Lafayette, Indiana, USA, conservation tillage is defined as: "any tillage or planting system in which at least 30% of the soil surface is covered by plant residue after planting to reduce erosion by water; or where soil erosion by wind is the primary concern, small grain residue on the surface during the critical wind erosion period." No tillage, minimum tillage, reduced tillage, mulch tillage and ridge tillage are terms synonymous with conservation tillage (Mannering and Fenster, 1983).

4.1.4 Incorporation vs surface retention

Surface placement of plant residues, such as conservation tillage mulch, usually results in slower, more variable rates of decomposition than where similar residues are incorporated into the soil by tillage. Nutrient elements mineralized from surface-applied residues are more susceptible to loss in runoff or by volatilization than those from incorporated residues. Compared to surface residue, incorporated residues experience much more constant moisture and temperature and are in intimate contact with soil moisture and soil organisms. The incorporated residues therefore, decompose more quickly and uniformly, and may release nutrients more easily by leaching (Turmel et al., 2015).

Management factors such as incorporating by tillage or leaving crop residue on the soil surface can additionally influence the effect of crop residue retention on SOC in the soil profile. Conventional tillage is usually considered responsible for C losses by increasing decomposition rates (Reicosky, 2003). Tillage disturbs soil structural stability (Kay, 1990) and redistributes organic matter, influencing microbial activity at the soil surface that releases carbon (Carter, 1986).

Salinas-Garcia et al. (2001) examined organic C sequestration under rain-fed maize production in two different regions in Mexico, Apatzingán and Casas Blancas, during a 6-year experiment. The results showed that conservation tillage, i.e. no tillage (NT) with 100%, 66% and 33% of crop residues left on the field, and minimum tillage (MT), significantly increased soil organic C than that of conventional tillage (disking and disk plowing) and NT/0% treatments on 0-5cm surface layer. Similarly, in Varanasi, India,

another region with high temperatures and decomposition rates, SOC and total N were highest under minimum tillage with residue retained on the surface (0-10cm) compared to incorporation (Kushwaha et al., 2001). Surface retention under no-tillage mainly attribute more SOC of surface soil (0-15cm) (Baker et al., 2007), but incorporation with tillage affects the distribution of SOC in deeper layers (150-30cm) (Yang and Wander, 1999; Jantalia et al., 2007). Dong et al. (2009) conducted a 5 year field study with five different tillage practices, the conventional tillage with and without surface residue, rotary tillage with incorporated chopped residue, no-tillage with surface chopped residue, and no-tillage with standing residue treatments, under the conventional tillage with residue treatment, SOC content was highest in the 5–10 cm layer below the surface. SOC content of other treatment was highest in the 0–5 cm layer, but decreased with depth up to 30 cm. In order to obtain more accurate assessment of the influence of residue management practices on SOC, it is thus recommended to sample the entire plow depth (VandenBygaart and Angers, 2006) so that shallow sampling does not present a bias towards no-till practices including residue retention (Baker et al., 2007). Moreover, many factors (e.g. time period, soil, climate, experiment) should be taken into account when comparing results from residue surface retention and incorporation (Ahuja et al., 2006).

4.1.5 Removal of rice straw

4.1.5.1 Anaerobic digestion of rice straw

Anaerobic digestion would be a more effective way, compared with other renewable ways, such as bioethanol, to treat rice straw and produce renewable energy

simultaneously (Fu et al., 2018). Methane production from rice straw through anaerobic digestion technology is growing worldwide and is considered ideal in many ways because of its economic and environmental benefits (Chandra et al., 2012). And biogas digestate, the by-product through co-digestion of rice straw with manure or other wastes was also utilized an effective fertilizer for crop plants. Several studies had already reported the beneficial effect of application of biogas digestate on soil chemical properties (Frąc et al., 2012), suppress soil borne-plant pathogens (Mawdsley et al., 1995) etc. In my study, biogas digestate was made from pig manure mixed with rice straw through anaerobic digestion, animal manure is preferably co-digested with organic wastes containing high amounts of carbon to improve the C/N ratio and to further increase biogas production (Nie et al., 2015).

4.1.5.2 Composting of rice straw

Co-composting of rice straw with other materials, such as different organic waste, manure, sewage sludge ect.is also an effective way for better recycling rice straw and wastes. There are also several studies had already reported effect of rice straw basis compost on soil plant system (Roca-Pérez et al., 2009), soil properties (Abdelhamid et al., 2004; Sodhi et al., 2009), soil microbial community structure (Tanahashi et al., 2004).

4.1.5.3 Animal feeding

Approximately 80% of the rice in the world is grown by small-scale farmers in developing countries, including South East Asia. The large amount of rice straw as a by-product of the rice production is mainly used as a source of feed for ruminant

livestock (Sarnklong et al., 2010).

4.1.5.4 Burning

Globally especially in Asian countries, burning of crop residues on field is commonly practiced. Burning also kills soil borne deleterious pests and pathogens. In most developing countries, farmers use burning practice to clear the land quickly of residues which could help with facilitating seed germination and establishment for the next crop establishment (Muhammad et al., 2018). In contrast, burning will emit high amount of CO_2, CH_4 and N_2O, thus, destructive effects of burning on soil productivity and probably on human health and environment suggest that crop residue would be well managed by other approaches (Kutcher and Malhi, 2010).

4.2 Life-cycle environmental effect of biogas digestate management

4.2.1 Biogas digestate application technique

4.2.1.1 Current options

Digestate must be integrated in the fertilization plan of the farm in the same way as mineral fertilizers and it must be applied at accurate rates, with equipment that ensures even applications throughout the whole fertilized area. The digestate application practice was similar to application of manure and slurry. The most suitable methods (trailing hoses, trailing shoes or injector) of application are therefore those that minimize the surface area of digestate exposed to air to ensure rapid incorporation of digestate into the soil (Nicholson et al. 2018). It is not recommended that splash plate spreading causes air pollution and loss of valuable nutrients (Lukehurst et al, 2010).

The fields where digestate is applied should be located close to the anaerobic

digester, to reduce transportation costs (Dahlin et al., 2015). When digestate has to be transported to longer distances, volume reduction through solid–liquid separation is considered. The simplest ways practiced for using these fractions are, the solid fraction to be composted and used as soil improver, while the liquid fraction is applied as nitrogen-rich fertilizer or further processed and sold as concentrated liquid fertilizer (Logan and Visvanathan, 2019). Therefore, considering of my study's results, dry digestate was more recommended to apply into market.

4.2.1.2 Merit and demerit of different applicable technique of biogas digestate

Considering the different applicable technique of biogas digestate, it is essential that their application does not adversely impact on the environment (i.e. soil, water and air quality) or human health. Low ammonia emission spreading techniques are recommended such as Trailing hose, trailing shoe applicator, and shallow injection, these techniques can be mounted to a vacuum or pumped tanker (Misselbrook et al., 2002). In order to comparing the effect of precision application techniques (shallow injection and trailing shoe) with the conventional surface broadcast (splash plates) method on NH_3 emissions, food-based digestate was applied, the results showed that both precision application methods reduced NH_3 emissions by 40–50% in comparison with the surface broadcast treatments (Nicholson et al., 2018). Moreover, the choice of applicable technique depends on the dry matter (DM) content in the biogas digetate, where the trailing hose and trailing shoe application are suitable for dry matter content between in between 6 to 9 % (Vettik &Tamm, 2013). In my study, I used two types of biogas digestate, wet biogas digestate (dry matter content 5%) and dry biogas digestat

(dry matter content 30%). Thus, the applicable method of dry biogas digestate should be conducted by convention tillage (incorporation) for reducing odor emission and soil fertility improvement in deep layer

4.2.2 Biogas digestate application on atmospheric pollution

4.2.2.1 Nitrous oxide emission

Several references have shown that the application of organic fertilizers to soils results in the loss of 0.2–1.5 % of the applied nitrogen as N_2O. The comparatively limited research that has focused on N_2O emissions after anaerobic digestate application shows in general that these emissions are low compared to what is found with other organic fertilizers, because of the low amounts of biodegradable organic matter in the digestates, which limit the extent of denitrification (Parkin, 1987; Rochette et al., 2008). Several comparative studies have shown lower N_2O emissions on land spread with digested slurries (Collins et al. 2011; Chantigny et al. 2007; Amon et al. 2006). Specifically, Börjesson and Berglund (2007) reported an average reduction of N_2O emissions from 40 (undigested) to 25 g (digested) N_2O per tonne of manure applied. Beside the aforementioned soil characteristics that influence N_2O emission, soil texture is a determinant as well. Thus, Chantigny et al. (2007) reported a 54–69 % lower N_2O emission with the digested than with undigested manure in a loam soil, as opposed to a 17–71 % lower emission in a sandy loam.

The N_2O model by Sommer et al. (2004) predicted that anaerobic digesiton (AD) could reduce N emissions by more than 50%. Nonetheless, Bertora et al. (2008) reported that the N_2O emission pattern derived from the liquid fraction of pig slurry

through anaerobic digestion was similar to that observed for the original slurry after soil application during a 58- day mesocosm incubation period. These different patterns in N_2O emissions could be due to various factors; the input of easily available organic C (measured as biological oxygen demand, BOD) seems to be the dominant factor driving the amount of N_2O emitted after digestate application (Clemens and Huschka, 2001). The higher the BOD, the more N_2O was emitted from the soil (Dosch and Gutser, 1996; Drury et al., 1998). The soil water content and the temperature, along with the ammonium concentration in the manure, were also have a significant impact on N_2O emission potential (Clemens and Huschka, 2001; Wulf et al., 2002). In my study, the application of dry biogas digestate application mixed with higher amount of rice straw on root-knot nematode showed obvious suppressiveness and also reduced nitrate leaching risk, but the green-house emission, especially N_2O emission should be taken into account. Further study should be focusing on green-house emission with the same rate of digestate application.

4.2.2.2 Ammonia emission

Ammonia emission from anaerobic digestates is affected by management and environmental factors such as storage conditions, methods of application, concentrations of ammonia in the digestate, pH, temperature, air velocity, surface areaand moisture (Sommer and Hutchings 2001; Sandars et al. 2003; Holm-Nielsen et al. 2009). Given the higher NH_3/NH_4 concentration and pH in anaerobic digestates relative to livestock manures (Haraldsen et al. 2011; Möller et al. 2008; Chantigny et al. 2008) and the intensification of biogas production across the world, biogas plants

and associated crop fields are expected to be major sources of emission of ammonia.

Several studies have found similar (Chantigny et al. 2004; Pain et al. 1990) or higher emissions than raw manures (Ni et al. 2012; Gericke 2009). In contrast, Rubaek et al. (1996) reported lower emissions with digested than with raw manure. Specifically, NH_3 emissions after anaerobic digestates application were estimated between 7 and 24 % of applied NH_4-N as opposed to 3 to 8 % for animal slurries (Gericke, 2010). Wulf et al. (2002) have precisely quantified these emissions at about 350, 275, 160 and 50 mg NH_3-N m^{-2} h^{-1} within the first 10 h following application of liquid digestate through splash plate, trailingshoe, harrow and injection methods, respectively.

Moller and Stinner (2009) found that ammonia losses could take place when the incorporation of digestate into soil occurs more than 12 h after field spreading. This suggests that digestates can provide particularly positive agronomic effects in terms of their N content if they stay on the soil surface for only a short time. Furthermore, weather conditions have been shown to notably affect NH_3 emissions when digestates are applied (Quakernack et al., 2012; Koster et al., 2014). These authors found that the major portion of ammonia was released during the first two days after land spreading, and they attributed these higher NH_3 emissions at low temperatures to the fact that the frozen soil could have restricted the infiltration of most of the applied digestate, thereby NH_3 remaining on the soil surface. In conclusion, the application of biogas digestates to soil should accurately follow precise agricultural practice bearing in mind the ready availability of nitrogen and the crop N demand, so to avoid N loss as NO_3, which could be drained to surface waters, leached to ground waters or denitrified into gaseous forms

(Bardgett and Wardle, 2010).

Appendix

Application of biogas digestate on soybean cyst nematode (*Heterodera glycines*) and potato rot nematode (*Ditylenchus destructor*)

1.1 Abstract

The objectives of this study were to investigate the application of biogas digestate to soil on soybean cyst nematode (*Heterodera glycines*) and potato rot nematode (*Ditylenchus destructor*). This study consisted of the following five treatments: i) control without any fertilizer (CONT), ii) chemical fertilizer (CF), iii) wet biogas digestate deriving from pig manure (WBD), iv, v) dry biogas digestate deriving from a mixture of pig manure and rice straw at an initial C/N ratio of 20 and 30 (DBD20 and DBD30), respectively. The application rate of CF and digestates was adjusted to 300 mg N kg^{-1} soil based on their inorganic nitrogen contents. By using real-time PCR method and baermann funnel method to investigate the population of sobybean cyst nematode and active nematode from soil after dry and wet digestate amendment, both of the results showed no suppressiveness after digestate amendment. The same results were also showed in potato rot nematode experiment.

1.2 Introduction

The soybean cyst nematode (SCN), is a plant-parasitic nematode and a destructive pest of soybean worldwide (Bajaj et al., 2015). The life cycle of the SCN has three stages: egg, juvenile, and adult. Soybeans are infected by the second-stage juveniles (J2s), the microscopic colorless worm stage that penetrates the roots with a stylet. After invading the roots, the nematodes migrate towards the vascular tissue where they feed and develop. While a number of highly effective chemical nematicides, including fumigants and non-fumigants, have been deployed over the past 30 years, various fungal and bacterial pathogens of nematodes have also been employed as potential biocontrol agents, with variable or limited success (Hildalgo-Diaz, 2008). There is a great need for development of nontoxic, inexpensive, and effective control methods.

Potato is the main host to *D. destructor*, but the nematode is occasionally found on over 70 crops and weeds including a similar number of fungal species (Sturhan et al., 1991). Ditylenchus destructor is favored by cool and moist soils which is favorable for development and movement of the nematode (Sturhan et al., 1991). The nematode overwinters in the soil as adults, juveniles or eggs, and multiplies by feeding on host plants, weeds and fungal mycelium (Jones et al., 2013). Shortly after juveniles hatch, the juveniles are immediately able to parasitize plants. Management of *D. destructor* once present in the field is a formidable task due to the wide host range and multiple generations per vegetative cycle of host crops (Chitwood, 2002). Several weed species are hosts to these nematodes making crop rotation a limited option (Nicol, 1972). Attempts to manage these nematodes using nematicides has not been adequate.

Additionally, there is pressure to minimize nematicides use due to health risks and enviromental contaminations.

Organic amendments can help to manage root-knot nematodes, so as biogas digestate. Previous studies have documented the use of compost or compost extracts as soil amendments for the control of root-knot nematodes, and reported reductions of nematode numbers following compost applications (Ntalli and Caboni, 2017, Xiang et al., 2018). There are reports that confirm a pesticidal effect of digestate on nematodes in tomatoes (Jothi et al., 2003). Therefore, the objective of this chapter is to determine if the application of dry and wet biogas digestate can suppress the density of soybean cyst nematode, potato rot nematode and in soil.

1.3 Materials and methods

1.3.1 Soil

Soybean cyst nematode (*Heterodera glycines*) and potato rot nematode (*Ditylenchus destructor*) infested soils were collected from Chiba and Aomori, Japan.

1.3.2 Biogas digestate

Two types of digestates, i.e., wet and dry biogas digestates, were used. The wet digestate was collected from a biogas plant in Aichi Prefecture, Japan, in which pig slurry was anaerobically digested at 35 °C with a hydraulic retention time of 15 to 20 days. Dry digestate was obtained from a dry thermophilic (55 °C) anaerobic digestion pilot plant that primarily used pig manure, and was supplemented with rice straw to adjust its C/N ratios to 20:1 or 30:1, with a sludge retention time of 40 days, in the Tokyo University of Agriculture and Technology, Institute of Engineering, Japan Both

digestates were directly taken from the effluent of the digester and stored at 4 °C until use. The chemical properties of the digestates and rice straw are shown in Table 1 and each sample was analyzed with three replicates. Ammonium-N content was measured using the indo-phenol blue method (Bolleter et al., 1961). Extraction was performed by: (i) mixing 5 mL of wet digestate or 5 g of dry digestate and rice straw with 25 mL of 2M KCl, (ii) shaking for 1 h at 120 rpm, and (iii) filtering through No. 5C filter paper (ADVANTEC Toyo Kaisha, Ltd., Tokyo, Japan). The water soluble total C (WSC) and N of the digestates were measured with a TOC-VCSH/CSN (Shimadzu, Kyoto, Japan) using the extracts. Carbon and N contents of the solid parts after extraction were measured with a CN coder (MT-700, YANACO New Science, Kyoto, Japan). Total C and N of digestate were then estimated from the sum of C and N in the water soluble and solid fractions. pH was determined in a 1:2.5 water-soluble extract.

Table 3-1. Chemical properties of digestates used in the present study.

	Water Content (%)	pH (H_2O)	Total C (g kg^{-1} or L^{-1})	Total N (g kg^{-1})	C/N Ratio	WSC*1 (g C kg^{-1})	WSN *2 (g N kg^{-1})	NH_4-N (g N kg^{-1})
WBD	97	6.2	12	5.0	2.4	2.81	3.88	4.2
DBD20	81	8.8	53	4.3	12.3	7.66	2.77	2.7
DBD30	80	8.7	56	3.4	16.5	5.06	1.78	1.6

WBD: wet biogas digestate, DBD20: dry biogas digestate adjusted to its original C/N ratio of 20 and then fermented (C/N ratio = 12), DBD30: dry biogas digestate adjusted to its original C/N ratio of 30 and then fermented (C/N ratio = 16). Data expressed on a fresh weight basis, data for WBD are expressed on a g L^{-1}. *1 WSC: water soluble C, *2 WSN: water soluble N, *3 ND: not determined.

1.3.3 Soybean cyst nematode (*Heterodera glycines*) suppressive experiment

I set up this experiment by using two methods (Real-time PCR and Bearmann funnel method) describe below to examine nematode suppressive effects. Two hundred g of soil (oven dry basis) was put in triplicate into 500 mL plastic bottles and the following four treatments were prepared: (i) control (no addition of biogas digestate and chemical fertilizer, CONT), (ii) compound chemical fertilizer (N:P:K = 8:8:8, Asahi Industries, Tokyo, Japan) (CF), (iii) wet biogas digestate (WBD), (iv) dry biogas digestate adjusted to its original C/N ratio of 20 and then dry fermented (C/N ratio = 12) (DBD20). (v) dry biogas digestate adjusted to its original C/N ratio of 30 and then dry fermented (C/N ratio = 16) (DBD30). Their application rates were adjusted to 300 mg ammonium (NH_4^+-N) kg^{-1} dry soil (equivalent to ~450 kg NH_4-N ha^{-1}) except for CONT, since this rate is maximum used for tomato cultivation. The actual added amounts of WBD, DBD20 and DBD30 were 100, 168 and 100 mg g^{-1} dry soil (equivalent to 150, 252 and 150 Mg ha^{-1}), respectively. The water content was adjusted to 60% of MWHC after addition of organic residues, including water contents in WBD,

DBD20 and DBD30. A total of 21 (7 treatments × 3 replicates) bottles were used. During the incubation period, the lid of each bottle was loosely closed to minimize water evaporation and to allow gas exchange. Every 7 days, the bottles were weighed, and moisture losses were replaced with deionized water to adjust to 60% of MWHC. Incubation was performed at 27 °C for 90 days. At 0, 2, 5 and 10 days later, soils were mixed thoroughly with a spatula and then 10 g (oven dry basis at 60 °C) was taken from each bottle for counting juvenile nematodes using Bearmann funnel method (Ingham, 1994). At 0, 7, 15 and 30 days later, soils were mixed thoroughly with a spatula and then 20 g (oven dry basis at 60 °C) was taken from each bottle for DNA extraction.

1.3.3.1 Nematode extractions by the Bearmann funnel method.

In a double-folded piece of a tissue paper (Kimwiper S-200., Nippon Paper Crecia Co. LTD., Tokyo Japan), 10 g of the soil was wrapped and put on a sieve (6.5 cm in diameter) with 1 mm-mesh window screen. The sieve was put on a funnel connected with a vinyl tube to 1.5 ml centrifuge tube. The funnel was filled with tap water and incubated at room temperature (24-26 °C) for 3 days. The tube was removed and added with 15µl of 0.02% Tween 20 (Wako Pure Chemical Industries, Ltd). After centrifugation (5000 rpm for 5 min, 25 °C) about 1 ml of supernatant was discarded and the rest was transferred on a glass slide for microscopic observation as described above. The extracted nematodes were counted based on the morphological patterns under light microscope with 100 × magnifications.

1.3.3.2 DNA extraction and real-time PCR

To extract soil DNA, soil samples was oven-dried at 60 °C for one night, and each

of the oven-dried soil (10 g) was pulverized in triplicated with a ball mill (Retsch MM 400, Tokyo, Japan) for 2 min at 20 frequencies/second. DNA was extracted in duplicated from 0.5g soil according to the method of Sato et al. (2010).

a) DNA extraction from soil

The homogenize soil (0.5 g) was put into a 2 ml DNA extraction tube with 500µl of 20% skim milk, 0.75 g zirconia beads (0.1 mm diam.) and 0.25 g glass beads, 10 ul of soybean cyst nematode DNA (SCN-10^{-4} concentration) was also added and the tube was centrifuged (12,000 × g for 1 min, 25 °C). Then, 600 µl of lysis buffer (0.5% SDS, 100 mM Tris, 50 mM EDTA, pH 8.0) was added. The soil was bead-beaten 2 times at 5,000 rpm for 1 min each, followed by centrifugation (12,000 × g for 5 min, 25 °C). Then, 600 µl of the supernatant was transferred to a new 2 ml tube, and 377 µl of 5 M NaCl and 270 µl of 10% CTAB were added to the tube. After a10-min incubation at 60℃ and shaken 3 times at 3, 5 and 7 min. After incubation and cool down the tube to room temperature level, 500 µl of chloroform was added, and the tube was centrifuged at 15,000 × g for 15 min at 25 °C. 1.1 ml of supernatant was transferred to a new 2 ml tube, 500 µl of chloroform was added, and the tube was centrifuged at 15,000 × g for 15 min at 25 °C. The supernatant was then mixed with 600 µl of 20% PEG solution and centrifuged at 15,000 × g for 20 min at 4 °C to collect DNA as a pellet. The DNA pellet was washed with 70% ethanol and centrifuged at 15,000 × g for 5 min at 4 °C. Then the tube was dried using VC-15Sp (TAITEC Co. Ltd., Koshigaya, Japan) for 20 min. The DNA suspended in 100 µl of TE buffer.

b) Real-time PCR

qPCR was performed in a StepOne Real time PCR System (Applied Biosystems Japan Ltd, Tokyo, Japan) with a final volume of 10 µl containing 2 µl of 10 times diluted template DNA, 0.4 µl of 10 µM soybean cyst nematode primer (*H. glycines*) (Goto et al., 2009) SCN44f (5'-GCGTCGTTGAGCGGTTGTT-3') SCN124r (5'-CCACGGACGTAGCACACAAG- 3') and 5µl of Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) under the manufacturer`s recommended conditions (95°C for 10 s, (95°C for 5 s and 60°C for 20 s, at increasing and decreasing rates of 0.2°C s^{-1}) for 45 cycles). A negative control was also included using distilled water instead of a template DNA. qPCR was done once per each DNA extract, since replicate samples showed almost identical values in qPCR. To know the DNA extraction efficiency of each soil, qPCR was performed as above procedure using 10 µM potato rot nematode primer PCN280f (5'-GCGTCGTTGAGCGGTTGTT-3') PCN398r (5'-CCACGGACGTAGCACACAAG- 3') under manufacturer's recommended conditions same as SCN.

1.3.4 Potato rot nematode (*Ditylenchus Destructor*) suppressive experiment

I set up this experiment by Real-time PCR to examine nematode suppressive effects. 70 g of soil (oven dry basis) was put in triplicate into 100 mL plastic bottles and treatments set up were followed with above (i) to (v). In order to estimate the suppressive effects, we inoculate 4000 J2 in each bottle. J2 was extract by using Baermann funnel method described as above. Just changed the soil into fresh infested garlic. Cutting two infested garlic into 1 mm slice by using a blade. Soil of each bottle

were inoculated by pouring 4000 J2 in 10 mL of sterile distilled water and mixed thoroughly with a spatula.

Their application rates were adjusted to 300 mg ammonium (NH_4^+-N) kg^{-1} dry soil (equivalent to ~450 kg NH_4-N ha^{-1}) except for CONT, since this rate is maximum used for tomato cultivation. The actual added amounts of WBD, DBD20 and DBD30 were 100, 168 and 100 mg g^{-1} dry soil (equivalent to 150, 252 and 150 Mg ha^{-1}), respectively. The water content was adjusted to 60% of MWHC after addition of organic residues, including water contents in WBD, DBD20 and DBD30. A total of 21 (7 treatments × 3 replicates) bottles were used. During the incubation period, the lid of each bottle was loosely closed to minimize water evaporation and to allow gas exchange. Every 7 days, the bottles were weighed, and moisture losses were replaced with deionized water to adjust to 60% of MWHC. Incubation was performed at 27 °C for 15 days. At 0, 7 and 15 days later, soils were mixed thoroughly with a spatula and then 20 g (oven dry basis at 60 °C) was taken from each bottle for DNA extraction (describe as above).

1.3.5 Statistical analysis

Statistical analysis of data was performed using SPSS version 22. Tukey multiple comparison analysis was used to determine significance of differences (presented as P values) between treatments or individual data points.

1.4 Results and discussion

1.4.1 Effect of fertilization on soybean cyst nematode (*Heterodera glycines*)

There was no significant difference ($p < 0.05$) between different fertilization treatments after 30 days incubation period (Figure 1.1 A). Number of soybean cyst

nematode was also showed the same results of no differences ($p < 0.05$) after 15 days incubation period (Figure 1.1 B). Even in my study, the digestate application failed to suppress the number of soybean cyst nematode in soil. But there is one study by comparing with raw manure, volatile fatty acids (VFA) enriched digestate and NH_4^+ enriched digestate, VFA enriched digestate showed the best result in reducing the egg counts of soybean cyst nematode compare with raw manure and NH_4^+ enriched digestate, suggesting the important role of volatile fatty acids in nematode suppression (Xiao et al. 2007). Several studies had demonstrated that organic amendments can successfully suppress soybean cyst nematode in soil (Widmer et al., 2002). The mechanism of suppressiveness might subject to different factors (Renčo, 2013), moreover, the different suppressive properties might affect in different stage of soybean cyst nematode development, such as egg hatching stage (Behm etal., 1995; Xiao et al., 2008), infective stage (Xiao et al. 2007). Considering fermentation process and ingredients differences of digestate, the suppressiveness of soybean cyst nematode after digestate amentdment would be lead to different results, the mechanism and reduction of soybean cyst nematode after digestate amendment should be further investigated.

1.4.2 Effect of fertilization on potato rot nematode (*Ditylenchus Destructor)*

There was no significant difference ($p < 0.05$) between different fertilization treatments after 15 days incubation period (Figure 1.2). The suppressive effect of wet and dry biogas digestate on *Ditylenchus Destructor* was not observed. Current management strategies of controlling nematodes are the use of chemical nematicides, organic amendments, resistant cultivars, soil solarization and biological control (Ntalli

and Caboni, 2017; Xiang et al., 2018). In this study, the digestate application was failed to suppress the population of *Ditylenchus Destructor* in soil. For controlling this species should be further investigate in more available strategies.

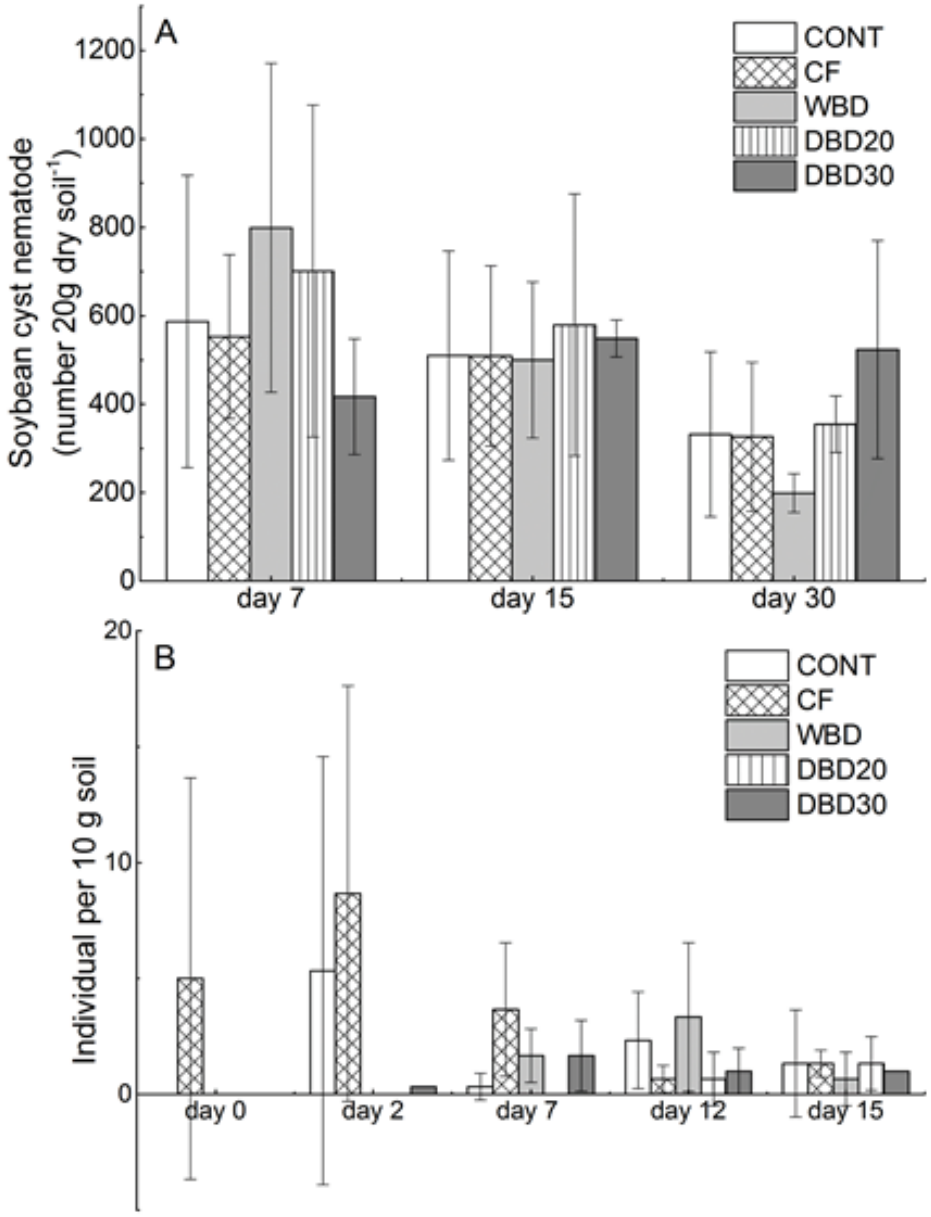


Figure 1.1 Effect of wet and dry biogas digestate application on soybean cyst nematode by real-time PCR (A) and Bearmann funnel method (B) during 30 days incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30. The values are the means $\pm$ SD. Bars represent standard deviations (n = 3).

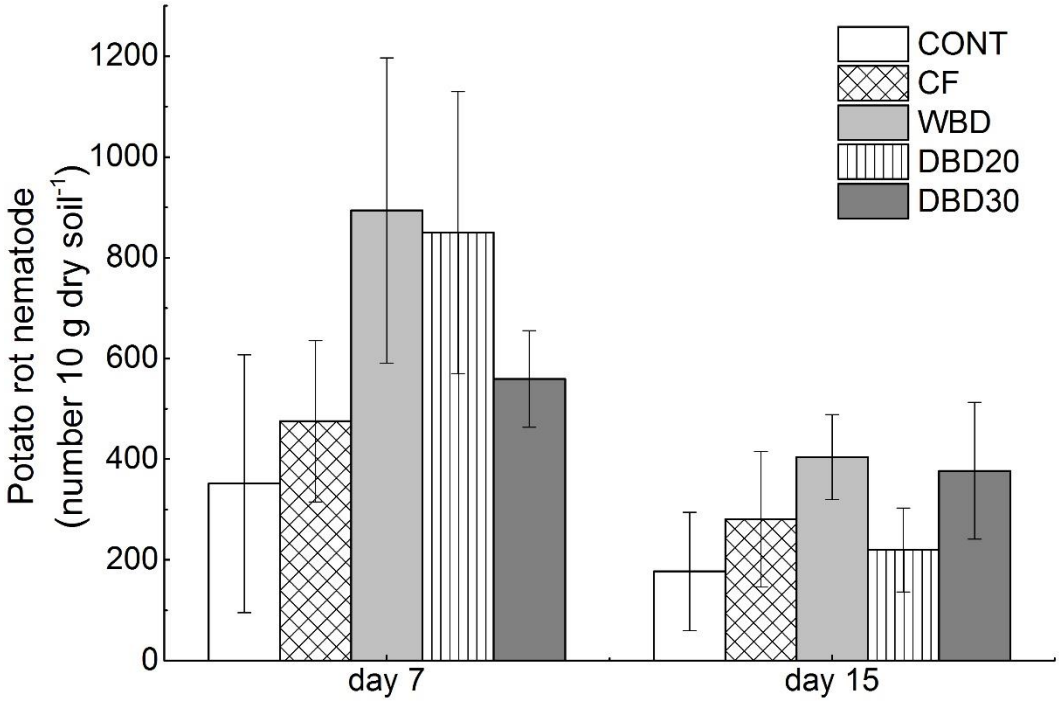


Figure 1.2 Effect of wet and dry biogas digestate application on potato rot nematode (*Ditylenchus Distructor)* by real-time PCR during 15 days incubation. WBD: wet biogas digestate, DBD20: dry biogas digestate with an initial C/N ratio of 20, DBD30: dry biogas digestate with an initial C/N ratio of 30. The values are the means $\pm$ SD. Bars represent standard deviations (n = 3).

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