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Application of Biogas Digestate with Rice Straw Mitigates Nitrate Leaching Potential and Suppresses Root-Knot Nematode (*Meloidogyne incognita*)

Yuexi Wang ¹, Seiya Chikamatsu ¹, Tuya Gegen ¹, Kozue Sawada ¹, Koki Toyota ^{1,*}, Shohei Riya ² and Masaaki Hosomi ²

¹ Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan; luna_wangyuexi@hotmail.com (Y.W.); s155422v@st.go.tuat.ac.jp (S.C.); gege15904@163.com (T.G.); kozue.do@gmail.com (K.S.)

² Graduate School of Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan; sriya@cc.tuat.ac.jp (S.R.); hosomi@cc.tuat.ac.jp (M.H.)

* Correspondence: kokit@cc.tuat.ac.jp; Tel.: +81-42-388-7915

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Abstract: This study investigated the effects of biogas digestate application to soil with rice straw on nitrate leaching potential and nematicidal activity toward root-knot nematodes *Meloidogyne incognita*. The following seven treatments were set up: (i) control (CONT); (ii) chemical fertilizer (CF); (iii) wet biogas digestate derived from pig manure (WBD); (iv) and (v) dry biogas digestate derived from a mixture of pig manure and rice straw at an initial C/N ratio of 20 and 30 (DBD20 and DBD30); (vi) and (vii) DBD20 mixed with rice straw to adjust the 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 the inorganic ammonium nitrogen contents. Nitrate contents readily increased in all the treatments with incubation, except for Mix2, and those at day 90 were decreased with increasing initial labile C contents. Garden balsam was grown as a test plant for root-knot nematodes using the soils at day 90 and the results showed that the gall index was significantly lower in Mix2 and Mix1 than in CF. These results suggest that dry digestate mixed with rice straw might have potential for lower nitrate leaching and nematicidal properties.

Keywords: anaerobic digestion; nitrification; nitrogen immobilization; nematode suppression

1. Introduction

Anaerobic digestion is a process which converts organic substrates, such as organic waste and energy crops, into biogas and digested residue, which is commonly named digestate. The digestate is used as a fertilizer in agriculture and thus anaerobic digestion is an environmentally useful technology [1]. The fermentation system consists of two types, wet and dry fermentation processes, based on the moisture conditions in biogas production [2]. Wet digestion processes are operated with total solids concentrations in the reactor below 10% and the digestate is pumpable and can be spread on fields for fertilization. Dry digestion processes are operated with total solids content in the reactor between 15% and 35%, and therefore the digestate has solid properties [2]. Both digestates contain higher NH₄-N contents and higher pH values than the original materials [3].

Biogas digestate is a highly valuable nutrient-rich and humus-rich fertilizer [4]. 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 [4,5]. However, when digestate is applied to soil, ammonium in the

biogas digestate can be readily nitrified [6]. Previous studies have demonstrated that biogas digestate application led to a higher soil nitrification rate than manure [7,8] and compost [9]. Thus, the use of biogas digestate may increase nitrate leaching risks [10–14], as reported in our previous study [15] 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 [16]. 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 [17,18]. Thus, the combined application of straw with manure [19,20] or mineral N fertilizer [21,22] reduces the superfluous accumulation of mineral N in soil and its losses [23]. Therefore, we 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.

Root-knot nematodes (RKN), *Meloidogyne* spp., are a major pest and cause significant losses in the yields or quality of crops [24,25]. Current management strategies are the use of chemical nematicides, organic amendments, resistant cultivars, soil solarization and biological control [26,27]. 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 [28] and to sugar beet by *Heterodera schachtii* [29]. Several toxic compounds are involved in nematode suppressive properties, such as ammonia [30,31], fatty acids [32], chitin, release of plant-specific toxins etc. [33]. 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 [28–30]. We 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.

As we describe above, previous studies have separately reported a decrease in nitrate leaching risk with rice straw and the effect of digestate application on nematode populations, and very few studies have focused on both nitrate leaching mitigation and nematode suppression [14]. Thus, we attempted to test the hypothesis that the application of dry biogas digestate with rice straw can mitigate nitrate leaching risk and suppress root knot nematodes due to the increase in labile C through mixing with rice straw.

The objectives of this study were to evaluate the effect of the application of dry and wet biogas digestates with rice straw on the dynamics of inorganic nitrogen in soil, in particular nitrate leaching potential, and to evaluate their effect on root-knot nematodes.

2. Materials and Methods

2.1. 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 [34]. 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 [35]. 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-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 1. Chemical properties of digestates used in the present study.

	Water Content (%)	pH (H ₂ O)	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
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.

2.2. 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 (H₂O) 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 (H₂O) 5.7, MWHC 0.81 g g⁻¹ and texture; sandy clay loam (23% clay, 27% silt and 50% sand) [36].

2.3. 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 (NH₄⁺-N) kg⁻¹ dry soil (equivalent to ~300 kg NH₄-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 NH₄⁺-N fraction in the digestates [37]. 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.

2.4. Dynamics of Inorganic Nitrogen

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 (NO₃⁻-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 K₂SO₄ 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 NH₄⁺ and NO₃⁻ in extracts were analyzed colorimetrically using the methods of Kandeler and Gerber [38] and Cataldo et al. [39], respectively. EOC was measured with a TOC-V_{CSH/CSN}. For measuring soil pH (H₂O), 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 (H₂O) 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. [6] as:

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

$$NC(\%) = 100 \times \frac{[(\text{NO}_3\text{-N}_{90d} - \text{NO}_3\text{-N}_{0d})_{\text{soil+digestate}} - (\text{NO}_3\text{-N}_{90d} - \text{NO}_3\text{-N}_{0d})_{\text{soil}}]}{(\text{added total N})} \quad (2)$$

2.5. Nematode Suppressive Experiment

We 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 [40].

Soil DNA was extracted in duplicate using the method of Sato et al. [41] from 0.5 g of 10 g oven-dried ball milled soil and finally dissolved in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was diluted 10-fold and used as a template in real-time PCR analysis. Specific primers for root-knot nematodes used in this study were RKNf (5'-GCTGGTGTCTAAGTGTGCTGATAC-3')-RKNr [(5'-GAGCCTAGTGATCCACCGATAAG-3')] [42]. Real-time PCR was performed in a StepOne Real-Time PCR System (Thermo Fisher Scientific, Tokyo, Japan) with a final volume of 10 µL containing 2 µL of template DNA, 0.4 µL of 10 µM primers and 5 µL of Fast SYBR®Green Master Mix (Life Technologies Japan, Tokyo, Japan) under the manufacturer's recommended conditions (95 °C for 10 s, (95 °C for 5 sec and 60 °C for 20 sec) for 45 cycles). A negative control was also included using distilled water instead of a template DNA. The population of root-knot nematodes was estimated based on the equation ($Ct \text{ value} = -0.9179 \times \log_2 (\text{No. of } M. \text{ incognita } 20 \text{ g}^{-1} \text{ soil}) + 37.37$) reported by Watanabe et al. [43].

2.6. 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 $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ 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. Results

3.1. Effect of Fertilization on the Dynamics of Inorganic N

Ammonium concentrations of Kikugawa and Fuchu soil at day 0 were 29 and 44 mg $\text{NH}_4\text{-N}$ 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 $\text{NH}_4\text{-N}$ kg^{-1} soil). With incubation, $\text{NH}_4\text{-N}$ concentration markedly decreased to less than 20 mg $\text{NH}_4\text{-N}$ kg^{-1} soil at day 7 of incubation in all of the treatments in Kikugawa soil, indicating the ready occurrence of nitrification (Figure 1). In Fuchu soil, it was still higher than 20 mg $\text{NH}_4\text{-N}$ kg^{-1} soil in CF, WBD, DBD20, and DBD30 at day 7, while it was less than 10 mg $\text{NH}_4\text{-N}$ kg^{-1} soil in Mix1 and Mix2 (Figure 1). After day 35, the $\text{NH}_4\text{-N}$ concentration was consistently lower than 10 mg $\text{NH}_4\text{-N}$ 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 1). This result indicated that N_m was higher in Kikugawa soil than in Fuchu soil. At day 7 of incubation, $\text{NO}_3\text{-N}$ 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 $\text{NO}_3\text{-N}$ 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, $\text{NO}_3\text{-N}$ 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 1. K_2SO_4 -extractable $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ 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.

3.2. 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 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 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.

Figure 2. 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 ($p < 0.05$).

Table 2. 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 ($p < 0.05$).

3.3. Factors Affecting the Nitrate Leaching Potential and Nematode Population

The pH (H₂O) 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 3). There was no significant relationship between initial soil pH and NO₃-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 3). 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 3A) or NC ($R^2 = 0.750$, $p < 0.05$ in Kikugawa soil and $R^2 = 0.762$, $p < 0.05$ in Fuchu soil, Figure 3B) 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 3C).

Table 3. Changes of soil pH and K₂SO₄-extractable organic C (EOC) content in Kikugawa and Fuchu soils after different fertilizer amendments on day 0.

Treatment	Kikugawa Soil		Fuchu Soil	
	pH	EOC (mg kg ⁻¹)	pH	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 3. 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. * $p < 0.05$.

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 4).

Figure 4. The relationship between root-knot nematode population at day 90 and EOC at day 0 in Kikugawa soil. ** $p < 0.01$.

4. Discussion

While net nitrification occurred in all the treatments in both soils (Figure 1), 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 [44], but there was no relationship between the initial nitrification rates and initial soil pH values in this study, unlike the study by Sawada and Toyota [15] 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 3). This phenomenon is the occurrence of N starvation due to microbial immobilization of N and has been already reported by many studies [45–48]. The size and C/N ratio of the easily degradable organic fraction of residues have critical roles in regulating N dynamics in soils [49]. According to Cheng et al. [11], 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 our 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 [50] and Alexander [51], 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 3).

Alburquerque et al. [6] 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 $\text{NO}_3\text{-N}$ 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, $\text{NO}_3\text{-N}$ 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 1). The period of N retention and N supply processes differ among soils [52]. According to Zhao et al. [48], 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. [23] who, using three long-term different fertilized soils, reported that N mineralization starts earlier in a fertile soil after the occurrence of N immobilization.

The populations of root-knot nematodes were drastically decreased in Mix2 and remained the lowest (Figure 2). This result was further supported by the significantly ($p < 0.05$) lower gall index in Mix2 than in CF (Table 2). 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 [53] 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 [54], 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 our study, all digestate amendment treatments were set up at the same level of $200 \text{ mg NH}_4\text{-N kg}^{-1}$ 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 [55,56]. 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 2), indicating that rice straw could have a nematode suppressive property. Maareg et al. [57] 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. [58] 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. [28], 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. [29] 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 2). 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 4). Several studies have already reported that not all types of organic amendments are beneficial in the suppression of root-knot nematodes [59,60]. In contrast, DBD30 significantly decreased populations of root-knot nematodes from day 60 to day 90 compared to WBD (Figure 2). DBD30 showed a significantly higher EOC at day 0 than WBD (Table 3), and therefore, $\text{NO}_3\text{-N}$ concentration was consistently lower in DBD30 than in WBD from day 14 to day 90 (Figure 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.

5. Conclusions

In conclusion, the addition of biogas digestate mixed with a higher amount of rice straw can effectively decrease nitrate leaching potential in soils by increasing the labile C contents of the amendment, which induces soil net N immobilization. Moreover, it also provides pronounced nematode suppression, and thus could be safely used as a soil amendment in nematode management programs.

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