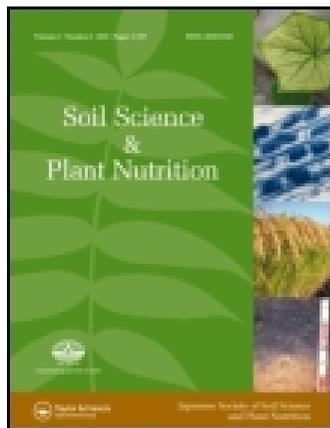


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Soil Science and Plant Nutrition

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tssp20>

Effects of soil aggregate size, moisture content and fertilizer management on nitrous oxide production in a volcanic ash soil

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Published online: 10 Oct 2011.

To cite this article: Farzana Diba, Mariko Shimizu & Ryusuke Hatano (2011) Effects of soil aggregate size, moisture content and fertilizer management on nitrous oxide production in a volcanic ash soil, *Soil Science and Plant Nutrition*, 57:5, 733-747, DOI: [10.1080/00380768.2011.604767](https://doi.org/10.1080/00380768.2011.604767)

To link to this article: <http://dx.doi.org/10.1080/00380768.2011.604767>

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ORIGINAL ARTICLE

Effects of soil aggregate size, moisture content and fertilizer management on nitrous oxide production in a volcanic ash soil

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*Soil Science Laboratory, Graduate School of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Hokkaido 060-8589, Japan***Abstract**

A laboratory incubation study was conducted to determine the effects of soil aggregate size, soil moisture content and manure application on nitrous oxide (N₂O) production through nitrification and denitrification. In Southern Hokkaido, soil samples were taken from a mineral soil layer (2.5–10 cm) of a grassland to which chemical fertilizer and manure had been applied. The soil aggregates were air-dried and sieved with 4.5 mm and 2 mm sieves, and the soil moisture was adjusted to 60% and 80% of field water capacity (FWC). Immediately after moistening, incubation was initiated and lasted for 9 days at 20°C. Following the start of incubation, a flush of N₂O, carbon dioxide (CO₂) and nitric oxide (NO) was observed. Production of all gases was higher in larger aggregates from the manure-applied soil. Productions of CO₂ and NO were not significantly influenced by soil moisture content, but N₂O production was considerably higher in 80% FWC as compared with 60% FWC. Based on the results of the N₂O–nitrogen (N)/NO–N ratio, the process of N₂O production was mainly due to nitrification in 60% FWC and denitrification in 80% FWC. Soil chemical properties, especially ammonium–N (NH₄⁺–N), nitrate–N (NO₃–N) and water extractable organic C (WEOC) and microbial biomass C (MBC) also changed immediately after moistening. These changes were higher in larger aggregates from the manure-applied soil. Potential denitrification enzyme activity (DEA) was significantly higher in larger aggregates from manure-applied soil with higher moisture content. The N₂O production in both 60% and 80% FWC correlated significantly with MBC and DEA. Regardless of soil moisture conditions, MBC correlated significantly with DEA, WEOC consumption and apparent N mineralization. These facts suggest that larger soil aggregates could have quickly developed suitable internal conditions for microbial activity inside the aggregates and consequently stimulated N₂O production through nitrification and denitrification.

Key words: Denitrification, manure application, nitrification, N₂O production, soil aggregate.

INTRODUCTION

Nitrous oxide (N₂O) is one of the most important “trace gases” in the atmosphere. It contributes to reactions that influence atmospheric chemistry and radiative properties. Nitrous oxide is reactive and participates in destruction of ozone in the stratosphere; it is stable for about 120 years and contributes to the greenhouse effect in the

troposphere (Crutzen 1981). Among the various sources of N₂O, about 13.5% of the gas is produced from agricultural soil (IPCC 2007). Accordingly, global attention has focused on agricultural soils for the amelioration of N₂O gas production.

Soil N₂O production is influenced by a range of microbiological, chemical and physical soil processes and properties. Generally N₂O is formed by two processes: nitrification and denitrification (Davidson and Swank 1990). There are natural sources of N₂O and there are anthropogenic sources of N₂O. The combined soil processes of mineralization, nitrification and denitrification convert applied organic and inorganic N-compounds to N₂O, which then diffuses from the soil to

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Received 10 April 2011.

Accepted for publication 8 July 2011.

the atmosphere and remains highly variable in space and time (Wrage *et al.* 2001; Davidson *et al.* 2004; Toma *et al.* 2010). Physical-chemical properties of soil strongly influence N₂O emission. Specifically, soil aggregate is an important controlling factor for soil properties through the modification of pore distribution affecting aeration, water retention and drainage.

Soil aggregates are defined as groups of soil particles that are bound to each other more strongly than to adjacent particles. Organic matter “glues” are produced when soil biota break down dead roots and litter holds the particles together. Threadlike strands of fungi also bind particles into aggregates. In fact, pore structure in aggregated soil is composed of intra- (micro) and inter-aggregate (macro) pores. Both types of pores affect the transport of water, solutes and gases. The macro pores are a major determining factor in the transport of substances through the soil, whereas the micro pores are important in the stock or supply of substances in the soil (Lefelaar 1993). Aggregation can physically protect soil organic carbon (C) from decomposition by trapping the C within the aggregates and thereby reducing direct contact between the organic matter and soil microorganisms (Angers *et al.* 1997; Gregorich *et al.* 1997). Aggregates not only physically protect soil organic matter (Tisdall and Oades 1982), but also influence microbial community structure (Hattori 1988), limit oxygen diffusion (Sexstone *et al.* 1985), regulate water flow (Prove *et al.* 1990), and determine nutrient adsorption and desorption (Linguist *et al.* 1997; Wang *et al.* 2001). Aggregate stability is a good indicator of the content of organic matter, biological activity, and nutrient cycling in the soil. Drury *et al.* (2004) separated the soil samples into six aggregate fractions (<0.25, 0.20–0.50, 0.50–1.0, 1.0–2.0, 2.0–4.0, 4.0–8.0 mm) and found that N₂O from denitrification increased with increasing aggregate size. Increased N₂O production with an increase in aggregate size was also reported by Khalil *et al.* (2005). Renault and Stengel (1994) found that small aggregates remained aerobic until saturated, but large unsaturated aggregates tended to have an anaerobic center. Aggregate size may be one of the significant factors that regulate the N₂O emission from soil.

The N₂O losses from manure plots extended over a longer period of time and were greater in magnitude than the losses from chemical nitrogen (N) fertilization (Jones *et al.* 2007). Other studies (Scott *et al.* 2000) have observed higher N₂O fluxes from manure and sewage applications as compared to chemical N fertilizer application. High N₂O fluxes from manure treatments can be partly explained by the higher total N input than chemical fertilizer treatments, providing more available N that can be mineralized over a longer period of time (Jin *et al.* 2009). Shimizu *et al.* (2010) stated that the cumulative

N₂O flux showed a significant positive correlation with mineral N surplus, which was calculated as the difference between the total mineral N supply and aboveground N uptake. Another reason for the increased N₂O fluxes could be the addition of organic C with the manure, which is known to stimulate denitrification (Bremner 1997).

Soil moisture content can also have a significant effect on N₂O emission. Dobbie and Smith (2003) found that rainfall (particularly around the time of N application), with its consequent effect on the water filled pore space (WFPS), was the main driving factor of N₂O emission during the growing season in a grassland. David and Josep (2005) demonstrated two events following precipitation into dry soils: a gradual increase of CO₂ from enhanced biological activity. Emissions of carbon dioxide (CO₂), N₂O and methane (CH₄) are affected by precipitation in at least two ways: (1) soil water content affects soil aeration which, in turn, affects microbial processes of production and consumption of these trace gases, and (2) the amount of precipitation can alter root turnover, litter-fall, decomposition, and mineralization, which, in turn, affect the availability of C and N substrates for trace gas production (Davidson and Schimel 1995; Davidson *et al.* 2000). However, Wang and Cai (2008) showed that N₂O production in soil increased slightly with an increase in soil moisture content from 40% to 70% of water holding capacity (saturated water content), but increased dramatically at 100% of water holding capacity in an incubation study with disturbed soil. The microbial activity could increase with soil water content until diffusion of oxygen (O₂) is restricted and the environment becomes anaerobic (Linn and Doran 1984). Thus, soil water content is one of the major controlling factors for denitrification and nitrification processes. Soil microbial C is an important indicator of soil fertility and biological quality. It also plays a vital role in soil nutrient transformations, acting as a labile nutrient pool available to plants (Jenkinson 1990). Denitrifying enzyme activity (DEA) was proposed by Smith and Tiedje (1979) as a way of assessing the potential optimum activity of existing denitrifying enzymes in soil. So, DEA is considered to be an important factor in identifying the main driver of denitrification among different aggregate size, water content and fertilizer management. Soil moisture, soil respiration, soil aggregation and soil compaction are key factors determining the aeration of soils and the formation of anoxic microsites (Granli and Bøckman 1994), and these factors may interact with and amplify each other in their effect on N₂O production. The effects of these soil variables on N₂O production remain unclear (Nishina *et al.* 2009). So, the main purpose of this study was to identify the interaction effects of different aggregate size, moisture content and fertilizer

management on N₂O production through N mineralization, nitrification and denitrification.

MATERIALS AND METHODS

Study site

Soil samples used in this experiment were taken from a managed grassland in Shizunai Experimental Livestock Farm of Hokkaido University in Southern Hokkaido, Japan (42°26'N, 142°29'E). The grassland soil was covered by reed canary grass (*Phalaris arundinacea*) and meadow foxtail grass (*Aleopurus pratensis*). The soil is derived from Tarumae (b) volcanic ash, and is classified as Thaptic Melanudands (Soil Survey Staff 2006; Mollic Andosol (IUSS 2006)). The characteristics of the study site are described in detail by Shimizu *et al.* (2009). On the grassland, the experimental plots were established in 2005 and treated with chemical fertilizer (fertilizer plot) and manure (manure plot) (Shimizu *et al.* 2009). Annual N application rate was 124 kg N ha⁻¹ as ammonium sulfate and ammonium phosphate in the fertilizer plot, and 296 kg N ha⁻¹ as beef cattle manure with bedding litter (bark) in the manure plot. Manure C at 6884 kg C ha⁻¹ was also applied in the manure plot. Soil samples were taken from a 2.5–10 cm depth in each plot on 27 June 2008 when the first harvest of grass was completed. The collected samples were brought to the laboratory and immediately stored in refrigeration at 4°C. The fresh soil samples were then spread on trays and air dried at room temperature.

Aggregate preparation

Air-dried soil samples were gently crushed by hand in order to pass through a 4.5 mm sieve; roots and stones were removed from the samples by hand. The soil aggregates of <2 mm were then separated using a 2 mm sieve. In order to measure the field water capacity (FWC), about 10 g of air dried soil of each aggregate size was saturated with water by draining loosely packed soil in a funnel of 0.55 g cm⁻³ for 24 h. The FWC of fertilizer-applied soil (hereinafter referred to as "FS") with 4.5 mm and 2 mm aggregate fractions were 0.96 and 0.93 g g⁻¹, and of manure-applied soil (hereinafter referred to as "MS") with 4.5 mm and 2 mm were 1.01 and 0.98 g g⁻¹, respectively. The FWC of aggregate fractions was determined to calculate the amount of distilled water that was added to achieve the desired moisture contents, which were 60% and 80% of FWC. Aggregate preparation and adjustment of moisture contents were performed at room temperature (20°C). The incubation procedure was initiated immediately after adding the distilled water to soil samples.

Incubation procedure

After adjusting the soil moisture content, 20 g dry-basis soil samples were loosely packed to a volume of 80 ml and to a bulk density of 0.52 g cm⁻³ in plastic cups and put into 1.8 L Mason jars. The jars were sealed tightly. A jar without a soil sample was also prepared and labeled as a blank. Ambient wet air was passed through a vinyl tube connected to the jar at a rate of 0.2 ml min⁻¹ for 30 min to replace the gas in the jar completely. About 250 ml of an air sample was extracted from the headspace of the blank jar into a Tedlar bag using a 50 ml syringe for nitric oxide (NO) and CO₂ analysis, and a sub-sample of 20 ml was taken from the Tedlar bag and injected into an evacuated 10 ml vial using a 25 ml syringe for N₂O analysis. These air samples were regarded as time 0 min. The Mason jars were left at 20°C for 24 h, and then air samples were again taken in the same manner. The jars were opened after the air sampling and were immediately closed tightly in order to avoid change to soil moisture by evaporation from the aggregates. Incubation was conducted for 9 days from 17 to 25 June 2010. Three replicates were conducted in the experiment.

Nitrous oxide concentration was determined by a gas chromatograph with electron capture detector (model GC-14B, Shimadzu, Kyoto, Japan). Carbon dioxide concentration was analyzed with an infrared CO₂ gas analyzer (ZFP9GC11, Fuji Electric System, Tokyo, Japan). Nitric oxide concentration was analyzed with a Chemiluminescence N Oxide Analyzer (Model 265 P, Kimoto Electric, Osaka, Japan). Gas production rate (F, μg kg⁻¹ h⁻¹) was estimated using the following equation:

$$F = \rho \times V/W \times \Delta c/\Delta t \times 273/T$$

where ρ is the density of gas at the standard condition (μg m⁻³), V is the volume of the jar (m³), W is the dry soil weight (kg), Δc (m³ m⁻³) is the gas concentration change in the jar, Δt is the incubation period (hour), and T is the absolute temperature (K). Positive values of F indicate gas production from soil while negative values indicate gas consumption from the atmosphere.

The cumulative gas production (F) during the incubation (mg kg⁻¹ d⁻¹) was estimated using the following equation:

$$\text{Cumulative F} = \Sigma(F_i \times D_i)$$

where F_i is the mean gas production rate of the two sampling intervals (mg kg⁻¹ day⁻¹) and D_i is the number of days in the sampling interval (1 day).

Soil properties analysis

Before and after incubation, concentrations of water extractable organic C (WEOC), NH₄⁺-N and NO₃⁻-N and

soil pH, total carbon (C) and total nitrogen (N) were analyzed. Also analyzed were soil microbial biomass C (MBC) before incubation and potential denitrification enzyme activity (DEA) after incubation. All analyses were conducted with three replicates. Wet soil samples were extracted with distilled water for measurement of WEOC, NO_3^- -N and soil pH, and with 2 mol L^{-1} KCl for measurement of NH_4^+ -N about two hours before and immediately after the incubation. Soil pH, NO_3^- -N and WEOC concentrations were analyzed using a combined electrode pH meter (F-8 pH meter, Horiba, Japan), an ion chromatographer (QIC analyzer, Dionex Japan, Osaka, Japan) and a total organic carbon analyzer (Model TOC-5000A, Shimadzu, Kyoto, Japan), respectively. The NH_4^+ -N concentration was analyzed using colorimetry with indophenol-blue (Uvmini-1240, Shimadzu).

Total C and N concentrations of the soil samples were measured using an N/C analyzer (Sumigraph NC-1000, Sumika Chemical Analysis Service, Ehime, Japan) after soil samples were air dried and ground.

Microbial biomass C was analyzed before incubation by the chloroform fumigation-extraction method. It was calculated by the following formula:

$$\text{MBC} = \Delta\text{C}/k$$

where ΔC is the difference in concentration of organic C extracted by 0.5 mol L^{-1} K_2SO_4 between fumigated and non-fumigated soil (mg kg^{-1}), and k is a factor (0.43, Martens 1995).

Potential DEA was determined by the acetylene block technique, which inhibits the final conversion of N_2O to N_2 gas (Tiedje 1994). Soil samples were incubated under an anaerobic condition at 25°C with solution treated with chloramphenicol (1 g L^{-1}) and NO_3^- -N ($200 \text{ mg KNO}_3\text{-NL}^{-1}$). Fifteen g of wet soil was placed in a 100 ml conical flask, and 15 ml treated solution was added to the flask. The flasks were evacuated and flushed with N_2 to ensure anaerobic conditions, and acetylene (C_2H_2) gas was added to a final concentration of 10% (10 kPa) in the headspace. The headspace gas was sampled with a syringe at 2 and 4 h and denitrification rates were calculated using regression coefficients obtained from plotting N_2O concentrations against sampling time.

Judgment of denitrification and nitrification

The $\text{N}_2\text{O-N/NO-N}$ ratio is the index of N_2O production from nitrification or denitrification (Lipschultz *et al.* 1981). The ratio is <1 during nitrification, 1–100 during both nitrification and denitrification, and >100 during denitrification. Using the N_2O and NO production rates during the incubation, the $\text{N}_2\text{O-N/NO-N}$ ratio was

calculated for the evaluation of denitrification and nitrification.

Statistical analyses

Statistical analyses were performed with SPSS 13.0 for Windows 2004 (SPSS Inc. Chicago, Illinois, USA). The differences between the treatments in gas productions, soil chemical properties, MBC and DEA were analyzed using analysis of variance (ANOVA). The differences of soil chemical properties before and after incubation were analyzed with paired t-test and simple linear regression analyses. Pearson's correlation coefficients (Pearson's R) were calculated between gas productions and soil chemical properties MBC and DEA.

RESULTS

Soil chemical properties

Table 1 shows the soil chemical properties (WEOC, NH_4^+ -N, NO_3^- -N, pH, total C and total N) measured before and after incubation, MBC measured before incubation, and DEA measured after incubation. Paired t-test for differences between soil chemical properties before and after incubation showed that WEOC, NH_4^+ -N, pH and total N were significantly different before and after incubation. In all treatments, fertilizer management (chemical fertilizer or manure), soil moisture content (60% or 80% of FWC) and aggregate size ($<2 \text{ mm}$ or 4.5 mm), WEOC, pH and total N decreased and NH_4^+ -N increased during the incubation. Nitrate-N concentration of fertilizer-applied soil decreased, but that of manure-applied soil increased during the incubation. However, there was no apparent trend in total C. These results indicate that soil properties, especially WEOC and NH_4^+ -N, changed immediately after the addition of water into soil and the influence of the change continued throughout the incubation. Results of ANOVA showed that all the soil chemical properties, MBC and DEA were significantly higher in 4.5 mm aggregates than in 2 mm aggregates. WEOC, NH_4^+ -N, NO_3^- -N, MBC and DEA were higher in 80% of FWC than in 60% of FWC, but pH, total C and total N were not influenced by soil moisture. Manure-applied soil showed higher MBC and soil chemical properties except for total N than FS. However, DEA was not influenced by the fertilizer managements.

Gas production rates

Figure 1 shows the change in N_2O production rates during the incubation. The N_2O production rate was highest immediately after the incubation started. However, the highest N_2O production rate was

Table 1 Soil properties before and after incubation from different treatments

Treatment	WEOC (mg kg ⁻¹)		NH ₄ ⁺ -N (mg kg ⁻¹)		NO ₃ ⁻ -N (mg kg ⁻¹)		pH		Total C (g kg ⁻¹)		Total N (g kg ⁻¹)		MBC (mg kg ⁻¹)		DEA (μg kg ⁻¹ h ⁻¹)			
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After		
60% of field water capacity																		
FS, 4.5 mm	633	362	69	121	29	23	5.6	5.5	40	39	4.3	3.9	1836			5.7		
FS, <2 mm	526	258	42	102	14	13	5.3	5.3	41	39	4.1	3.9	1234			1.5		
MS, 4.5 mm	1036	518	104	300	26	37	6.1	5.6	43	41	4.3	4.0	2407			7.1		
MS, <2 mm	806	288	75	152	8	12	5.8	5.3	33	32	3.5	3.2	1365			2.0		
80% of field water capacity																		
FS, 4.5 mm	1286	677	134	216	70	46	5.6	5.3	39	40	4.3	4.0	3499			11.6		
FS, <2 mm	833	397	60	153	27	20	5.5	5.1	40	39	4.0	3.9	1944			3.2		
MS, 4.5 mm	2315	1100	219	613	65	72	6.8	5.5	43	42	4.3	4.1	5217			13.0		
MS, <2 mm	973	437	112	220	17	17	5.8	5.2	32	34	3.7	3.3	2095			3.3		
The difference in each soil chemical property before and after incubation (Paired <i>t</i> -test)																		
<i>P</i> value	0.001		0.014		0.59		0.009		0.184		<0.001		—		—		DEA	
<i>P</i> value of ANOVA	WEOC		NH ₄ ⁺ -N		NO ₃ ⁻ -N		pH		Total C		Total N		MBC		DEA			
Management	<0.001		<0.001		<0.001		<0.001		<0.001		0.209		<0.001		0.521			
Moisture	<0.001		<0.001		<0.001		0.061		0.117		0.836		<0.001		0.012			
Aggregate size	<0.001		<0.001		<0.001		0.001		<0.001		0.008		<0.001		<0.001			
Management × Moisture	<0.001		<0.001		0.333		0.304		0.658		0.594		0.007		0.959			
Management × Aggregate	<0.001		0.001		0.152		0.054		<0.001		0.156		<0.001		0.664			
Moisture × Aggregate	<0.001		<0.001		<0.001		0.255		0.289		0.922		<0.001		0.115			
Management × Moisture × Aggregate	<0.001		0.002		0.623		0.072		0.783		0.671		0.009		0.964			

Three replication data of each treatment before incubation were used in ANOVA; pair *t*-test analysis was performed using an average value of the data obtained before and after incubation. C, carbon; DEA, denitrification enzyme activity; FS, fertilizer-applied soil; MBC, microbial biomass C; MS, manure-applied soil; N, nitrogen; NH₄⁺-N, ammonium N; NO₃⁻-N, nitrate N; WEOC, water extractable organic C.

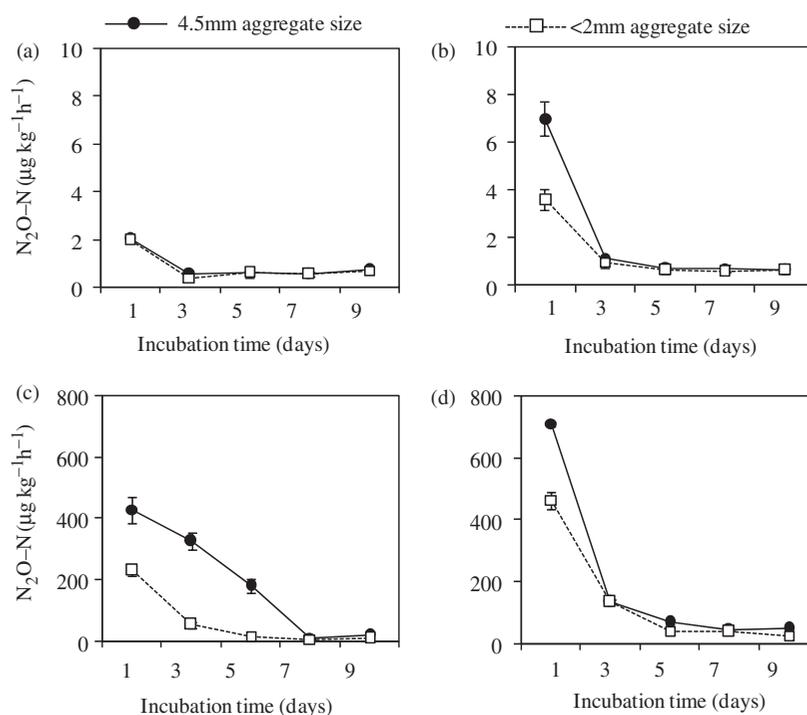


Figure 1 Nitrous oxide (N_2O) production rate during incubation from (a) fertilizer and (b) manure-applied soils with 60% of field water capacity (FWC), and from (c) fertilizer and (d) manure-applied soils with 80% of FWC. Data of every treatment represents means \pm standard deviation. ($n = 3$). N, nitrogen.

considerably different between 60% and 80% of FWC. In 60% of FWC, the highest production rate ranged from 1.97 (FS and 2 mm aggregates) to 7.01 (MS and 4.5 mm aggregates) $\mu\text{g kg}^{-1}\text{h}^{-1}$, while in 80% of FWC, it ranged from 229 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (FS and 2 mm aggregates) to 711 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (MS and 4.5 mm aggregates). There was a tendency for MS to show a higher N_2O production rate than FS in both soil moisture contents. In MS, N_2O production rate decreased dramatically after the first flush, while it decreased slowly in FS. N_2O production rate was higher in 4.5 mm aggregates compared to 2 mm aggregates for each fertilizer management with the same soil moisture content, except in the FS with 60% of FWC. The CO_2 production rate showed a peak ranging from 2134 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (FS 2 mm aggregate with 60% of FWC) to 3817 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (MS 4.5 mm aggregate with 60% of FWC) until the fourth day after incubation started, and decreased gradually to 743 to 1816 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (Fig. 2). In each field management with each soil moisture content, CO_2 production rate was higher in 4.5 mm aggregates compared to 2 mm aggregates. There was a tendency for CO_2 production rate in both aggregate sizes to be higher in manure-applied soil than in fertilizer-applied soil. However, in manure-applied soil, it was higher in 80% than that in 60% of FWC, while there was no such tendency in fertilizer-applied soil.

The production rate of NO during the incubation is illustrated in Fig. 3. The NO production rate was highest immediately after the incubation started, ranging from 0.90 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (FS and 2 mm aggregates with 80% of FWC) to 5.02 $\mu\text{g kg}^{-1}\text{h}^{-1}$ (MS and 4.5 mm aggregates with 60% of FWC). The first flush of NO production was larger in 60% than that in 80% of FWC, and it was larger in 4.5 mm aggregates than in 2 mm aggregates in 60% of FWC, but similar between 2 mm and 4.5 mm aggregates in 80% of FWC. The production rate of NO in 60% of FWC decreased dramatically after the first flush, while in 80% of FWC it decreased only slightly after the first flush, then increased gradually. There was a tendency for the NO production rate in each aggregate size with the same soil moisture content to be higher in MS than that in FS.

Ratio of $N_2O-N/NO-N$

The ratio of $N_2O-N/NO-N$ in 60% of FWC mostly ranged from 1–100 and decreased with an increase in N_2O production (Fig. 4a). However, the ratio with 80% of FWC was >100 or near 100 and increased with an increase in N_2O production (Fig. 4b). The results indicate that the N_2O production during incubation in 60% of FWC was derived mainly from nitrification while N_2O production in 80% of FWC was derived from denitrification.

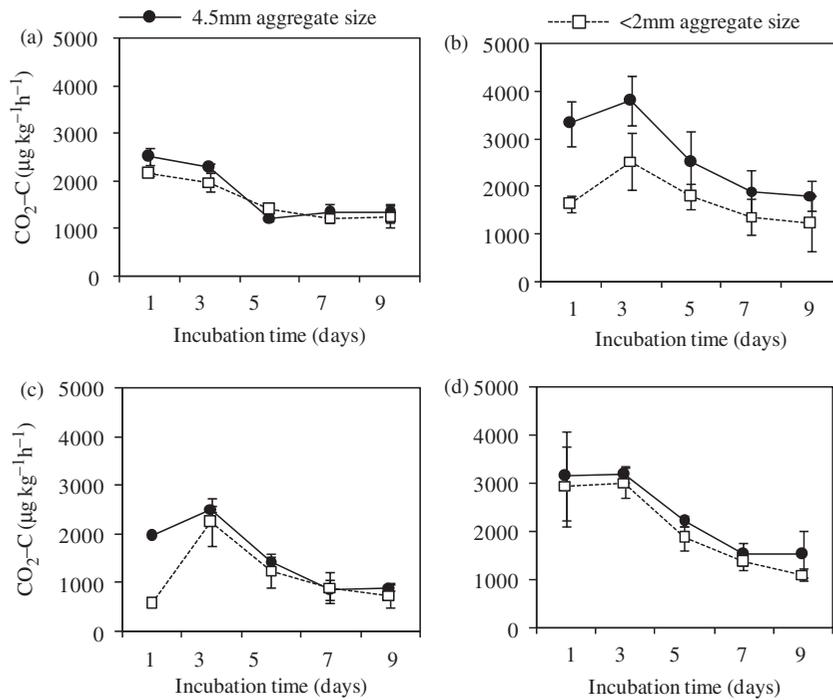


Figure 2 Carbon dioxide (CO₂) production rate during incubation from (a) fertilizer and (b) manure-applied soils with 60% of field water capacity (FWC), and from (c) fertilizer and (d) manure-applied soils with 80% of FWC. Data of every treatment represents means \pm standard deviation. ($n=3$). C, carbon.

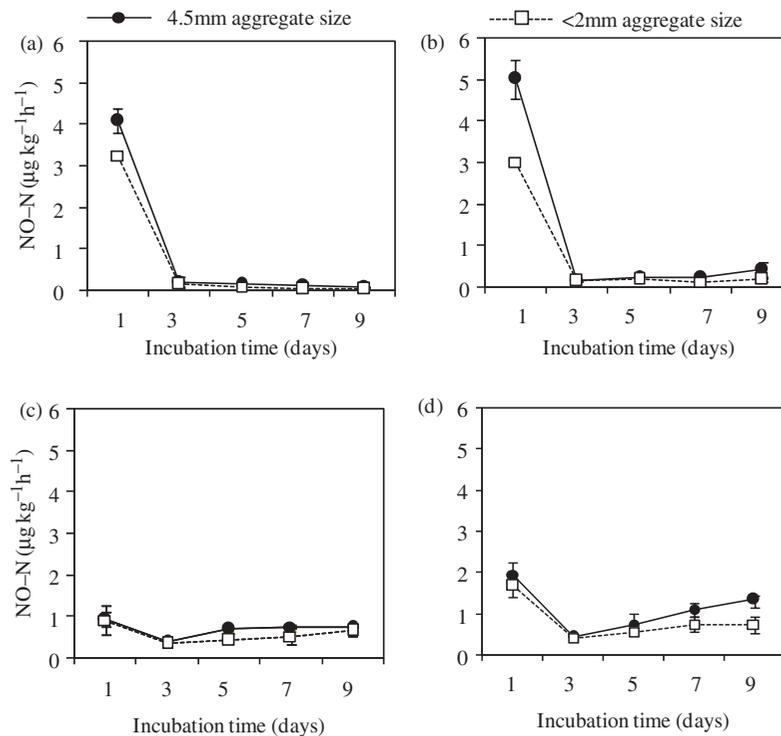


Figure 3 Nitric oxide (NO) production rate during incubation from (a) fertilizer and (b) manure-applied soils with 60% of field water capacity (FWC), and from (c) fertilizer and (d) manure-applied soils with 80% of FWC. Data of every treatment represents means \pm standard deviation. ($n=3$). N, nitrogen.

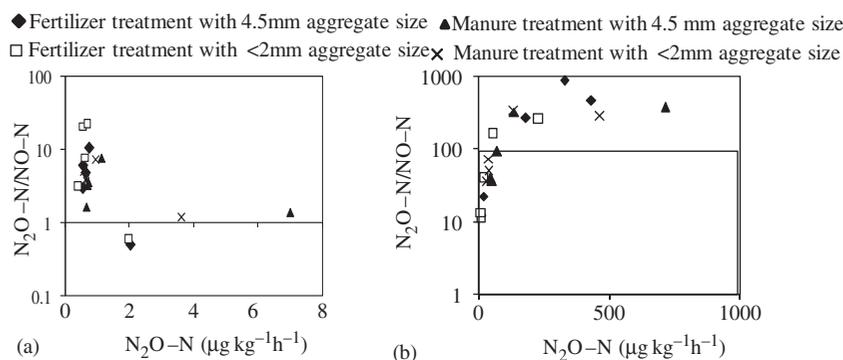


Figure 4 Relationship between nitrous oxide (N_2O) production rate and the N_2O -nitrogen (N)/nitric oxide-N ($N_2O-N/NO-N$) ratio in (a) 60% and (b) 80% of field water capacity.

Table 2 Cumulative productions of carbon dioxide (CO_2), nitrous oxide (N_2O) and nitric oxide (NO) from different treatments

Management	Moisture	Aggregate size	N_2O-N	CO_2-C	$NO-N$
			($mg\ kg^{-1}\ 9\ day^{-1}$)	($mg\ kg^{-1}\ 9\ day^{-1}$)	($mg\ kg^{-1}\ 9\ day^{-1}$)
			Mean	Mean	Mean
Fertilizer	60% of field water capacity	4.5 mm	0.182 ± 0.02	363 ± 19	0.169 ± 0.002
		<2 mm	0.169 ± 0.01	348 ± 14	0.129 ± 0.006
Manure	60% of field water capacity	4.5 mm	0.400 ± 0.02	611 ± 107	0.222 ± 0.011
		<2 mm	0.316 ± 0.01	396 ± 72	0.135 ± 0.001
Fertilizer	80% of field water capacity	4.5 mm	40.7 ± 3.1	332 ± 18	0.146 ± 0.009
		<2 mm	12.0 ± 0.9	257 ± 65	0.114 ± 0.005
Manure	80% of field water capacity	4.5 mm	39.1 ± 0.3	499 ± 72	0.227 ± 0.010
		<2 mm	27.6 ± 0.7	433 ± 68	0.168 ± 0.028
Sources of variation		Df	P value	P value	P value
Management		1	<0.001	<0.001	<0.001
Moisture		1	<0.001	0.090	0.995
Aggregate size		1	<0.001	0.003	<0.001
Management \times Moisture		1	<0.001	0.592	0.002
Management \times Aggregate		1	<0.001	0.099	0.002
Moisture \times Aggregate		1	<0.001	0.357	0.086
Management \times Moisture \times Aggregate		1	<0.001	0.051	0.332

Data represent mean \pm sd. Three replication data of each treatment were used in the analysis. C, carbon; Df, degrees of freedom; N, nitrogen.

Cumulative gas productions

Cumulative N_2O production was considerably different between 60% of FWC and 80% of FWC (Table 2). In 60% of FWC, cumulative N_2O production was similar among fertilizer managements and aggregate sizes, ranging from $0.170\ mg\ kg^{-1}\ 9\ day^{-1}$ (FS and 2 mm aggregates) to $0.400\ mg\ kg^{-1}\ 9\ day^{-1}$ (MS and 4.5 mm aggregates), while it ranged from $12.0\ mg\ kg^{-1}\ 9\ day^{-1}$ (FS and 2 mm aggregates) to $40.7\ mg\ kg^{-1}\ 9\ day^{-1}$ (FS and 4.5 mm aggregates) in 80% of FWC. The cumulative N_2O production was significantly influenced by fertilizer management, aggregate size, and soil moisture content. There was an interaction between each pair of treatments and among the three treatments,

due to a large difference in N_2O production between moisture contents.

Cumulative CO_2 production ranged from $258\ mg\ kg^{-1}\ 9\ day^{-1}$ (FS and 2 mm aggregates with 80% of FWC) to $611\ mg\ kg^{-1}\ 9\ day^{-1}$ (MS and 4.5 mm aggregates with 60% of FWC) (Table 2). The cumulative CO_2 production was significantly influenced by fertilizer management and aggregate size, but not influenced by soil moisture content. There was no significant interaction among the treatments.

Cumulative NO production ranged from $0.114\ mg\ kg^{-1}\ 9\ day^{-1}$ (FS and 2 mm aggregates with 80% of FWC) to $0.227\ mg\ kg^{-1}\ 9\ day^{-1}$ (MS and 4.5 mm aggregates with 80% of FWC) (Table 2). The cumulative NO production was not influenced by soil

moisture content but was significantly higher in the larger MS aggregate. However, there was a significant interaction between fertilizer management and soil moisture or management and aggregate size.

Table 3 presents the Pearson's correlation coefficients of the relationship between gas productions and each of the soil chemical properties before and after incubation, MBC and DEA. Since there was a significant difference in WEOC, NH₄⁺-N, NO₃⁻-N, MBC, DEA, and cumulative N₂O between 60% of FWC and 80% of FWC (Table 1 and 2), regression analysis was separately conducted for each soil moisture treatment.

The results of regression analysis for cumulative N₂O production in 60% of FWC showed a significant positive correlation with WEOC, NH₄⁺-N, and pH before and after incubation and MBC. However, it was significantly correlated with NO₃⁻-N only after incubation. On the other hand, the results for cumulative N₂O production in 80% of FWC had a significant positive correlation with WEOC, NH₄⁺-N, NO₃⁻-N, pH and MBC before incubation and WEOC, NO₃⁻-N, pH and DEA after incubation. Regardless of the moisture content, N₂O production significantly correlated with WEOC, NH₄⁺-N, NO₃⁻-N before and after incubation, MBC before incubation and DEA after incubation. N₂O production did not strongly correlate with pH, total N and total C.

Cumulative CO₂ production in 60% of FWC showed a similar tendency to that of cumulative N₂O production. In other words, cumulative CO₂ production in 60% of FWC showed a significant positive correlation with WEOC, NH₄⁺-N, and pH before and after incubation and MBC, and it was significantly correlated with NO₃⁻-N concentrations only after incubation. On the other hand, cumulative CO₂ production in 80% of FWC was different from that of the cumulative N₂O production in that 80% of FWC showed significant and positive correlation with NO₃⁻-N, and pH before and after incubation. Although there was a significant correlation with WEOC before incubation, correlation with WEOC after incubation was not significant. Without considering moisture content, CO₂ production showed significant correlation with NH₄⁺-N and pH before and after incubation, while it did not show significant correlation with other soil properties.

Cumulative NO production in 60% of FWC showed a significant correlation with MBC, DEA and all soil properties except for total N before incubation. While cumulative NO production in 80% of FWC showed a significant correlation with WEOC, NH₄⁺-N and pH before and after incubation, it was significantly correlated with NO₃⁻-N after incubation. Regardless of the moisture content, NO production showed a significant correlation with WEOC, NH₄⁺-N, pH, total C before and after incubation, MBC before incubation and DEA

after incubation. Nitric oxide production showed a significant correlation with NO₃⁻-N only after incubation.

Interaction of gases

The relationship between cumulative N₂O and CO₂ production was significant in 60% of FWC ($r=0.860$, $P<0.01$, $n=12$) while there was no significant relationship in 80% of FWC ($r=0.536$, NS, $n=12$). The relationship between NO and CO₂ production was also significant in 60% ($r=0.762$, $P<0.01$, $n=12$) and 80% of FWC ($r=0.776$, $P<0.01$, $n=12$).

DISCUSSION

Soil properties

Soil properties, especially WEOC and NH₄⁺-N, changed immediately after adding the water for both aggregates and this situation continued throughout the incubation process (Table 1) because the added water could activate the microbial processes inside the soil aggregate. In a lab incubation study, Martin and David (1996) found a rapid change in WEOC when moistening the dry and sieved soil samples and there was a 40% decline from the initial WEOC values after 9 weeks. The results of this study also showed a trend of decrease in WEOC during the 9-day incubation for all treatments (Table 1). On the one hand, the concentrations of NH₄⁺-N increased and that of NO₃⁻-N decreased with incubation time in fertilizer-applied soil (Table 1). A similar result was reported by Zhang and Wienhold (2002). They showed that at soil moisture levels greater than 80% of WFPS, NO₃⁻-N concentration declined rapidly and NH₄⁺-N concentration increased; this is likely due to anaerobic conditions existing at higher WFPS levels during an incubation study. Mori *et al.* (2010) also reported a similar result from an incubation study with different moisture contents. On the other hand, our results showed that the NO₃⁻-N content of MS increased after incubation, indicating that the consumption of NO₃⁻-N by denitrification was slower than the production of NO₃⁻-N from nitrification. Actually, the NH₄⁺-N content after incubation is the net result of the mineralization of organic N and nitrification, and the NO₃⁻-N content is the net result of NO₃⁻-N production from nitrification and consumption by denitrification. Wang and Cai (2008) reported a similar result where NO₃⁻-N content increased after incubation even at a soil moisture content of 100% of FWC. In our study, larger aggregates always showed higher concentration of soil properties, especially WEOC, NH₄⁺-N, NO₃⁻-N and MBC compared to those of smaller aggregates (Table 1). Several studies showed

Table 3 Pearson's correlation coefficient (r) of nitrous oxide (N₂O), carbon dioxide (CO₂) and nitric oxide (NO) productions to soil chemical properties, microbial biomass carbon (C) (MBC) and denitrification enzyme activity (DEA)

Gas	Incubation time	WEOC		NH ₄ ⁺ -N		NO ₃ ⁻ -N		pH		Total C		Total N		MBC		DEA	
		r	n	r	n	r	n	r	n	r	n	r	n	r	n	r	n
N ₂ O	Before	0.969**	12	0.890**	12	0.233	12	0.750**	12	0.244	12	0.069	12	0.740**	12	—	—
	After	0.834**	12	0.974**	12	0.752**	12	0.683*	12	0.192	12	0.167	12	—	—	0.569	12
	Before	0.682*	12	0.810**	12	0.771**	12	0.499	12	0.217	12	0.419	12	0.753**	12	—	—
	After	0.730**	12	0.566	12	0.748**	12	0.832**	12	0.378	12	0.341	12	—	—	0.712**	12
60% and 80% of FWC	Before	0.758**	24	0.793**	24	0.794**	24	0.403	24	0.004	24	0.166	24	0.795**	24	—	—
	After	0.777**	24	0.600**	24	0.698**	24	-0.142	24	0.261	24	0.221	24	—	—	0.621**	24
CO ₂	Before	0.830**	12	0.802**	12	0.350	12	0.643*	12	0.426	12	0.161	12	0.771**	12	—	—
	After	0.817**	12	0.889**	12	0.773**	12	0.705**	12	0.361	12	0.340	12	—	—	0.389	12
	Before	0.638*	12	0.748**	12	0.168	12	0.708**	12	-0.025	12	-0.009	12	0.560	12	—	—
	After	0.588	12	0.700*	12	0.477	12	0.672*	12	0.003	12	-0.059	12	—	—	0.456	12
60% and 80% of FWC	Before	0.365	24	0.440*	24	0.040	24	0.578**	24	0.216	24	0.078	24	0.311	24	—	—
	After	0.343	24	0.525*	24	0.365	24	0.689**	24	0.166	24	0.124	24	—	—	0.285	24
NO	Before	0.770**	12	0.829**	12	0.727**	12	0.630*	12	0.601*	12	0.420	12	0.942**	12	—	—
	After	0.987**	12	0.877**	12	0.983**	12	0.970**	12	0.622*	12	0.605*	12	—	—	0.866**	12
	Before	0.858**	12	0.897**	12	0.397	12	0.933**	12	0.258	12	0.268	12	0.793**	12	—	—
	After	0.819**	12	0.894**	12	0.727**	12	0.862**	12	0.299	12	0.233	12	—	—	0.473	12
60% and 80% of FWC	Before	0.636**	24	0.688**	24	0.360	24	0.793**	24	0.410*	24	0.340	24	0.624**	24	—	—
	After	0.655**	24	0.767**	24	0.680**	24	0.807**	24	0.450*	24	0.403	24	—	—	0.528**	24

FWC, field water capacity; N, nitrogen; WEOC, water extractable organic C; *, significant at the 0.05 level; **, significant at the 0.01 level; n , sample size.

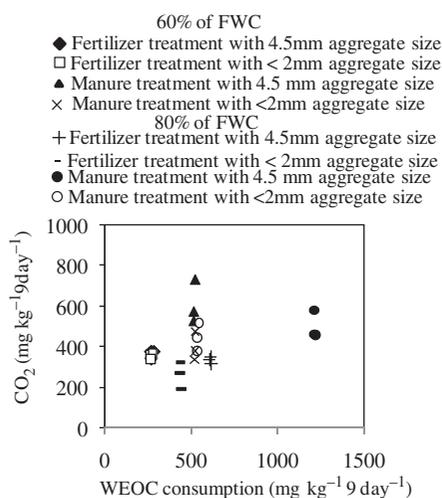


Figure 5 Relationship between cumulative carbon dioxide (CO₂) production and water extractable organic carbon (WEOC) consumption. Data of three replications were used. FWC, field water capacity.

increasing organic C, MBC and potentially mineralized organic matter with increasing aggregate sizes (Puget *et al.* 1995; Jastrow *et al.* 1996; Semenov *et al.* 2010). Therefore, macro aggregates are generally associated with larger concentrations of soil organic C, mineralizable nutrients and microbial biomass as compared with micro aggregates (Gupta and Germida 1988).

Carbon mineralization

The effect of different moisture contents on CO₂ production was not significant in this study (Table 2). Different fertilizer managements and aggregate sizes might have more prominent effects on CO₂ productions, possibly suppressing the effect of moisture content. Application of chemical fertilizer adds only N to the soil while manure application adds both N and C to the soil. For this reason, more WEOC was produced in the manure-applied soil (Table 1). Therefore, more CO₂ production was observed in MS in this study (Table 2). Steven *et al.* (2006) found similar results. Zou *et al.* (2005) defined soil labile organic C as the fraction of organic C with most rapid turnover which is degradable during microbial growth with its oxidation that drives the CO₂ production. Therefore, the higher amount of MBC in larger aggregates shown in Table 1 may induce higher labile C resulting in greater CO₂ production. Semenov *et al.* (2010) demonstrated that a higher content of potentially mineralized organic matter occurred in larger aggregates (3–1 mm, 1–0.25 mm) than in the smaller aggregates (<0.25 mm). Puget *et al.* (1995) and Jastrow *et al.* (1996) also observed increasing proportions of labile C with increasing aggregate size. These

results indicate higher CO₂ emission from larger aggregates as compared to emissions from smaller aggregates in our study (Table 2).

Several studies have implied that the amount of WEOC was a measure of a readily available resource for microbial growth and biological decomposition, often being considered as a good index of C availability (Paul and Beauchamp 1989; Liang *et al.* 1996; Jensen *et al.* 1997). Carbon dioxide is released largely from microbial decay (Janzen 2004; Smith 2004). Results from regression analysis showed a significant positive correlation between CO₂ production and WEOC before incubation (Table 3). The slope value of the regression equation between CO₂ production and MBC was higher in 60% of FWC ($y = 0.189x + 105.5$) than in 80% of FWC ($y = 0.044x + 241.4$), indicating that MBC can produce CO₂ more efficiently in 60% of FWC than in 80% of FWC. On the other hand, WEOC consumption was significantly correlated to MBC regardless of soil moisture content ($r = 0.906$, $P < 0.01$). Comparing WEOC consumption (reduction of WEOC during incubation) and CO₂ production, all samples in 80% of FWC produced less CO₂ than WEOC consumption (Fig. 5). These results suggest that WEOC was consumed not only by decomposition but also by assimilation in the higher soil moisture condition, and the microbial activities are higher in larger aggregates. Larger aggregates can produce water saturated zones inside the aggregates (Khalil *et al.* 2005). This may contribute more effectively to the decomposition of WEOC in the soil desiccation process by supplying water from the saturated zone. Microbial biomass C and WEOC were considered to be important limiting factors for CO₂ production. Soil aggregates control the microbial activities by producing the water saturated zones among aggregates.

Results from this study also showed significant positive correlation between CO₂ production and NH₄⁺-N or NO₃⁻-N (Table 3). The availability of mineral N may influence the decomposition of organic matter and further CO₂ production when N becomes a limiting factor for microbial activities (Hadas *et al.* 1998). Soil pH was significant factor for CO₂ production in all cases (Table 3). An incubation study showed significant positive effects of soil pH on soil respiration (Andersson and Nilsson 2001), particularly since microbial activity like denitrification increases with rising pH values (Ellis *et al.* 1998).

Nitrous oxide production

This study showed a high initial peak of N₂O production immediately after the addition of water (Fig. 1). An increase in N₂O production after moistening of dry soil has also been reported by Kester *et al.* (1997). They

ascribed this to the presence of nitrifiers which can produce N_2O in soil quickly after the rainfall event. Davidson *et al.* (2004) observed N_2O flush immediately after precipitation in a field study. They ascribed this to the change in the aeration status of soil which affects the microbial processes for the production of N_2O . This suggests that the denitrification process may be a significant process for N_2O production immediately after rainfall events.

In our study, cumulative N_2O production in 60% of FWC revealed a significant correlation with NH_4^+-N both before and after incubation (Table 3), and the N_2O production rate increased with the decrease in $N_2O-N/NO-N$ ratio from 100 to 1 (Fig. 4a). These results indicate that nitrification was the main driver of N_2O production, and NH_4^+-N was the reactant in this nitrification process (Lipschultz *et al.* 1981; Hadar *et al.* 2010). On the other hand, cumulative N_2O production in 80% of FWC showed significant correlations with $NO_3^- -N$ (Table 3), and the N_2O production rate increased with an increase of $N_2O-N/NO-N$ ratio from 1 to 100 (Fig. 4b). These facts suggest that denitrification is the main process of N_2O production, and $NO_3^- -N$ was the reactant in the denitrification process (Lipschultz *et al.* 1981; Livesley *et al.* 2009; Toma and Hatano 2007). Actually, denitrification requires an anaerobic environment, while aerobic conditions are necessary for nitrification. Furthermore, high soil moisture content favors anaerobic conditions for denitrification and N_2O appeared to be the main product of denitrification. Therefore, it is clear that the relative importance of each process to N_2O production depends largely on soil water content and the aeration status of the soil. Granli and Bøckman (1994) reported that when nitrification was the main process of N_2O production, emission of N_2O from soil tended to increase with an increase in soil pH. In our study, the significant positive relationship between N_2O production and pH was found in 60% of FWC (Table 3). Sirwan *et al.* (1997) demonstrated that N_2O and NO fluxes decreased remarkably with an increase in soil acidity. Later on they concluded that the microbial community of the soil had adjusted to the low pH and this microbial community was responsible for the entire production of N_2O from nitrification-denitrification processes and for much of the NO release. Our results also showed a significant positive correlation between NO and pH especially in 60% of FWC (Table 3). Production of N_2O in each soil moisture condition showed a significant positive correlation with MBC (Table 3). The MBC was greater in the larger size of soil aggregates and in higher soil moisture content, and it also increased with manure application (Table 1). Furthermore, MBC was significantly correlated with apparent N mineralization (increase in

$NH_4 + NO_3$ during the incubation process) regardless of soil moisture ($r=0.795$, $P<0.01$, $n=24$). Also, as described above, MBC was significantly correlated with WEOC consumption regardless of soil moisture content. Moreover, there was a significant relationship between MBC and DEA ($r=0.796$, $P<0.01$, $n=24$). This suggests that all samples used in this experiment were able to quickly develop the conditions suitable for denitrification, producing more N_2O immediately after moistening. Therefore, these findings indicate that an increase in soil aggregate size increases N_2O production due to the development of suitable conditions for microbial activity inside aggregates, which is enhanced by precipitation and manure application.

Most soil microorganisms get their energy from organic materials. Nitrogen mineralization and the following transformation, i.e., nitrification and denitrification whereby N_2O is produced as an immediate product, are intimately linked to organic C decomposition (Wrage *et al.* 2001). A positive relationship between N_2O and CO_2 production was found in this study, especially in 60% of FWC. This suggests that N_2O production is strongly affected by organic matter decomposition. Toma *et al.* (2010) found a very strong significant relationship between N_2O and CO_2 emissions, which were stimulated more by application of crop residues with a lower C/N ratio. This could be a factor explaining why manure-applied soil with high organic matter might have been responsible for higher N_2O production. Jin *et al.* (2009) found higher N_2O production from MS than the FS in a field experiment conducted for three years. Relationship between N_2O and CO_2 productions in 80% of FWC was not significant. In our study, CO_2 production did not significantly vary with moisture content, but N_2O production significantly varied with moisture content. In other words, higher moisture content resulted in extremely high N_2O production. Linn and Doran (1984) observed a linear relationship between CO_2 and N_2O productions at 60% WFPS, where water content below 60% WFPS limited microbial activities but water content above 60% WFPS decreased only aerobic microbial activities. They also found that the relative activity of anaerobic denitrification was negligible at 60% WFPS, but that it increased with increasing water and reached a maximum level at saturation.

In this experiment, larger aggregates of both fertilizer and manure-applied soils showed higher N_2O flux than smaller aggregates at both moisture contents because of the association of higher amounts of NH_4^+-N and $NO_3^- -N$ with larger aggregates. Khalil *et al.* (2005) used an incubation study and reported an increase in N_2O production in aggregated soil. They suggested that the increase is dependent on aeration within the aggregate.

Drury *et al.* (2004) also revealed that larger aggregate size fractions resulted in greater N₂O production from denitrification where soil aggregates were incubated anaerobically using a C₂H₂ inhibition technique. Lefelaar (1993) used an experimental respirometer system and developed a theoretical simulation model to study the dynamics of water movement, oxygen supply and biological processes on the aggregate scale in unsaturated soil. It was suggested that larger aggregates contain more intra-(micro) and inter-aggregate (macro) pores, which determine the transport properties of water, solutes and gaseous substances through the soil. The transport properties are important in determining the biological activity of the soil. Macro pores are a major determinant in the transport of substances through the soil from and to the atmosphere. Micro pores are important in the transport of substances from the larger pores in the soil into and out of the aggregates, structural elements, or more generally, the denser structure in the soil. Moreover, larger aggregates of both fertilizer and manure-applied soil showed higher FWC, indicating that larger aggregates are able to hold more water inside the soil pores and decrease the O₂ concentration in the soil. Larger aggregates have greater anoxic volumes for which the amount of denitrified N is higher within the larger aggregates, resulting in higher N₂O production by larger soil aggregates.

Conclusions

There was a strong and rapid effect of soil wetness on N₂O production, which can induce the flush of N₂O emission immediately following precipitation. This was enhanced by increase in aggregate size and manure application. Moistening also increased the production of CO₂ and NO, and induced a rapid change in NH₄⁺-N, NO₃⁻-N, WEOC and MBC. These effects of moistening continued for a while (at least 9 days). Larger aggregates produced more gases, and induced more WEOC consumption and N mineralization, which was stimulated by manure application. In this study, the effect of soil moisture content on N₂O production was stronger than that on CO₂ and NO productions. Considerably larger N₂O production was found in 80% of FWC than in 60% of FWC. The result of N₂O-N/NO-N ratio suggested denitrification in 80% of FWC, but nitrification in 60% of FWC is considered to be the main process of N₂O production.

ACKNOWLEDGEMENTS

We would like to thank the technical staff of the Shizunai Livestock Farm for their help in collecting

the soil samples. This study was partly supported by a research grant provided by the project entitled 'Establishment of good practices to mitigate greenhouse gas emissions from Japanese grasslands' funded by the Racing and Livestock Association.

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