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Title

Effect of chemical fertilizer and manure application on N_2O emission from reed canary grassland in Hokkaido, Japan

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Abstract

We evaluated the effect of chemical fertilizer and manure applications on N_2O emission from a managed grassland by establishing three treatment plots of chemical N fertilizer (chemical fertilizer), manure combined chemical N fertilizer (manure), and no N fertilizer (control) at the Shizunai Experimental Livestock Farm in southern Hokkaido, Japan. N_2O fluxes from soils were measured by a closed-chamber method from May 2005 to April 2008. Soil denitrifying enzyme activity (DEA) in root-mat layer (0-2.5cm) and mineral soil layer (2.5-5cm) of each treatment plot was measured by an acetylene inhibition method after treatment with NO_3^--N addition, glucose addition, both NO_3^--N and glucose addition and neither NO_3^--N nor glucose addition, respectively. Annual N_2O emission ranged from 0.6 4.9 kg N_2O-N $ha^{-1}yr^{-1}$, with the highest observed in manure plot and lowest in control plot. Chemical fertilizer–induced emission factor (EF) (range: 0.85 - 1.32%) was significantly higher than manure-induced EF (range 0.35 - 0.85%). Denitrification potential of soil horizons was measured with addition of both NO_3^--N and glucose, which was significantly higher in root-mat soil than that in mineral soil. Soil DEA in root-mat in NO_3^--N addition with and without addition of glucose had a significantly positive correlation with soil pH ($P < 0.05$). Soil pH was significantly

influenced by N source, which was significantly lower in chemical fertilizer plot than that in control and manure plot. For a fixed quantity of available N, application of manure could result in higher N_2O emission compared to chemical fertilizer owing to high soil pH values under manure application than under chemical fertilizer application.

Key words: chemical fertilizer, grassland, manure, N_2O emission, soil DEA.

INTRODUCTION

N_2O is one of the most important radiatively active trace gases in the atmosphere that contributes at least 5% to the observed global warming at present (Myhre *et al*. 1998). The atmospheric concentration of N_2O has increased from a pre-industrial value of about 270 ppb to 319 ppb in 2005 and continues to increase as a result of human activities (IPCC 2007). Agriculture as a whole (i.e. animal excreta, denitrification of leached nitrate, etc.) contributes about 80% of the anthropogenic N_2O emissions (Brown *et al.* 2001). Direct and indirect emissions from agricultural systems are now thought to contribute approximately 6.2 Tg N_2O-N yr^{-1} to the total global source strength of 17.7 Tg N_2O-N yr^{-1} (Kroeze *et al.* 1999). About 57% of the global atmospheric sources of N_2O are estimated to be related to emissions from soils (Mosier *et al*. 1998).

N_2O is produced in soils primarily by microbial processes of nitrification and

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denitrification (Tiedje 1988; Conrad 1996). Nitrification is the biological oxidation of ammonium to nitrite or nitrate under aerobic conditions, while denitrification is the reduction of nitrate to N_2O and N_2 when the supply of oxygen is limited. Increasing soil N availability as a result of increased N inputs by the application of chemical fertilizer and manure and atmospheric deposition have greatly enhanced N_2O emissions from soils (Kroeze *et al*. 1999) by influencing nitrifying and denitrifying enzyme activity. Chemical fertilizer and animal wastes are the two most important sources of direct N_2O emissions from agricultural soils (Mosier *et al*. 1998). The default IPCC emission factor, i.e. the percentage of applied N emitted as N_2O, is 1.0%, regardless of the fertilizer type (IPCC 2006). However, the type of N inputs to the fields may affect the N_2O emission rate in different ways, leading to different patterns of N_2O emissions from inorganic and organic N fertilizers. Addition of inorganic N increases N_2O emission through affecting the process of nitrification and denitrification by increasing the available NH_4^+-N and NO_3^--N substrates. Organic fertilizers with a high and easily mineralizable organic C content stimulate microbial activity and thus N_2O emissions (Chadwick *et al*. 2000). The application of chemical N fertilizer resulted in short-lived N_2O peaks (Dobbie and Smith 2003). However, N_2O losses from manure plots extended over a longer period of time and were greater in magnitude than from chemical N fertilization (Jones *et al*. 2007). Higher

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N_2O fluxes from manure and sewage applications compared to that from chemical N fertilizers were also observed in other studies (Scott *et al.* 2000). High N_2O fluxes from manure treatments can be partly explained by the higher total N input than chemical fertilizer treatments, providing more available N that was mineralized over a longer period of time. Another reason for the increased N_2O losses could be an addition of organic C by the manures, which is known to stimulate denitrification. McTaggart *et al.* (1997) reported that C supply from slurry spread onto grasslands stimulated N_2O production, resulting in a four times larger N_2O loss compared to the application of NH_4NO_3 although the total rate of N application was similar in both treatments.

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Grassland is an important ecosystem to support the production of herbivorous livestock (Soussana *et al.* 2007). Application of chemical fertilizer and animal manure to grasslands has been conducted to increase grass production, especially in developed countries where grassland-based livestock production is important (Bouwman *et al.* 2002). However, N application to grasslands also poses a risk of N loss to the environment in the form of N_2O emission. The objective of this study is to clarify the effect of fertilizer and manure application on N_2O emission and to identify the factors controlling N_2O emission from the grassland.

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MATERIALS AND METHODS

Study site

This study was conducted on a managed grassland located at the Shizunai Experimental Livestock Farm, Field Science Center for Northern Biosphere of Hokkaido University in Southern Hokkaido, Japan (42°26’N, 142°29’E). The site is characterized by a humid continental climate with cold winters and cool summers. During 1979 to 2000, the mean annual precipitation and air temperature for this region were 1365 mm and 7.9°C, respectively. The soil is derived from Tarumae (b) volcanic ash, and is classified as Thaptic Melanudands (Soil Survey Staff, 2006; Mollic Andosol (IUSS Working Group, WRB, 2006)). A layer of three cm thick root-mat was found on the top, and a 21 cm thick Ap-layer was found under the root-mat in a survey conducted in August 2004 (Shimizu *et al*. 2009). The C and N contents in the Ap-layer were 3.7% and 0.33%, respectively, and the C:N ratio was 11.1. Dominant grass species at this site were reed canary grass (*Phalaris arundinacea L.*) and foxtail grass (*Alopecurus pratensis L.*). The harvesting of grass was carried out twice a year (21st June and 11th August in 2005, 27th June and 23rd August in 2006 and 18th June and 18th August in 2007) in accordance with the local practice.

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Experimental setup

Three experimental plots were set up on the study site; one for treatment with chemical fertilizer (chemical fertilizer plot), another with beef cattle manure and chemical fertilizer (manure plot), and the other with no N fertilizer or manure (control plot). Setting of the treatments was initiated in the spring of 2005. Eighteen subplots (5 m × 4 m) were established for the chemical fertilizer, manure, and control plots with six and four replications from May 2005 to April 2007 and from May 2007 to April 2008, respectively.

Table 1 shows the information on the date of application and the application rates of chemical fertilizer and manure. The N application rates in the chemical fertilizer plot were at the recommended level for this site on the basis of soil tests, and were 164 kg N ha^{-1} year^{-1} in 2005 and 184 kg N ha^{-1} year^{-1} in 2006 as ammonium sulfate and ammonium phosphate (Table 1). For learning the N_2O emission from grassland soil under the local N fertilization level, N fertilization decreased to 74 kg N ha^{-1} year^{-1} in chemical fertilizer plot according to the recommends of Shizunai Experimental Livestock Farm staffs. The pH value of manure that used in 2005, 2006 and 2007 were 8.3, 8.8 and 9.1, respectively. The manure application rates were the optimum rates used by farmers in the region, and were based on adequate amounts of potassium (K) application to the fields. Beef cattle manure with bedding litter (bark) was applied to the manure plot, and the application rates were 44 Mg FM ha^{-1} (236 kg N ha^{-1} and 5.8 Mg C ha^{-1}) in May 2005, 43 Mg FM ha^{-1} (310 kg N ha^{-1} and 6.0 Mg C ha^{-1}) in May 2006, and 43 Mg FM ha^{-1} (331 kg N ha^{-1} and 7.7 Mg C ha^{-1}) in May 2007 (Table 1). In the manure plot, the N supply rates from manure were estimated by multiplying the application rates by the N mineralization rate, and the differences between the supply rates in manure and the application rates in the fertilizer plot were supplied by chemical fertilizer. The N mineralization rates were estimated based on Uchida's model (Shiga *et al*. 1985) which was developed in Japan and were 13.2%, 7.0% and 5.5%, respectively in the first, second and third years after application. The mineralization rates of P and K from the manure were estimated based on the handbook of animal waste management and utilization in Hokkaido 2004 (Anon. 2004). The P mineralization rate was 20%, 10%, and 0% and the K mineralization rate was 70%, 10%, and 0% in the first year, second year, and third year after application, respectively.

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N_2O and NO fluxes

We defined the crop growing season as a 7-day moving average of daily air temperature above 5 ˚C and the non-growing season as the rest (Shimizu *et al*. 2009). The growing season was 215 days in 2005 (From 10th April 2005 to 10th November 2005), 218 days in

2006 (From 15th April 2006 to 18th November 2006) and 220 days in 2007 (From 13th April 2007 to 18th November 2007. N_2O and NO fluxes from the soil to the atmosphere were measured by the static closed chamber method on the control, fertilizer, and manure plots (Shimizu *et al*. 2009). The flux measurements were conducted in 2 - 28 day intervals during the crop growing season and 10 - 30 day intervals during the non growing season and between 8:00 and 11:00 h in each measuring day to minimize the effect of diurnal temperature variation. The stainless steel chambers were 40 cm in diameter and 30 cm high in the chemical fertilizer and manure plots, and 20 cm in diameter and 25 cm high in the control plots. The chambers were placed directly into the soil to a depth of about 3 cm, 12 hours before the measurement of each subplot, and contained no aboveground biomass in the chemical fertilizer, manure, and control plots. Before closing the chamber, a 250 ml gas sample from the headspace of each chamber was extracted into a Tedlar bag for NO analysis, and a 20 ml gas sample was injected into an evacuated vial (10 ml) for N_2O analysis. This measurement was regarded as time 0 min. After 20 min or 30 min under a closed-chamber condition, 250 ml of the headspace gas sample was extracted from each chamber into a bag, and 20 ml was injected into a vial. From these bag samples, NO gas concentrations were determined in a laboratory within 16 hours using a Chemiluminescence N Oxide Analyzer (Model 265P, Kimoto Electric, Osaka, Japan).

N_2O gas concentrations were determined in a laboratory within 1 month using an ECD (Electron capture detector) gas chromatograph (model GC-14B, Shimadzu, Kyoto, Japan) from the samples of vials.

Gas fluxes were calculated from the change in gas concentration in the chamber against closure time:

$$F = \rho \times h \times (\Delta c/\Delta t) \times [273/(273+T)]$$

where F is the gas flux (μg N m^{-2} h^{-1} for N_2O), ρ is the gas density (N_2O-N = 1.26 × 109 μg m^{-3}), h is the height of the chamber from the soil surface (m), Δc/Δt is the change in gas concentration inside the chamber during the sampling period (m^3 m^{-3} h^{-1}), and T is the average air temperature during the sampling period (°C). A positive flux denotes the emission from the soil, whereas a negative flux denotes the uptake from the atmosphere.

The cumulative gas flux was calculated as follow:

$$\text{Cumulative gas flux} = \sum_{i=1}^{n} (\boldsymbol{Ri} \times 24 \times \boldsymbol{Di})$$

where Ri is the mean gas flux (mg m^{-2} hr^{-1}) of the two successive sampling dates, Di is the number of days in the sampling interval, and n is the number of sampling times. The cumulative period of 2005, 2006 and 2007 were calculated from 10th April 2005 to 14th April, from 15th April 2006 to 18th April 2007 and from 19th April 2007 to 4th April 2008, respectively.

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Emission factor

N_2O Emission factor (EF) for chemical fertilizer and manure (kg N_2O–N (kg N input)$^{-1}$) was calculated as follows:

Chemical fertilizer-induced EF ＝ {[N_2O emission (chemical fertilizer plot)]－[N_2O emission (control plot)]}／[chemical fertilizer N application rate (chemical fertilizer plot)]

Manure-induced EF＝{[N_2O emission (manure plot)]－[chemical fertilizer N application rate (manure plot)]×chemical fertilizer-induced EF－[N_2O emission (control plot)]}／[manure N application rate (manure plot)]

Environmental variables

Daily precipitation was obtained at the Sasayama AMeDAS (Automated Meteorological Data Acquisition System) station by the Japan Meteorological Agency. Air temperature and soil temperature at a 5 cm depth were measured at the same time with the flux measurements using a thermistor thermometer (CT220, CUSTOM, Tokyo, Japan), and soil moisture content at a 0 - 6 cm depth was measured using the Frequency Domain Reflectometry (FDR) method (DIK-311A, Daiki, Saitama, Japan). Soil core samples (14 cm diameter, 13 cm height) were collected in April 2007, and calibration curves were made to calculate water-filled pore space (WFPS) from the FDR device reading (m^3 m^{-3})

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and percent total porosity (Linn and Doran 1984). The percent total porosity was measured using a 100 ml soil core collected in April 2007 and was regarded as constant throughout the study period because of no tillage.

Soil chemical analyses

Soil samples at a depth of 0 to 5 cm from ground surface were collected during a period from April to November at three replicates in all treatment plots. Within 48 hours of soil sampling, soil samples were sieved through the 2 mm sieve and stones and roots were removed. Soil samples were then immediately extracted in deionized water (1:5) and in 2 M KCl (1:10), and the extracts were stored at 4 °C until analysis for dissolved nutrients after filtered through 0.2-μm membrane filters. Water-soluble organic carbon (DOC) content in the deionized-water-extract solution was analyzed using a TOC analyzer (TOC 5000A, Shimadzu). The concentration of NO_2-N and NO_3-N in the deionized-water-extract solution was analyzed by ion chromatography (Dionex QIC Analyzer, Dionex Japan, Osaka, Japan). The concentration of NH_4^+-N in the 2 M KCl extracted solution was determined by the indophenol-blue method (UV mini 1240, Shimadzu, Kyoto, Japan). Soil pH was measured in the deionized-water-extract solution with a combined electrode pH meter (F-8 pH meter, Horiba, Japan).

Measurement of soil denitrifying enzyme activity (DEA)

For measuring soil denitrifying enzyme activity (DEA), soil samples were taken from all treatment plots in the root-mat layer (0 - 2.5cm depth) and mineral soils layer (2.5 - 5cm depth) with 3 replications in April, June, and August 2007. The root-mat soil samples were cut into small pieces with 1cm diameter, and stones or roots were removed from the mineral soil samples by passing through the 2mm sieve within 48 h after sampling. Then we mixed three replicates of soil samples and kept in refrigerator at 4 °C until analysis. The DEA was determined by an acetylene block technique, which inhibits the final conversion of N_2O to N_2 gas (Tiedje, 1994). Soil samples were incubated under anaerobic condition at 25°C with a solution treated with 1) chloramphenicol (1g L^{-1}) (Chl), 2) with chloramphenicol (1g L^{-1}) and NO_3^--N (200 mg N L^{-1} as KNO_3) (Chl+N), 3) with chloramphenicol (1g L^{-1}) and organic-C (2 g C L^{-1} as glucose) (Chl+C), and 4) with chloramphenicol (1g L^{-1}), NO_3^--N (200 mg N L^{-1} as KNO_3) and organic-C (2 g C L^{-1} as glucose) (Chl+N+C). Fresh soil of 15g was placed into a 100 conical flask, and 15 ml treated solution was added to the flask. The flasks were evacuated and flushed four times 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 by a syringe at 2 and 4 h and denitrification rates were calculated from the linear increment of N_2O production against time. Denitrification potential of the soil horizons was measured with the addition of both NO_3^--N and soluble C source as proposed by D'Haene *et al.* (2003).

Statistical analyses

Analysis of variance (ANOVA) and Pearson correlation analysis were performed using SPSS 13.0. Linear regression and other statistical analyses were carried out by using Excel 2003. Two-way ANOVA and Tukey test were used to compare the mean difference ($P < 0.05$) of a given variable between treatment plots and years. Three-way ANOVA and Tukey test were used to compare the mean difference ($P < 0.05$) in soil N_2O fluxes among the seasons, treatment plots of field experiment, and years; and in soil DEA among the treatment plots of field experiment, soil layers, and incubation treatments.

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RESULTS

Soil temperature and moisture

Daily precipitation is shown in Fig. 1a. Annual precipitations were 1176 mm from the mid April 2005 to the mid April 2006, 1047 mm from the mid April 2006 to the mid April 2007, and 879 mm from the mid April 2007 to the beginning of April 2008. These values are smaller than that the mean annual precipitation (1365 ± 215 mm) from 1989 to 2000. Soil moisture content expressed as WFPS in a 0 to 6 cm depth is shown in Fig. 1b. The

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WFPS from April to November was influenced by precipitation and the low soil moisture was observed with low precipitation in August 2006 and June 2007. From December to March, soil moisture was not observed because of soil freezing. In the winter of 2006/2007, soil freezing began from beginning of December 2006, reaching the maximum depth of 17.75 cm in 9th March 2007, and then thawed in early April in 2007. In the winter of 2007/2008, soil freezing also began from beginning of December 2007, but reaching the maximum 29 cm in 12th March 2008, and then thawed in early April in 2008. The soil freezing depth was not observed in the winter of 2005/2006.

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Soil temperature at a 5 cm depth is shown in Fig. 1c. The soil temperature increased from April, reaching its maximum from July through August, and then decreased gradually. The soil temperature was around 0 ˚C from December to March. There was no difference in soil temperature between the chemical fertilizer and manure plots, but soil temperature was higher in the control plot than in the chemical fertilizer and manure plots ($P < 0.05$).

N_2O fluxes

The seasonal patterns of N_2O fluxes were mainly driven by a seasonal variation as varieties of air and soil temperature which were higher in summer and lower in winter, and influenced by fertilization (Fig. 2a). N_2O fluxes in the chemical fertilizer and manure

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The annual N_2O emission in control, chemical fertilizer, and manure plots ranged from 0.6 to 0.7, 1.4 to 3.0 and 2.1 to 4.9 kg N_2O-N $ha^{-1}yr^{-1}$ during 2005 to 2007, respectively

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(Table 4). Application of both chemical fertilizer and manure stimulated the annual cumulative N_2O emissions, and the significantly highest annual cumulative N_2O emission was observed in the manure plot, followed by the chemical fertilizer plot. Application of chemical fertilizer contributed to 76.9, 79.2 and 47.2% of the total N_2O emission from the chemical fertilizer plot in 2005, 2006, and 2007, respectively. In the manure plot, N_2O emission from the applied chemical fertilizer and manure contributed to 81.8, 87.4, and 67.6% of the total N_2O emission in 2005, 2006, and 2007, respectively. Chemical fertilizer–induced EF was 1.32, 1.30, and 0.85 % in 2005, 2006, and 2007, respectively. Manure-induced EF was significantly lower than the fertilizer–induced EF ($P < 0.001$), and was 0.51, 0.85, and 0.35 % in 2005, 2006, and 2007, respectively (Table 5).

NO fluxes showed a seasonal variation that similar with the seasonal pattern of N_2O fluxes, which were higher in summer and lower in winter and also influenced by fertilization (Fig. 2b). The NO fluxes ranged from -1.2 to 91.3 ug NO-N m-^{2}h^{-1} which was smaller than the N_2O fluxes (-3.6 to 1290.7ug N_2O-N m-^{2}h^{-1}). Large NO fluxes were observed mainly after the manure and chemical fertilizer application (Fig. 2b). Most of the values of N_2O/NO ratio were distributed from 1 to 100, and a significant positive correlation was found ($P < 0.01$) between the N_2O/NO ratio and the N_2O fluxes (Fig. 3).

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Soil chemical properties

During 2005 to 2007, soil pH in chemical fertilizer plot was obviously lower than that in control and manure plot (Fig.4a). Chemical fertilizer application could lead to the soil pH decreasing not only in chemical fertilizer plot but also in manure plot (Fig.4a). The mean soil pH during 2005 to 2007, in control, chemical fertilizer and manure plots were 5.2, 4.6, and 5.1, respectively. The soil pH in the chemical fertilizer plot was significantly lower than that in the manure and control plots ($P < 0.001$). There was no significant difference in soil pH between the manure and control plots.

The mean NH_4^+-N concentrations, during 2005 to 2007, in control, chemical fertilizer, and manure plots were 4.4, 23.7, and 17.5 mg kg^{-1}, respectively. Soil NH_4^+-N concentration in chemical fertilizer and manure plots exhibited a wide range of 0.4 - 245 mg kg^{-1}(Fig. 4b). In contrast, soil NH_4^+-N concentration in the control plot was stable, and was always below 12 mg kg^{-1}. The pattern of soil NH_4^+-N concentration was not influenced by manure application only; however, it was influenced by the chemical fertilizer application (Fig. 4b). Soil NH_4^+-N concentration in chemical fertilizer and manure plots increased rapidly right after chemical fertilizer application, but then decreased within a few days (Fig. 4b). In 2005 and 2006, peak concentrations of soil NH_4^+-N were always observed in both chemical fertilizer and Manure plot after chemical

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fertilizer application. But in 2007, only one small peak was observed in the chemical fertilizer plot following the base fertilizer application in May. After chemical fertilizer application in July 2007, soil NH_4^+-N concentration peak was not observed may caused by low precipitation and low soil moisture that limited the additional NH_4^+-N by chemical fertilizer application go into more deep soil layer by water dynamics before it was assumed on the soil surface and in root mat through NH_4^+-N volatilization or microbial, chemical and physical reaction.

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The mean NO_3^--N concentrations, during 2005 to 2007, were 1.4, 2.7, and 2.2 mg kg^{-1} in the control, chemical fertilizer, and manure plot, respectively. The pattern of soil NO_3^--N concentration was also influenced by N application, and the peaks were observed slightly later than those of soil NH_4^+-N concentration (Fig. 4c).

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During the study period, the soil DOC concentration ranged from 48 to 121 mg kg^{-1} in the control plot, from 23 to 116 mg kg^{-1} in the chemical fertilizer plot, and from 43 to 199 mg kg^{-1} in the manure plot (Fig. 4d). The mean soil DOC concentrations in control, chemical fertilizer, and manure plots were 73.3, 59.4, and 97.8 mg kg^{-1}, respectively. The soil DOC concentration in the manure plot was significantly higher than that in the control and chemical fertilizer plots ($P < 0.01$), but application of chemical fertilizer had no significant influence on the soil DOC concentration compared with that in the control

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plot. Continuous 3 years manure application significantly increased the soil DOC concentration, which was significantly higher in 2007 than in 2005 and 2006 ($P<0.01$).

Pearson correlation analysis showed that instantaneous N_2O flux had a strong positive correlation with soil temperature ($P < 0.01$), soil NO_3^--N concentration ($P < 0.01$), and soil NH_4^+-N concentration ($P < 0.01$) (Table 6).

Soil denitrifying enzyme activity (DEA)

Table 7 shows the result of DEA. Result of a 3-way ANOVA shows that there was a significant difference in soil DEA among soil layers (root-mat and mineral) ($P < 0.001$) and treatments (with and without NO_3 and glucose) ($P < 0.001$), but there was no significant difference in soil DEA among plots (control, chemical fertilizer, and manure) ($P = 0.058$) (Table 8). However, there was a significant interaction between the soil layer and treatments ($P < 0.001$). In the root-mat layer, soil DEA was significantly increased by the addition of NO_3-N with ($P < 0.001$) or without ($P < 0.001$) the addition of glucose. But there was no significant effect on soil DEA by the addition of only glucose. There was no significant difference in soil DEA between the treatments Chl+N and Chl+N+C. On the other hand, there was no significant effect of single addition of NO_3-N (Chl+N) or glucose (Chl+C) on soil DEA in the mineral soil. However, a combination of NO_3-N and glucose addition (Chl+N+C) increased the soil DEA significantly ($P < 0.05$). The soil

DEA with the addition of both NO_3-N and glucose in the root-mat soil was significantly higher than that in the mineral soil ($P < 0.001$). The soil DEA in the root-mat soil with the addition of NO_3-N and both NO_3-N and glucose had a significantly positive correlation with soil pH ($P < 0.05$) (Fig. 5).

DISCUSSION

Seasonal pattern of N_2O emission

Soil N_2O fluxes were significantly higher in the growing season than that in the non-growing season. This is attributed to the high soil temperature (Table 6) in the growing season. Granli and Bøckman (1994) found an increased rate of N_2O production with an increase in soil temperature up to 20-40 °C. High peaks of N_2O fluxes were usually observed in both chemical fertilizer and manure plots within a few weeks after the application of manure or chemical fertilizer in our study (Fig. 2a). This can be attributed to the result of rapid increase in soil NH_4^+-N and NO_3^--N concentrations immediately after the application of fertilizer, which decreased within a few days (Fig 4). It is well established that the rate of N_2O emission usually increases with an increase in soil available N (Skiba and Smith 2000; Sehy *et al*. 2003). Several studies reported that N_2O fluxes significantly increased after the application of N fertilizers. Mu *et al*. (2008)

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reported that N_2O fluxes increased rapidly to higher emission levels in soils cultivated with wheat (from 242 to 433 μg N m^{-2} h^{-1}) and onion (from 47.2 to 157μg N m^{-2} h^{-1}) after N fertilization and that the fluxes lasted for about three weeks. Schils *et al*. (2008) also reported high N_2O fluxes occurred in the first week after the application of chemical fertilizer or cattle slurry.

The bacterial processes of nitrification and denitrification are the most important sources of N_2O in soil (Granli and Bøckmann 1994). According to Davidson (1992) and Skiba *et al*. (1993), nitrification produces more NO than N_2O; conversely, dentrification produces more N_2O than NO. The ratio of N_2O-N/NO-N is the index of N_2O production from nitrification or denitrification (Lipschultz *et al*. 1981). Lipschultz *et al.* (1981) reported that the ratio of production of N_2O-N / NO-N ranged from 0.2 to 1.0 in nitrification and 100 in denitrification. A significant positive correlation between the N_2O fluxes and the ratio of N_2O-N / NO-N and was found (Fig. 3) in our study ($P < 0.01$), indicating that the high N_2O emissions are primarily due to denitrification.

Cumulative N_2O emission

Chemical fertilizer and animal wastes are the two most important sources of direct N_2O emissions from agricultural soils (Mosier *et al.* 1998). Increasing soil N availability associated with application of N by chemical fertilizer and manure has greatly enhanced

N_2O emissions from agricultural soils (Kroeze *et al*. 1999). Meng *et al*. (2005) found that chemical fertilizer and manure contributed to 74–82% of the total N_2O emissions. Mori *et al*. (2008) also reported the N_2O emission predominantly derived from the manure and the chemical fertilizer N application on a volcanic grassland soil in Nasu, Japan. Generally, emissions of N_2O increase with an increase in N application rates (Granli and Bøckman 1994; MacKenzie *et al*. 1997). In our study, the N_2O emission from applied chemical fertilizer and manure contributed to 77-85% of the total N_2O emission in 2005 and 2006. The contribution of chemical fertilizer and manure to N_2O emission in 2007 decreased to 47-65% due to the lower application rates than that in 2005 and 2006. The chemical fertilizer–induced EF ranged from 0.85 to 1.32%, which was comparable to the IPCC default value 1% (IPCC 2006), but was higher than that reported by Akiyama and Tsuruta (2003) from the Japanese Andisols amended with chemical fertilizer (ranging from 0.06% to 0.29%). The manure-induced EF of our study ranged from 0.35 to 0.85 %, which was significantly lower than the chemical fertilizer-induced EF and the IPCC default value, but close to that reported by Akiyama and Tsuruta (2003), which was 0.55%.

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Soil DEA

The soil DEA with an addition of NO_3^--N and glucose in the root-mat soil was

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significantly higher than that in the mineral soil, indicating that the soil denitrification potential in the root-mat soil was significantly higher than that in the mineral soil. Microbial activities in the surface soil are reported to be higher than in the deeper soil (Speir *et al*. 1984; Higashida and Takao, 1985). Parkin and Meisinger (1989) reported that total viable bacteria and numbers of denitrifying bacteria were found to decrease exponentially with an increase in soil depth on a well-drained silt loam soil.

Soil DEA in the root-mat soil significantly increased by the addition of NO_3-N with ($P < 0.001$) or without ($P < 0.001$) the addition of glucose, indicating that the availability of soil NO_3^--N could be the major limiting factor for soil DEA in our study grassland. In the mineral soil, only addition NO_3-N or glucose could not increase the soil DEA, but addition NO_3-N and glucose together increased the soil DEA that means not only the NO_3-N but also the carbon is the limited factor for soil DEA in mineral soil. The soil DEA in the root-mat soil with NO_3-N addition and both NO_3-N and glucose addition had a significantly positive correlation with soil pH ($P < 0.05$, Fig. 5). The soil pH is supposed to be a major variable of soil, controlling the microbial community in general and the community of denitrifiers in particular (Simek and Hopkins 1999). Simek and Hopkins (1999) detected an optimum pH value for denitrification in soils as a range from 7 to 8. Simek and Cooper (2002) reported that both the overall rates of denitrification under field

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conditions (i.e. the formation of N_2O, N_2 and NO and their subsequent emission) and DEA were influenced by soil pH, and they were less in acidic soils than in neutral or slightly alkaline soils. Ellis *et al*. (1998) observed that the production of N_2O decreased with decreasing pH under anaerobic conditions through an incubation experiment. These results suggest that the highest N_2O emission in the manure plot in our study was resulted from the soil DEA that could have been controlled by the soil pH.

The application of chemical fertilizer significantly decreased the soil pH in the chemical fertilizer plot than in the control plot. However, the soil pH in manure plot was not significantly different with control plot. That maybe because higher pH of manure (8.3-9.1) input decrease the effect of chemical fertilizer application on the soil pH. Soil acidity is controlled by the amount of H^+ and Al^{3+} which is either contained in or generated by the soil and soil components. According to Kirikae *et al*. (2001), nitrification is a source of H^+ through two nitrification pathways of NH_4^+ origin and organic N origin. They reported that the ratio of H^+ to NO_3^- was 2 in the pathway of NH_4^+ origin and 1 in the pathway of organic N origin. In the meanwhile, NO_3^- uptake by vegetation was the sink of H^+. Therefore, the organic N has less effect on H^+ production than NH_4^+-N. On the other hand, application of manure increased the value of cation exchange capacity (CEC) compared to that of chemical fertilizer (Bulluck *et al*. 2002).

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The soils with a high CEC have a greater capacity to contain or generate sources of acidity. The soil pH is higher in soils with manure than that with chemical fertilizer as reported by several studies (Bulluck *et al*. 2002 and *Gil et al*. 2008).

Conclusions

The application of both chemical fertilizer and manure to grassland stimulated the annual N_2O emission. The chemical fertilizer–induced EF (range: 0.85 - 1.32%) was significantly higher than the manure-induced EF (range 0.35 - 0.85%). However, annual N_2O emission was significantly higher in the manure plot than that in the chemical fertilizer plot. The soil DEA in the NO_3-abundant root-mat layer significantly decreased with a decrease in soil pH. Moreover, application of chemical fertilizer could significantly decrease soil pH, but the manure application had no significant effect on soil pH. Therefore, for the a fixed quantity of available N, application of manure could result in higher N_2O emission compared to chemical fertilizer owing to high soil pH values under manure application than under chemical fertilizer application.

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We would like to thank the technical staff of Shizunai Livestock Farm for their help in the field measurements. This study was partly supported by a research grant provided by the

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Figure captions

Figure 1 Seasonal patterns of precipitation (a), WFPS at 6 cm depth (b), and soil temperature at 5 cm depth (c). Data of WFPS and soil temperature represent means ± SD (n = 4 to 6).

Figure 2 Seasonal patterns of soil N_2O fluxes (a) and soil NO fluxes (b). Data represent means ± SD (n = 4 to 6). Full arrow indicates date of the chemical fertilizer application, and dotted arrow indicates date of manure application. The growing season was 215 days in 2005 (From 10th April 2005 to 10th November 2005), 218 days in 2006 (From 15th April 2006 to 18th November 2006) and 220 days in 2007 (From 13th April 2007 to 18th November 2007).

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Figure 3 Relationships between N_2O fluxes and the ratio of N_2O to NO.

Figure 4 Seasonal patterns of Soil pH (a), soil NH_4^+-N (b), soil NO_3^--N (c), and soil DOC (d) at a depth of 0 - 5 cm. Data represent means ± SD (n = 3). Full arrow indicates date of the chemical fertilizer application, and dotted arrow indicates date of manure application. The arrows indicate the date of fertilizer application.

Figure 5 Relationship between the soil DEA and the soil pH in root-mat soil.

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We evaluated the effect of chemical fertilizer and manure applications on N_2O emission from a managed grassland by establishing three treatment plots of chemical fertilizer, manure, and control at the Shizunai Experimental Livestock Farm in southern Hokkaido, Japan. Seasonal N_2O and NO fluxes were measured by a closed-chamber method at 4 to 6 replications in each treatment plot from May 2005 to April 2008. Soil samples were collected from a 0-5 cm top soil layer at three replications on each gas sampling date for measuring pH, NO_3^--N, NH_4^+-N, and DOC. Soil samples were collected from the root-mat layer (0-2.5cm) and the mineral soil layer (2.5-5cm) of each treatment plot in April, June, and August 2007, which was followed by measuring soil denitrifying enzyme activity (DEA). The soil DEA was measured by an acetylene inhibition method under the four treatments with and without the addition of NO_3^--N and glucose. The cumulative N_2O emission in control, chemical fertilizer, and manure plots ranged from 0.6 to 0.7, 1.4 to 3.0, and 2.1 to 4.9 kg N_2O-N $ha^{-1}yr^{-1}$, respectively. The application of both chemical fertilizer and manure significantly increased the cumulative N_2O emission, and the highest cumulative N_2O emission was observed in the manure plot. N_2O fluxes were positively correlated with the N_2O:NO ratio ($P < 0.01$), indicating high N_2O fluxes resulting from the increased denitrification activities. The denitrification potential of the soil horizons was significantly higher in the root-mat soil than that in the mineral soil. The soil DEA in the root-mat soil in NO_3^--N addition with and without the addition of glucose had a significantly positive correlation with soil pH ($P < 0.05$). The soil pH was significantly influenced by N fertilization, which was significantly lower in the chemical fertilizer plot than that in the control and manure plots. For the similar quantity of available N, application of manure could result in higher N_2O emission compared to chemical fertilizer owing to high pH values in manures.

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Table 1 The applied date and the application rates of chemical fertilizer and manure during the study period

Treatment	Date	Fertilizer type	Application rates (kg ha^{-1}) C	N	P_2O_5	K_2O
Control	2005/5/11	Chemical fertilizer [a]	0	0	0	0
	2005/7/4	Chemical fertilizer [a]	0	0	0	0
	2006/5/9	Chemical fertilizer [a]	0	0	0	0
	2006/7/10	Chemical fertilizer [a]	0	0	0	0
	2007/5/12	Chemical fertilizer [a]	0	0	14	73
	2007/7/5	Chemical fertilizer [a]	0	0	7	37
Chemical fertilizer	2005/5/11	Chemical fertilizer [a]	0	103	23	168
	2005/7/4	Chemical fertilizer [a]	0	61	23	97
	2006/5/9	Chemical fertilizer [a]	0	124	50	177
	2006/7/10	Chemical fertilizer [a]	0	59	18	97
	2007/5/12	Chemical fertilizer [a]	0	49	14	73
	2007/7/5	Chemical fertilizer [a]	0	25	7	37
Manure	2005/5/11	Manure [b]	5833	236	191	266
	2005/7/4	Chemical fertilizer [a]	0	133	7	70
	2006/5/9	Manure [b]	5958	310	212	167
	2006/5/9	Chemical fertilizer [a]	0	71	0	33
	2006/7/10	Chemical fertilizer [a]	0	59	6	97
	2007/5/12	Manure [b]	7714	331	342	336
	2007/7/5	Chemical fertilizer [a]	0	21	0	0

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[a] Chemical fertilizer is comprised of ammonium sulfate, ammonium phosphate, potassium sulfate, and potassium magnesium sulfate.

[b] Beef cattle manure with bedding litter was applied in the manure plot.

Table 2 ANOVA results for mean N_2O fluxes

Source	df	Mean Square	F	P value
Season	1	179,974.04	17.521	0
Year	2	13,990.75	1.362	0.258
Treatment	2	39,922.53	3.887	0.022
Season * Year	2	15,307.66	1.49	0.228
Season * Treatment	2	38,549.47	3.753	0.025
Year * Treatment	4	7,659.25	0.746	0.562
Season * Year * Treatment	4	7,454.57	0.726	0.575
Error	210	10,271.71		

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Table 3 Mean N_2O fluxes from the control, chemical fertilizer, and manure plots

Season	Treatment	Mean N_2O fluxes (ug N_2O-N m-^{2}h^{-1})		
		2005	2006	2007
Growing season	Control	12.3 (12.7)	12.9 (15.23)	16.7 (27.6)
	Chemical fertilizer	85.3 (94.1)	83.9 (66.3)	36.5 (53.2)
	Manure	101.9 (102.1)	187.6 (301.6)	50.6 (48.9)
Non-growing season	Control	0.3 (0.6)	2.6 (6.0)	4.7 (3.1)
	Chemical fertilizer	2.7 (2.3)	6.0 (11.4)	7.4 (13.7)
	Manure	2.7 (3.9)	3.6 (5.1)	3.5 (8.0)

Data represent means (SD), n=4 to 6. We defined the crop growing season as a 7-day moving average of daily air temperature above 5 ˚C and the non-growing season as the rest (Shimizu *et al*. 2009). The growing season was 215 days in 2005 (From 10th April 2005 to 10th November 2005), 218 days in 2006 (From 15th April 2006 to 18th November 2006) and 220 days in 2007 (From 13th April 2007 to 18th November 2007.

Table 4 Cumulative N_2O emissions from the control, chemical fertilizer, and manure plots

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Treatment	Cumulative N_2O emissions (kg N_2O-N ha^{-1} yr^{-1})		
	2005	2006	2007
Control [a]	0.7 (0.4)	0.6 (0.3)	0.7 (0.5)
Chemical fertilizer [b]	2.8 (0.7)	3.0 (0.8)	1.4 (0.5)
Manure [c]	3.6 (1.2)	4.9 (2.8)	2.1 (0.6)

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Data represent means (SD), n=4 to 6, and different small letters denote the significant difference at the 0.05 level between each treatment.

Table 5 N_2O Emission factor for chemical fertilizer and manure (kg N_2O–N (kg N input)$^{-1}$)

	N_2O emission factor (%)		
	2005	2006	2007
Chemical fertilizer	1.32 (0.43)	1.30 (0.44)	0.85 (0.97)
Manure	0.51 (0.42)	0.85 (0.89)	0.35 (0.23)

Data represent means (SD), n=4 to 6.

Table 6 Relationships (Pearson correlation coefficient, r) between instantaneous N_2O fluxes and environmental factors using the whole data

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	N_2O	Soil T.	WFPS	pH	NO_3^-	NH_4^+	DOC
N_2O	1						
Soil T.	0.330 **	1					
WFPS	0.020	-0.288 **	1				
pH	-0.117	-0.275 **	0.378 **	1			
NO_3^-	0.307 **	0.378 **	-0.061	-0.201 *	1		
NH_4^+	0.329 **	0.156 *	-0.048	-0.273 **	0.380 **	1	
DOC	-0.104	-0.173 *	-0.204 *	0.341 **	-0.282 **	-0.040	1.

*$p<0.05$; **$p<0.01$

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Table 7 Denitrification enzyme activity (DEA) of soil samples from three treatment plots of control, chemical fertilizer, and manure

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Date	Soil layer	Treatment	DEA ($mgN_2O\text{-}N\ kg^{-1}h^{-1}$) Control plot		Chemical fertilizer plot		Manure plot	
2007/4/29	Root-mat	Chl	0.04	(0.04)	0.04	(0.01)	0.04	(0.02)
		Chl+N	9.99	(1.07)	3.66	(0.50)	16.30	(2.98)
		Chl+C	0.06	(0.01)	0.07	(0.02)	0.14	(0.07)
		Chl+N+C	10.98	(6.71)	3.08	(0.49)	13.56	(3.16)
	Mineral	Chl	2.68	(3.84)	0.16	(0.11)	0.58	(0.34)
		Chl+N	0.52	(0.64)	0.16	(0.03)	1.56	(0.25)
		Chl+C	0.02	(0.12)	0.22	(0.01)	0.07	(0.14)
		Chl+N+C	1.00	(0.20)	2.21	(1.50)	1.97	(0.36)
2007/6/11	Root-mat	Chl	0.15	(0.02)	0.01	(0.00)	0.10	(0.05)
		Chl+N	14.05	(5.03)	2.63	(0.68)	15.05	(2.28)
		Chl+C	0.08	(0.02)	0.02	(0.01)	0.13	(0.03)
		Chl+N+C	9.47	(0.33)	4.66	(0.40)	17.35	(0.81)
	Mineral	Chl	0.09	(0.03)	0.07	(0.02)	0.71	(0.09)
		Chl+N	1.43	(0.00)	0.35	(0.10)	1.24	(0.55)
		Chl+C	0.03	(0.00)	0.04	(0.01)	0.05	(0.14)
		Chl+N+C	2.14	(0.21)	0.54	(0.15)	1.71	(2.15)
2007/8/20	Root-mat	Chl	0.12	(0.05)	0.20	(0.06)	0.04	(0.01)
		Chl+N	27.84	(3.87)	9.80	(0.18)	13.58	(1.83)
		Chl+C	0.13	(0.06)	0.14	(0.05)	0.03	(0.02)
		Chl+N+C	21.33	(0.23)	35.19	(37.29)	19.91	(1.73)
	Mineral	Chl	1.14	(0.31)	0.76	(0.37)	3.01	(0.20)
		Chl+N	1.34	(0.17)	0.55	(0.09)	2.49	(1.83)
		Chl+C	0.37	(0.03)	0.64	(0.33)	1.03	(1.22)
		Chl+N+C	2.92	(0.23)	0.83	(0.33)	7.65	(0.87)

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Data represent means (SD), n=3.

Table 8 ANOVA results for soil denitrification enzyme activity (DEA)

Source	df	Mean Square	F	P value
Plot	2	94,796,698	3.351	0.058
Soil layer	1	991,237,940	35.040	0.000
Treatment	3	832,401,859	29.426	0.000
Plot * Soil layer	2	9,030,352	0.319	0.390
Plot * Treatment	6	53,775,775	1.901	0.174
Soil layer * Treatment	3	451,061,076	15.945	0.000
Plot * Soil layer * Treatment	5	25,358,833	0.896	0.258
Error	37	28,288,420		

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1

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0.330 **

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0.020

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-0.117

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0.307 **

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0.329 **

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-0.104

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-0.288 **

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-0.275

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0.378 **

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0.156 *

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-0.173 *

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0.378 **

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-0.061

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-0.048

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-0.204 *

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-0.201 *

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-0.273 **

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0.341 **

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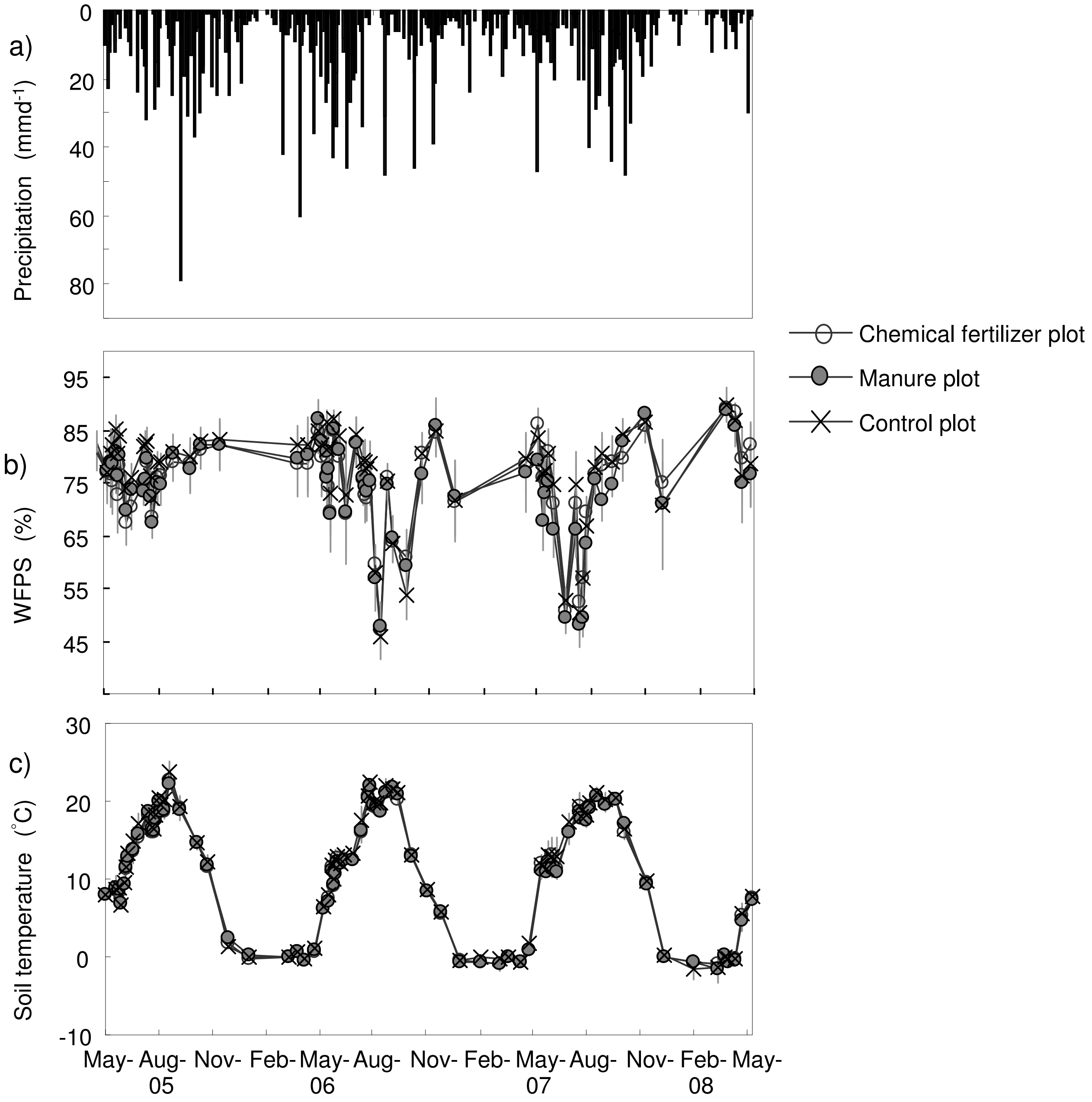
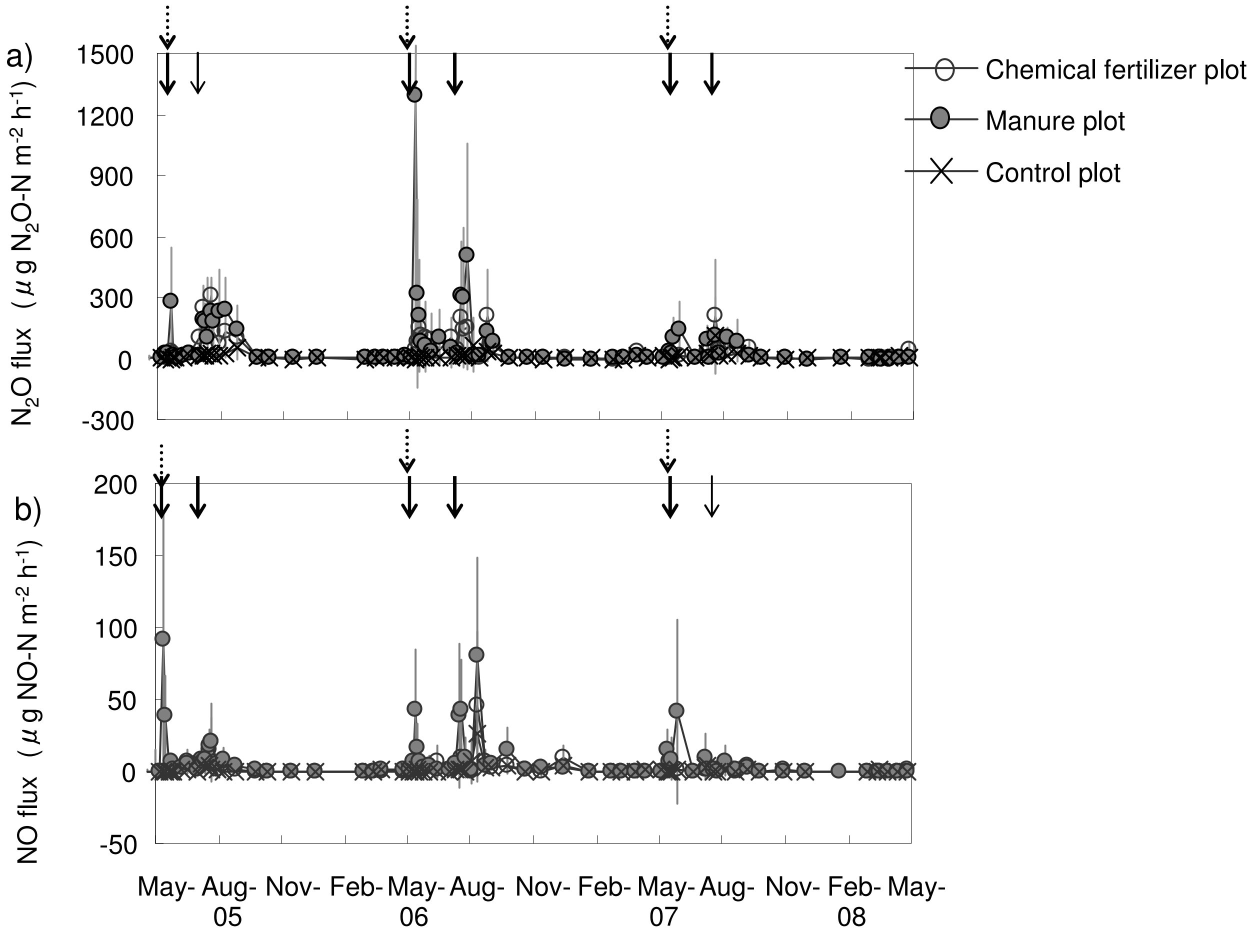
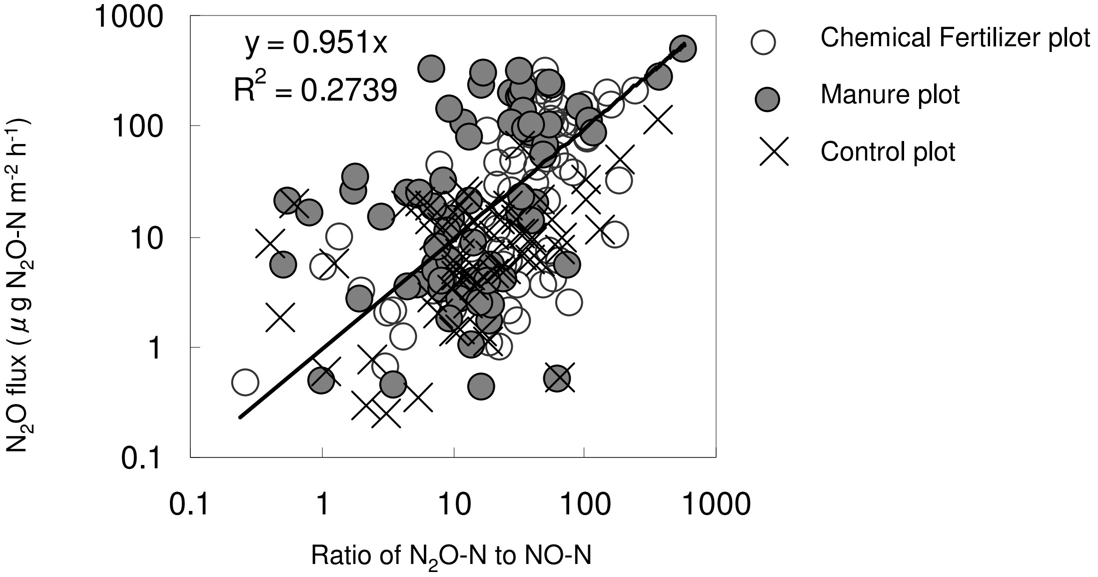
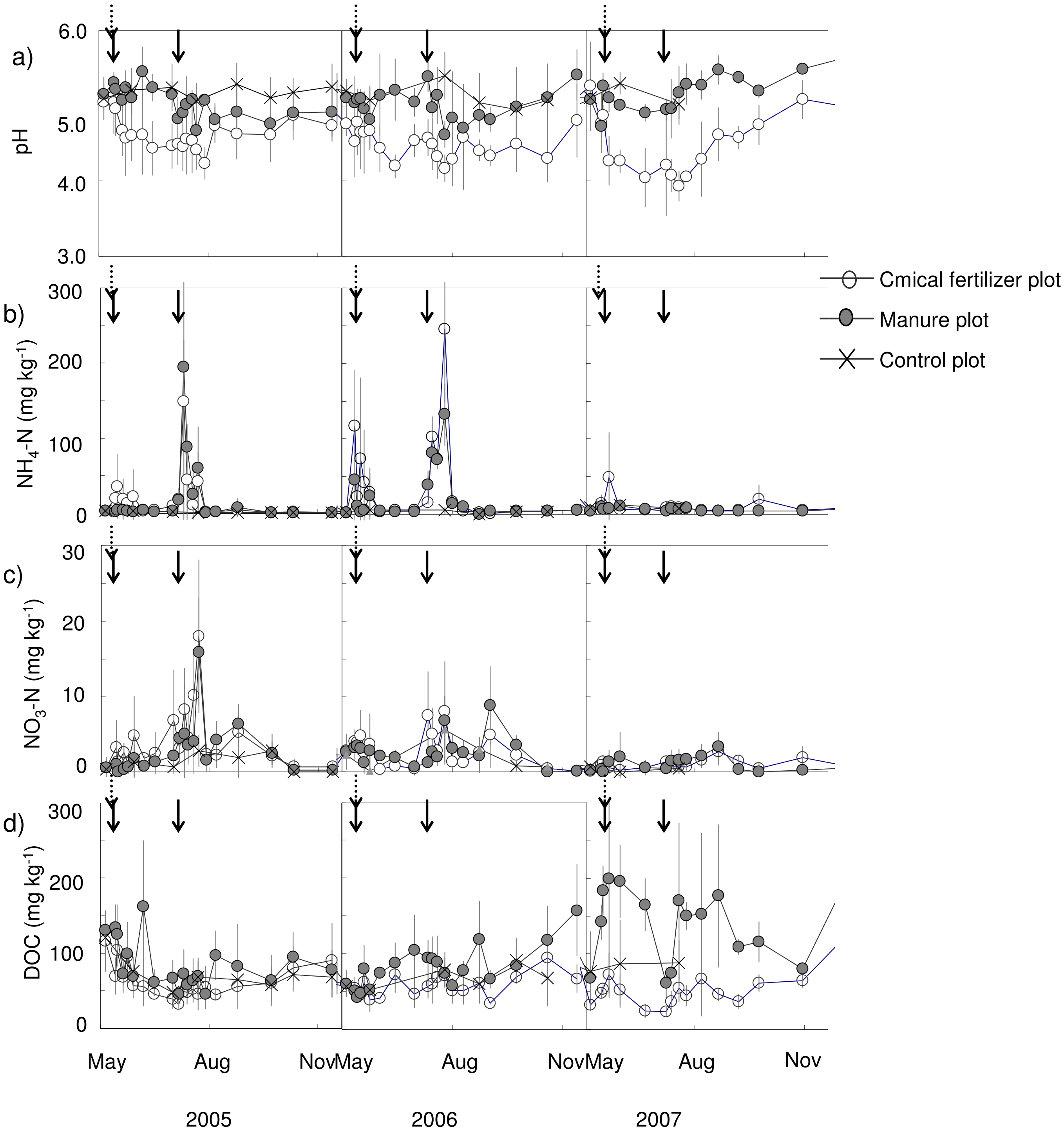
-0.040

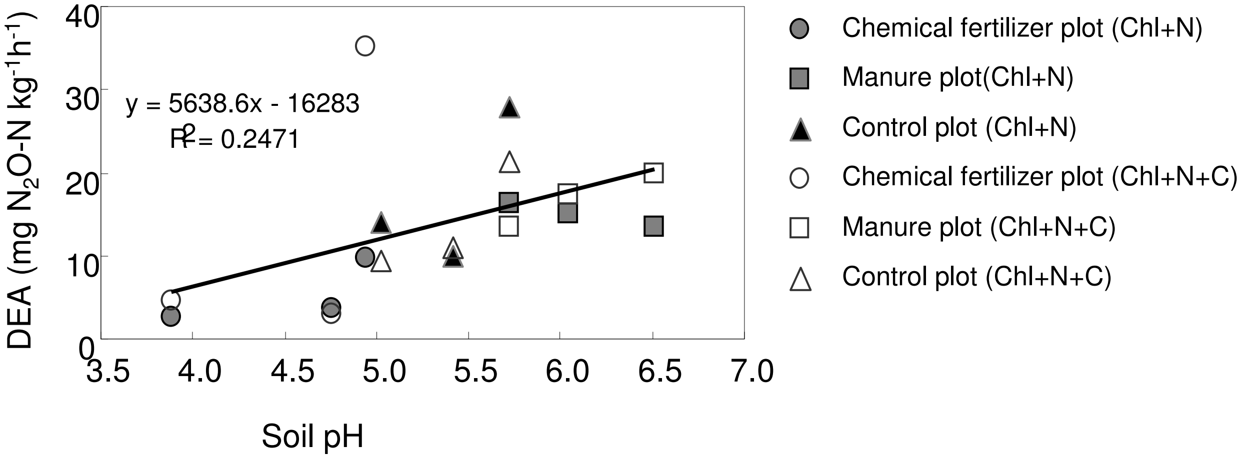


Follow the ‘Figure captions’ in the manuscript.


a)
b)
N₂O flux (μg N₂O-N m⁻² h⁻¹)
NO flux (μg NO-N m⁻² h⁻¹)
1500
1200
900
600
300
0
-300
200
150
100
50
0
-50
Chemical fertilizer plot
Manure plot
Control plot
May-
Aug-
05
Nov-
Feb-
May-
06
Aug-
Nov-
Feb-
May-
07
Aug-
Nov-
Feb-
08
May-


y = 0.951x
$R^2 = 0.2739$
Chemical Fertilizer plot
Manure plot
Control plot
1000
100
10
1
0.1
0.1
1
10
100
1000
N_2O flux (μ g N_2O-N m^{-2} h^{-1})
Ratio of N_2O-N to NO-N


a)
pH
6.0
5.0
4.0
3.0
b)
NH$_4$-N (mg kg^{-1})
300
200
100
0
c)
NO$_3$-N (mg kg^{-1})
30
20
10
0
d)
DOC (mg kg^{-1})
300
200
100
0
Cmical fertilizer plot
Manure plot
Control plot
May
Aug
Nov
May
Aug
Nov
May
Aug
Nov
2005
2006
2007


DEA (mg N_2O-N $kg^{-1}h^{-1}$)
40
30
20
10
0
y = 5638.6x - 16283
R^2 = 0.2471
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
Soil pH
● Chemical fertilizer plot (Chl+N)
■ Manure plot(Chl+N)
▲ Control plot (Chl+N)
○ Chemical fertilizer plot (Chl+N+C)
□ Manure plot (Chl+N+C)
△ Control plot (Chl+N+C)