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1   Title

2   N_2O emissions during the freezing and thawing periods from six fields in a livestock

3   farm, southern Hokkaido, Japan

4

5   Running title

6   N_2O emissions during the freezing-thawing period

7

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10

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21

1   Abstract

2   In many countries, high nitrous oxide (N_2O) emissions have been observed during

3   soil freezing and thawing periods. Quantification of those emissions is crucial to

4   evaluate annual N_2O emissions. For this study, we measured N_2O and NO fluxes along

5   with soil N_2O concentrations at a corn field and five grasslands during a winter-spring

6   period in Southern Hokkaido, Japan. We also measured denitrification activities of the

7   soils from those sites. During the observation period, the soils froze to a maximum

8   depth of 370 mm under saturated conditions and the lowest soil temperature at a 50

9   mm depth was -4.5 °C. After 6 March 2005, daily air temperature rose above 0 °C, but

10   the soil temperature remained approximately 0 °C for about two weeks. These two

11   weeks were defined as the 'transition period,' while the periods before and after the

12   transition period were defined as the 'freezing' and the 'thawing' periods, respectively.

13   During the freezing and transition periods, N_2O concentration increased in the frozen

14   soils relative to the unfrozen soils and the highest values were observed in the frozen

15   soils during the transition period. During the thawing period, the N_2O concentration in

16   the soils decreased. N_2O emissions were much higher during the thawing period than

17   during the freezing and transition periods, and remarkably higher N_2O emissions were

18   observed at the corn site compared to those at the grassland sites. NO emissions were

19   also observed during the thawing period but at much lower levels than N_2O emissions

20   at all the sites. N_2O-N/NO-N ratio exceeded one at all the sites during the entire period,

21   indicating N_2O production through denitrification. At the corn site, denitrification

22   activity was much lower and $N_2O/(N_2O+N_2)$ was much higher than at the grasslands.

23   The result indicated that high N_2O emissions at the corn site were caused by

24   complimentary processes: (1) high accumulated N_2O through denitrification in the

25   frozen soil during the freezing and transition periods; and (2) low N_2O reduction rate

26   during the thawing period.

3

1

2　Key words: corn field, denitrification activity, freezing and thawing periods, grassland,

3　nitrous oxide

4

1 **INTRODUCTION**

2 Nitrous oxide (N_2O) is a major greenhouse gas that is responsible for destruction

3 of stratospheric ozone (Crutzen 1970). Global N_2O emissions are estimated at 17.7 Tg

4 N y^{-1} and of those, an estimated 6.7 Tg N y^{-1} are from anthropogenic sources (Denman

5 et al. 2007). Agricultural soils are the main source of N_2O emissions, at an estimated

6 42% of total anthropogenic N_2O emissions (Denman et al. 2007).

7 In soils, N_2O is produced mainly by nitrification and denitrification. These

8 biological processes are influenced by soil environmental factors, such as moisture

9 condition, oxygen level, soil temperature, nitrogen (N) availability, organic matter

10 content, and pH (Mosier 1998; Sahrawat and Keeney 1986). The soil freeze-thaw cycle

11 has also been recognized as having an influence on soil N_2O emissions, since Bremner

12 et al. (1980) observed N_2O emissions during a winter period. Kaiser and Ruser (2000)

13 summarized annual N_2O emission data and winter N_2O losses observed on arable soils

14 in Germany from 1995 to 1999. The annual N_2O losses ranged from 0.53 to 16.8 kg N

15 ha^{-1} and N_2O losses during the winter ranged from 7% to 89%. We also summarized

16 the reported data and the N_2O losses during the winter ranged from 0% to 93% (Table

17 1). These results indicate a high concentration of N_2O emissions during the winter

18 period and uncertainty of N_2O emissions.

19 It has been assumed that N_2O would be produced in an unfrozen soil layer

20 beneath a frozen layer and released from the soil surface during a thawing period once

21 the frozen soil cover has disappeared in situ (Bremner et al. 1980, Burton and

22 Beauchamp 1994, and Kaiser et al. 1998). But based on a soil column experiment,

23 Teepe et al. (2001) and Wagner-Riddle et al. (2007) hypothesized that N_2O was

24 produced and trapped in the frozen soil during a freezing period and then released

25 during a thawing period. Recently, Yanai et al. (2011) found an increase in N_2O

26 concentration with a decrease in O_2 concentration in frozen soil during the freezing and

1 thawing periods.

2 To investigate the N_2O production process during the freezing and thawing

3 conditions, soil incubation experiments have been conducted by many researchers.

4 Yanai et al. (2004b) evaluated the nitrification potential of frozen soils and reported

5 that soil freeze-thaw cycles did not inhibit the nitrification potential of soils that

6 experienced a smaller decrease in microbial biomass following the freeze-thaw cycles.

7 The source of ammonium (NH_4^+), which is the base of nitrification, may be soil

8 organic matter (McGarity 1962, Groffman and Tiedje 1989, Neilson et al. 2001) or

9 destructed microbes or substrates from the destructed microbes (McGarity 1962,

10 Groffman and Tiedje 1989, Schimel and Clein 1996, Yanai et al. 2004a). Christensen

11 and Christensen (1991) evaluated soil carbon availability by denitrifiers, using an

12 acetylene block method under freeze-thaw conditions. Müller et al. (2002) and Ludwig

13 et al. (2004) reported that high N_2O emissions were caused by the enrichment of NO_3^-

14 through $^{15}NO_3^-$ addition experiments. Based upon an experiment with a ^{15}N tracer,

15 Wagner-Riddle et al. (2008) concluded that denitrification activity during a thawing

16 period was the main process of N_2O production. Mørkved et al. (2006) also conducted

17 a $^{15}NO_3^-$ addition experiment and reported that denitrification was the main N_2O source

18 in freeze-thaw-affected soil and that soluble carbon could play a significant role in the

19 freeze-thaw cycle. Öquist et al. (2004) observed higher N_2O emissions after freezing at

20 -4 ^{o}C in soils with a high moisture content compared to soils with a low moisture

21 content.

22 Despite significant research on N_2O emissions from soil, there are only a few

23 studies that have measured both N_2O concentration in soil and N_2O flux at a field and

24 evaluated denitrification potentials as well. The objective of this study was to

25 determine the cause of high N_2O emissions from soils during a thawing period. We

26 measured N_2O fluxes and N_2O concentrations in soils at a livestock farm in southern

1   Hokkaido, Japan. The study was conducted after a cropping period in 2004, because

2   high N_2O emissions were observed from 2000 to 2003 (Table 1). Furthermore, an

3   incubation experiment was conducted just after soil thawing to measure denitrification

4   activities in soils.

5

6   **MATERIALS AND METHODS**

7   **Study area**

8   The study was conducted at the 458-ha Shizunai Experimental Livestock Farm

9   of the Field Science Center for the Northern Biosphere, Hokkaido University, Japan

10   (42.4317N,142.4817E). The site is an experimental station, but also a working

11   production facility where young animals are reared to maturity (e.g., for beef

12   production and preserving native species of Hokkaido horses) and crops (primarily

13   corn and grass) are grown to support the animals. The farm is located in the watershed

14   of the Kepau River, which flows through the farm from an upstream forested area. The

15   average annual mean temperature at the study site is 7.9 °C, the minimum monthly

16   temperature is −8.1 °C in February, and the maximum monthly temperature is 23.6 °C

17   in August. The average annual precipitation is 1365 mm.

18   Measurements were taken at a corn field (C_2), a grassland that was recently

19   converted from a corn field in 2003 (CG), and four grasslands (G_{2s}, G_{2c}, G_{2n} and G_3)

20   which have been used as a meadow for more than three years. Three grasslands G_{2s},

21   G_{2c}, and G_{2n} were divided from G_2 and named G_{2s} for the southern site, G_{2c} for the

22   center site, and G_{2n} for the northern site (see Supporting Information, Figure S1).

23   These three grasslands were adjacent to one another and they were all managed in the

24   same way. The soil types of the research sites (Katayanagi et al. 2008) are Vitric

25   Andosols at CG, G_{2s}, G_{2c}, and G_{2n} and Histosols at C_2 and G_3 (FAO 1988) (Table 2).

26   The dominant vegetation type is corn (*Zea mays* L.) at C_2, reed canary grass (*Phalaris*

1 *arundinacea* L.) at G_{2s}, G_{2c}, G_{2n} and G_3, and timothy-grass (*Phleum pratense* L.) at CG.

2 Grass was harvested twice annually and corn was harvested once a year.

3   After the cropping season in 2004, manure was applied to CG, G_3, and C_2 on 27

4 October 2004 and slurry was applied to C_2 on 18 October 2004 (Table 3). Nitrogen

5 surpluses at G_2, G_3, CG, and C_2 calculated by Katayanagi et al. (2008) are also

6 provided in Table 3.

7

8 **Temperature, precipitation, and snow depth**

9   Daily precipitation measurements from the farm's Sasayama Weather Station

10 (42.4333N, 142.4817E) (Fig. 1) were used for the analysis of gas data. Daily air

11 temperature was also observed at the station but there were many missing data due to

12 equipment problems. Snow depth was not observed at the station. Therefore, the data

13 measured at the Shizunai Weather Station (42.3433N, 142.3617E) were used instead.

14 For calculation of gas fluxes, air temperature inside a static chamber was measured by

15 a digital thermometer during the gas measurement. Soil temperature at a depth of 50

16 mm was measured continuously during the study period at hourly intervals using a

17 small waterproof temperature logger (TR-52, T&D, Nagano, Japan) for one replicate

18 per field. Depth of soil freezing was measured using an acrylic tube that was filled with

19 a 0.03% methylene blue solution. A polyvinyl chloride (PVC) pipe was used to protect

20 the acrylic tube. Petroleum jelly was spread thinly on the acrylic tube to prevent it from

21 sticking to the PVC pipe.

22

23 **Soil sampling**

24   Disturbed soil samples for measurements of soil chemical properties were

25 collected from a 0−50 mm depth. The sampling was conducted with three to six

26 replicates per site before and after freezing (08 or 18 December 2004 and 19 April

27 2005, respectively). Only a single sample was collected on 31 March 2005 when the

1   soil started to thaw because only surface soil from approximately 0 to 10 mm depth

2   had thawed, and it was difficult to collect a large volume of soil samples at that time.

3   The collected soil samples were preserved at 4 °C and analyzed within a week of

4   sampling.

5

6   **Analysis of soil chemical properties**

7   The disturbed soil samples from each site were extracted in deionized water (at a

8   1:5 ratio of soil to water) in bottles and filtered through a 0.2 μm membrane filter using

9   suction. Nitrate-N (NO_3^--N), NH_4^+-N and dissolved organic carbon (DOC)

10   concentrations in the extracted solution were determined using an ion-exchange

11   chromatography (QIC analyzer, Dionex, Sunnyvale, CA, USA), colorimetry with

12   indophenol-blue, and total organic carbon analysis (TOC-5000A, SHIMADZU, Kyoto,

13   Japan), respectively. Soil pH was measured with a glass electrode (F-22, Horiba, Kyoto,

14   Japan) using infiltrated samples remaining in the bottle after the filtration.

15

16   **Measurement of denitrification enzyme activity**

17   Using the disturbed soil samples collected on 31 March 2005, denitrification

18   activity was measured by the acetylene block technique proposed by Tiedje (1994). A

19   total of 15 g of fresh soil sample was thoroughly mixed with a 15 mL chloramphenicol

20   solution (1 g L^{-1}) in a 200-mL serum bottle to prevent enzyme production (Tiedje 1994,

21   Lowrance 1992, Murray and Knowles 1999). To determine the denitrification activity

22   under field conditions, available C and N were not added to the soil samples. The

23   serum bottle was evacuated and flushed four times with N_2 to ensure anaerobic

24   conditions. For this study, the incubations were conducted using non-acetylene and

25   acetylene treatments to determine the denitrification activities of N_2O production and

26   N_2+N_2O production, respectively. N_2O production was estimated using N_2O emissions

27   from the non-acetylene-treated samples and N_2+N_2O production was estimated using

1 N_2O emissions from the acetylene-treated samples because acetylene inhibits N_2O

2 reduction to N_2. Acetylene gas was added to a final concentration of 10% (10 kPa) in

3 the headspace for the acetylene treatment. Incubations were conducted in triplicate at

4 5 °C, which was the soil temperature at the time of collection from the field. At 1 and 2

5 hours after the incubation start time, 15 mL of headspace gas was sampled from each

6 bottle into an evacuated glass vial using a 25 mL syringe. N_2O concentration of the gas

7 samples, kept in evacuated glass vials, was analyzed by gas chromatography with an

8 electron capture detector (GC 14B, Shimadzu, Kyoto, Japan) at 375 °C. PorapackQ

9 filled in a stainless steel column (2 m length and 3 mm inner diameter) was used as

10 stationary compound and 5% methane in argon was used as the carrier gas. The

11 minimum detectable concentration of N_2O was 0.007 ppmv, as determined from the

12 deviation obtained by repeatedly analyzing the concentration of N_2O standard gas

13 (atmospheric level).

14

15 **Measurement of N_2O and NO fluxes**

16 Measurements of N_2O and NO were conducted once a month in December 2004

17 and February 2005 and once a week in March and April 2005. Each flux measurement

18 was replicated at four locations per site for G_3, CG, and C_2 and at six locations per site

19 for G_{2s}, G_{2c}, and G_{2n}.

20 The measurements were conducted using a closed-chamber technique (Rolston

21 1986). A cylindrical stainless steel chamber (diameter 300 mm, height 350 mm) was

22 used for measurements. Detailed information about this chamber design is reported in

23 a previous study (Katayanagi et al. 2008). The deepest snow depth on the monitoring

24 days was only 150 mm, and the chambers were placed on the snow during the freezing

25 period. During the soil freezing conditions, gas samples were collected at 0 and either

26 40 or 60 minutes after closing the lid; during the other periods, samples were collected

1   at 0 and 20 minutes after closing the lid. The chamber height from the snow surface to

2   the top of the chamber was measured for four replicates to calculate gas flux. A

3   500-mL gas sample was taken using a 50 mL syringe and injected into a 500 mL

4   Tedlar® bag. The bags were then brought to the laboratory and 20 mL of each gas

5   sample was immediately transferred into a 10 mL evacuated glass vial, using a 25 mL

6   syringe, for N_2O analysis.

7   N_2O concentrations of the samples kept in evacuated glass vials were analyzed

8   using the gas chromatograph as described earlier. The gas samples kept in Tedlar® bags

9   were analyzed by a chemoluminescence nitrogen oxide analyzer (MODEL-265P,

10   Kimoto Electric, Osaka, Japan) within 24 hours of sampling to measure NO

11   concentrations in the samples.

12

13   **Measurement of soil N_2O concentration**

14   The soil N_2O concentration was measured concurrently with the gas flux

15   measurement. Stainless steel tubes (inner diameter 10 mm and outer diameter 12 mm;

16   the lengths of the tubes were the target depth plus 100 mm; there was no side hole on

17   the pipe) were installed in the soil at 100, 200, 300, 400, 500, and 600 mm depths with

18   three replications. A stainless steel stick (20 mm longer than the pipe) was inserted in

19   the pipe during installation, in order to avoid clogging the pipe with soil and to provide

20   air space (ca. 2 mL) at the bottom of the pipe. The stick was removed after installation.

21   A three-way cock was connected to the aboveground part of an installed pipe using a

22   vinyl tube (100 mm), a straight connector and a silicone tube (50 mm); 50 mL air was

23   drained from inside the pipe through the cock just after the installation. The pipe was

24   left in the place throughout the freezing and thawing periods, and the cock was closed

25   and covered using a PET cap to avoid freezing the cock from snow and ice exposure. A

26   50 mL soil gas sample was collected from each pipe. The gas samples were collected

27   slowly to prevent contamination by gas from non-target depths. However, the volumes

1   of the pipes were small and the gas under the target depth would be collected together

2   with the gas at the target depth. The samples that were collected from the same depth

3   were placed into a Tedlar® bag together. A 300 mL air sample from the soil surface was

4   collected near the pipes at the same time. N_2O was analyzed using the gas

5   chromatography, as described earlier. Because of those operations, we had only one

6   replicate sample per depth at each site; therefore, standard variations could not be

7   reported in this paper.

8

9   **Calculation of N_2O and NO fluxes**

10   N_2O and NO fluxes were calculated by a linear regression using the following

11   equation:

12

13   $F = \rho \times (V/A) \times (\Delta c/\Delta t) \times [273/(273 + T)] \times P/760$

14

15   where F is the flux (mg N m^{-2} h^{-1}), ρ is the gas density ($\rho N_2O\text{-}N = 1.26 \times 10^6$ and

16   $\rho NO\text{-}N = 0.63 \times 10^6$ mg N m^{-3}), V is the volume of the chamber (m^3), A is the area of

17   the chamber (m^2), $\Delta c/\Delta t$ is the ratio of change in the gas concentration inside the

18   chamber (10^{-6} m^3 m^{-3} h^{-1}), T is the air temperature inside the chamber (°C) and P is air

19   pressure (mm Hg). For this study, we defined positive fluxes as emissions and negative

20   fluxes as uptake by soil or snow and calculated $N_2O\text{-}N/NO\text{-}N$ when both N_2O and NO

21   fluxes were positive during the observation period.

22

23   **Statistical analysis**

24   Statistical analyses were performed with Kyplot 5.0 (KyensLab Inc. Tama, Tokyo,

25   Japan). The $NH_4^+\text{-}N$, $NO_3^-\text{-}N$, and DOC concentrations, measured before freezing and

26   at the end of thawing, were compared using paired t-tests. Differences between N_2O

27   and N_2+N_2O fluxes, which were the results of denitrification enzyme activity

1 measurement, were also compared using paired t-tests and differences of N_2O and

2 N_2+N_2O fluxes among each site were compared by Tukey-Kramer test. Correlation

3 analyses between the NH_4^+-N, NO_3^--N, and DOC concentrations and the manure plus

4 slurry N application and surplus N were performed using the same software.

5

6 **RESULTS**

7 **Temporal variability of air temperature, soil temperature, precipitation, snow**

8 **depth and frozen soil layers**

9 The daily air temperature decreased from December to February and increased

10 from March to April (Fig. 1). Daily mean soil temperature at 50 mm depth dropped

11 below zero from 20 December 2004 and remained below zero until around 06 March

12 2005 (Fig. 2). The lowest recorded daily soil temperatures were -4.5, -1.8, -2.3, -4.7,

13 -3.2, and -3.3 °C at C_2, CG, G_{2s}, G_{2c}, G_{2n}, and G_3, respectively. After 06 March 2005,

14 the soil temperature did not increase and remained approximately 0 °C for about two

15 weeks, even though air temperature rose above 0 °C (Fig. 2a). We defined these two

16 weeks as the 'transition' period while the periods before and after were defined as the

17 'freezing' and 'thawing' periods, respectively. The freezing period started from 20

18 December under the definition and the period prior to 20 December 2004 was defined

19 as the 'unfreezing' period.

20 The maximum snow depth during the whole period was 250 mm (Fig. 1) and

21 soil freezing was observed from 24 January 2005. The depth of the frozen soil layer

22 was getting deeper after soil freezing started (Fig. 2e); the maximum depths of the

23 frozen layers were observed in March and were 370, 230, 220, 220, 340, and 350 mm

24 at C_2, CG, G_{2s}, G_{2c}, G_{2n}, and G_3, respectively. The frozen soils were very hard during

25 the freezing period due to the saturated soil conditions caused by high soil water

26 content before freezing (water-filled pore space was 72, 92, 79, 81, 82 and 79% at C_2,

13

1 CG, G_{2s}, G_{2c}, G_{2n}, and G_3, respectively) and also by water supply from rainfall and

2 melted snow when air temperature increased above 0 °C. Soil thawing was observed

3 from 24 March 2005 at both the soil surface and the bottom of the frozen layers (Fig.

4 2e). Frozen soil layers were not observed after 19 April 2005 at all sites.

5

6 **N_2O and NO fluxes**

7   N_2O emissions during the thawing period were much higher than the emissions

8 during other periods (Fig. 2b). During the thawing period, fluxes at all sites showed

9 three patterns: i) remarkably high N_2O emissions were observed at C_2 and CG; ii) N_2O

10 emissions increased through the transition and thawing periods at G_{2s}, G_{2c}, and G_3, and

11 the fluxes at those sites were much lower than fluxes at C_2 and CG; and iii) N_2O peaks

12 were observed during the transition and thawing periods and high N_2O uptake

13 (negative N_2O flux) was also observed at G_{2n}. N_2O emissions were also observed

14 during the unfreezing and freezing periods at all sites and were higher at C_2 than at the

15 other sites (Fig. 2b), but the emissions were lower during the freezing period than

16 during the thawing period.

17   During the study period, NO emissions at all sites showed a similar temporal

18 variation: low NO emissions were observed at the beginning of the transition period

19 and higher NO emissions were observed during the latter part of the transition and

20 thawing periods (Fig. 2c). NO emissions were also observed at all sites during the

21 unfreezing and freezing periods as well as during the thawing period (Fig. 2c).

22 However, while NO emissions were observed during the whole observation period, the

23 emission level was much lower than N_2O (Fig. 2b, c).

24   N_2O-N/NO-N during the unfreezing and freezing periods was lower than the

25 values during the thawing period (Fig. 2d). The values were distributed from 2.0 to

26 13.6 except at C_2 during the unfreezing period. The highest value at C_2 during the

14

1 unfreezing was 69.8. During the thawing period, higher N_2O-N/NO-N was observed at

2 C_2 than at the other sites and the highest value at C_2 was 151. The values at the other

3 sites were distributed from 8.5 to 63.3 during the thawing period. The temporal

4 variation of N_2O-N/NO-N was similar to that of N_2O at C_2, CG, G_{2s}, and G_{2c} (Fig. 2b,

5 d). At G_{2n} and G_3, there was no apparent pattern due to a few samples and negative

6 N_2O emissions.

7

8 **Soil N_2O concentrations**

9 During the freezing and transition periods, the N_2O concentrations increased in

10 the frozen soils and were higher during those periods than concentrations during the

11 unfreezing and thawing periods at all sites except G_{2c}, where gas samples in the frozen

12 soil could not be collected (Fig. 2e). The highest N_2O concentrations were observed in

13 the frozen soils during the transition period at all the sites except G_{2c}. N_2O

14 concentrations also increased in the unfrozen soil layers below the frozen layers at C_2,

15 CG, G_{2s} and G_{2n}, but, the concentrations in the unfrozen soils were lower than those in

16 the frozen soils (Fig. 2e). At G_{2c} and G_3, the N_2O concentration in the unfrozen soil

17 layers just below the frozen soil layers and at the edge of the frozen soil layers was

18 lower than that of the atmosphere (i.e., approximately 0.3 ppmv).

19 After the start of soil thawing, N_2O concentrations in the thawed and unfrozen

20 layers decreased at C_2, CG, G_{2s} and G_{2n}. At G_{2c} and G_3, the N_2O concentrations

21 increased to and above the level in the atmosphere (Fig. 2e).

22

23 ***Soil NH_4^+-N, NO_3^--N and DOC concentrations during soil freezing***

24 Figure 3 shows the soil NH_4^+-N, NO_3^--N and DOC concentrations during the

25 unfrozen period measured on 08 or 18 December 2004, at the beginning of the thawing

26 period measured on 31 March 2005, and at the end of thawing period measured on 19

27 April 2005.

1   Soil NH_4^+-N values were remarkably high at the beginning of the thawing period

2 compared with the values during the unfreezing period and at the end of the thawing

3 period for all sites (Fig. 3); however, the data couldn't be statistically compared with

4 the other data. The value at the end of the thawing period ($p = 0.004$) was significantly

5 higher than before freezing at C_2, while there was not a significant difference in the

6 values at the other sites (Fig. 3). The NH_4^+-N concentration did not show any

7 correlation with slurry + manure N and N surplus, as shown in table 3.

8   Soil NO_3^--N values at the beginning of the thawing period were higher than those

9 before the freezing period at C_2, CG, and G_{2n}; the values at G_{2s}, G_{2c} and G_3 did not

10 show appreciable differences (Fig. 3) but the data couldn't be statistically compared

11 with the other data. The highest NO_3^--N concentrations were observed at the end of the

12 thawing period for all sites, and the values at C_2, CG, G_{2n} and G_3 were significantly

13 higher than those before the freezing period (Fig. 3; the p values were 0.0005, 0.0384,

14 0.0499, and 0.0034, respectively). The NO_3^--N concentration showed a positive

15 correlation with the application rates of slurry + manure N (Table 3) for all sampling

16 dates ($r = 0.9031$ and $p = 0.0136$ on 08 and 18 December 2004, $r = 0.9272$ and $p =$

17 0.0078 on 31 March 2005, and $r = 0.9035$ and $p = 0.0135$ on 19 April 2005,

18 respectively).

19   The soil DOC concentrations measured at the beginning of the thawing period

20 were higher than those measured during the unfreezing period and at the end of the

21 thawing period for all sites (Fig. 3). There was no significant difference between the

22 values for the freezing period and those for the end of the thawing period. The DOC

23 concentrations did not show any correlation with slurry + manure N and N surplus.

24

25 **Denitrification activities**

26   N_2O production was significantly lower at C_2 and CG and higher at G_3 ($p <$

27 0.05); N_2O+N_2 production was significantly lower at C_2 and CG and higher at G_{2c} and

1   G_3 compared with the other sites (Table 4). $N_2O + N_2$ production was significantly

2   higher than N_2O production at G_{2c} and G_3 and lower than that at CG ($p < 0.05$) (Tables

3   4). $N_2O + N_2$ production was also higher than N_2O production at G_{2s} and G_{2n}, but it

4   was not significant because of the high standard deviation (Table 4). The ratio of

5    $N_2O/(N_2O+N_2)$ was lower at G_{2c} and G_3 than at the other sites, but it was not

6   significant (Table 4). A regression analysis of N_2O and $N_2O + N_2$ productions and the

7   amount of manure + slurry N and surplus N was also conducted, but the results did not

8   show any relationship.

9

10   **DISCUSSION**

11   **N_2O production and accumulation in frozen soil during the freezing and**

12   **transition periods**

13   During the freezing period, production and accumulation of N_2O in the frozen

14   soils resulted from inhibition of gas diffusion by the frozen soil lids and from N_2O

15   reduction to N_2. Higher N_2O concentrations in the soils, relative to the atmosphere (Fig.

16   2), indicated N_2O accumulation in the soils and disturbance of gas exchange by the

17   frozen saturated soil, which functioned as a "lid" for all the sites. In the soil layers, the

18   highest N_2O concentration was observed in frozen rather than unfrozen soils (Fig. 2);

19   this indicated that higher N_2O production and/or lower N_2O reduction occurred in the

20   frozen soils compared to unfrozen soils and that the produced or remaining N_2O in the

21   frozen soils accumulated there. The results show that N_2O production occurs in the

22   frozen soil *in situ* as well as in a laboratory (Christensen and Christensen 1991, Müller

23   et al. 2002, Yanai et al. 2004a and b, Öquist et al. 2004, Öquist et al. 2007), and

24   support the hypothesis of Teepe et al. (2001) and Wagner-Riddle et al. (2007) that N_2O

25   is produced and trapped in frozen soils during a freezing period.

26   Denitrification was identified as an important N_2O production process in the

1   frozen soils, as demonstrated by the higher-than-one ratio of N_2O-N/NO-N during the

2   freezing period (Fig. 2).   Lipschultz et al. (1981) reported that the N_2O-N/NO-N ratio

3   is considered an indicator of the contribution of nitrification and denitrification; when

4   N_2O-N/NO-N is less than one, the main process of N_2O production is considered to be

5   from the nitrification process. Alternatively, when N_2O-N/NO-N is larger than 100, the

6   main process of N_2O production is considered to be the denitrification process. The lid

7   mentioned before must disturb O_2 diffusion from the atmosphere into the soils and O_2

8   concentration in the soils must be low during the freezing period. Under such

9   conditions, nitrification must be restrained by deficiency of O_2 as an electron acceptor

10   and N_2O production by denitrification would be accelerated. Yanai et al. (2011)

11   observed an increase in N_2O concentration corresponding with a decrease in O_2

12   concentration in frozen soil during freezing and thawing periods and Wrage et al.

13   (2001) reported that low O_2 concentration inhibited N_2O reduction to N_2.

14   Nitrate, which is a substrate of the denitrification, was supplied from slurry and

15   manure, because application rates of slurry + manure N (Table 3) showed a positive

16   correlation with NO_3^--N on all the sampling dates. NH_4^+-N concentrations were higher

17   in the surface soils just after thawing than before freezing (Fig. 3); this indicates that

18   NH_4^+, which is a substrate of nitrification, was supplied through mineralization during

19   the freezing period and high NO_3^--N was caused by the nitrification activity. The

20   source of the NH_4^+ was not determined in this study. However, it would likely be the

21   applied slurry and manure, soil organic matter (McGarity 1962, Groffman and Tiedje

22   1989, Neilson et al. 2001), destructed microbes or substrates from destructed microbes

23   (McGarity 1962, Groffman and Tiedje 1989, Schimel and Clein 1996, Yanai et al.

24   2004a). Yanai et al. (2004b) evaluated the nitrification potential of frozen soils and

25   reported that a decrease in microbial biomass after freeze-thaw cycles was less

26   substantial and that the nitrification potential was not inhibited by the soil freeze-thaw

18

1   cycles. Neilson et al. (2001) reported that soil freezing did not disturb nitrification. The

2   nitrification process would also produce N_2O, but Mørkved et al. (2006) observed a

3   low contribution of nitrification: N_2O produced through nitrification was -0.7% to

4   4.35% of N_2O emissions during the thawing period.

5

6   **N_2O production and emission during the thawing period**

7   High N_2O emissions during the thawing period were caused by diffusion of

8   accumulated N_2O after disappearance of the frozen soil lids and a low N_2O reduction

9   rate during the thawing period. At C_2 and CG, higher N_2O concentrations in the surface

10   soils were observed during the freezing and transition periods, and high N_2O emissions

11   were observed during the thawing period after disappearance of the frozen soil lid (Fig.

12   2). However, the denitrification activity was much lower and $N_2O/(N_2O+N_2)$ was much

13   higher at those sites compared with other sites (Table 4). From these results, it is

14   evident that the accumulated N_2O was not reduced to N_2 by denitrification activity and

15   high N_2O emissions were caused by the high N_2O concentration in soils and the high

16   gas diffusion coefficients (Table 2) at the sites. Compared with the N_2O emissions at

17   C_2 and CG, N_2O emissions at G_{2s} and G_3 were much lower, even though N_2O

18   concentrations in the surface soils were high (Fig. 2). The low emissions must be

19   attributed to high denitrification activities, which reduced N_2O to N_2 and resulted in

20   low $N_2O/(N_2O+N_2)$ (Table 4). The low gas diffusion coefficient at the sites (Table 2)

21   would also accelerate N_2O reduction to N_2 before the emission. The N_2O concentration

22   in the frozen soil at G_{2c} was not clear, but the low N_2O emissions during the thawing

23   period would be attributed to the same reason as G_{2s} and G_3; the N_2O profile (Fig. 2),

24   the low gas diffusion coefficient (Table 2), the high denitrification activity and the low

25   $N_2O/(N_2O+N_2)$ (Table 4) at G_2 were similar to the values at G_3. At G_{2n}, low N_2O

26   emissions as well as N_2O uptake were observed during the thawing period (Fig. 2b).

27   This would be caused by a relatively low N_2O concentration in the frozen soil (Fig. 2)

1 and high denitrification activity (Table 4). In a soil column experiment, Teepe et al.

2 (2001) and Wagner-Riddle et al. (2007) showed that N_2O trapped in frozen soil was

3 released during a thawing period, which is consistent with our findings.

4   N_2O produced through nitrification and denitrification during the thawing period

5 would also contribute to the N_2O emissions during the period, but the contribution

6 must be low at the sites. The N_2O-N/NO-N values above one resulted from an increase

7 in N_2O emissions during the thawing period (Fig. 2) indicated a low contribution of

8 nitrification. The contribution of denitrification to N_2O emissions was considered from

9 the results, but high denitrification activity decreased N_2O to N_2 in the sites. Therefore,

10 the contribution of N_2O production through denitrification during the thawing period

11 would be low. Nitrification and the diffusion of accumulated N_2O was the main source

12 of N_2O emissions during the period.

13

14

15 **CONCLUSION**

16   N_2O produced through denitrification activity during the freezing and transition

17 periods accumulated in the frozen soils; this resulted from inhibition of N_2O diffusion

18 by frozen soil lids and from N_2O reduction to N_2. N_2O emissions during the thawing

19 period were caused by diffusion of the accumulated N_2O in the surface soil. During the

20 thawing period, the contribution of N_2O produced by nitrification and denitrification to

21 the emissions was low at the study sites.

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26

1   Figure legends

2   Figure 1. Temporal variability of precipitation observed at the Sasayama Weather

3   Station in the Shizunai Experimental Livestock Farm and air temperature and snow

4   depth observed at the Shizunai Experimental Station from December 2004 to April

5   2005.

6

7   Figure 2. Temporal variability of a) soil temperature at a 50 mm depth, b) N_2O flux, c)

8   NO flux, d) N_2O-N/NO-N ratio, and e) N_2O concentration in soil with soil freezing

9   depths at C_2, CG, G_{2s}, G_{2c}, G_{2n}, and G_3 from December 2004 to April 2005. The lines in

10   e) indicate the edge of the frozen soil layers and the soil between the lines froze.

11

12   Figure 3. NH_4^+-N, NO_3^--N, and dissolved organic carbon (DOC) concentrations in soil

13   at a 0−50 mm depth at the research sites in the Shizunai Experimental Livestock Farm.

14   Measurements were carried out on 8th (C_2, CG and G_3) or 18th (G_{2s}, G_{2c}, and G_{2n})

15   December 2004 before soil freezing, on 31st March 2005 just after the start of soil

16   thawing, and on 19th April 2005 at the end of the thawing period. Paired t-test was used

17   to test for statistically significant differences between the data observed on 8th Dec

18   2004 and 19th April 2005 for each site. Levels of significance was as follows: * <0.05,

19   ** <0.01, *** <0.001.

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Table 1 N$_2$O emissions during the winter and cropping periods and N$_2$O losses during the winter period for the investigated experiments

Reference	Country	N$_2$O emissions during winter period (kg N ha^{-1})	Annual N$_2$O emissions (kg N ha^{-1} y^{-1})	Emission during winter (%)
Grassland				
Wagner-Riddle et al. 1997	Canada	-0.1 – 0.09	-0.07 – 0.12	-75 – 143
Kaiser et al. 1998	Germany	0.57 – 0.92	1.29 – 2.37	39 – 53
Goossens et al. 2001	Belgium	0.59 – 2.36	2.55 – 31.73	7 – 23
Regina et al. 2004	Finland	0.06 – 6.7	2.6 – 9.9	1 – 68
Syvasalo et al. 2004	Finland	0.54 – 2.24	1.5 – 3.9	33 – 59
Katayanagi et al. 2008[†]	Japan	0.35 – 2.39	7.30 – 82.1	2 – 33
Cropland				
Goossens et al. 2001	Belgium	0.17 – 0.59	0.78 – 1.54	11 – 76
Wagner-Riddle et al. 2007	Canada	0.42 – 2.91	0.89 – 3.32	24 – 88
Regina et al. 2004	Finland	3.30 – 18.9	6.20 – 24.1	53 – 79
Syvasalo et al. 2004	Finland	1.55 – 6.98	3.70 – 7.5	42 – 93
Flessa et al. 1995	Germany	1.09 – 4.63	9.36 – 16.78	11 – 46
Röver et al. 1998	Germany	1.43 – 2.34	1.84 – 3.5	67 – 78
Kaiser et al. 1998	Germany	1.66 – 2.01	3.33 – 6.16	30 – 61
Teepe et al. 2000	Germany	2.80	4.80	58
Ruser et al. 2001	Germany	0.73 – 3.32	1.34 – 6.93	40 – 58
Sehy et al. 2003	Germany	0.30 – 1.7	3.10 – 10.1	10 – 20
Koga et al. 2004	Japan	0.00 – 2.1	0.09 – 2.36	0 – 89
Bremner et al. 1980	US	0.03 – 0.44	0.34 – 1.97	8 – 26
Katayanagi et al. 2008[†]	Japan	0.01 – 0.77	1.12 – 11.7	1 –24

[†] The winter period emission and the emission rate during the winter are the unpublished data.

Table 2 Soil properties at a 0-100 mm depth at the study site in Shizunai Experimental Livestock Farm.

Site	Land-use type[†]	Total C[‡] (g kg^{-1})	Total N[‡] (g kg^{-1})	Bulk density[‡] (Mg m^{-3})	D/D$_0$[§]
C$_2$	C	60.8	4.5	0.60	0.0635
CG	G	41.8	3.5	0.78	0.00684
G$_{2s}$	G	32.2	3.0	0.81	0.00113
G$_{2c}$	G	42.4	3.4	0.68	0.00429
G$_{2n}$	G	49.7	4.3	0.63	0.00132
G$_3$	G	75.2	5.4	0.65	0.00143

[†]Cornfield: C; Grasland: G

[‡]The values were reported by Katayanagi et al. (2008)

[§]Relative gas diffusion coefficients (D/D$_0$) of soil core samples were measured using the method proposed by Osozawa (1998). The data was measured using soils collected on 19 April 2005.

Table 3 Slurry and manure application after the cropping season and nitrogen surplus in 2004 at the study site in Shizunai Experimental Livestock Farm. Slurry was applied on 19 October 2004 and manure was applied on 1st November 2004.

Site	Slurry (kg N ha^{-1})	Manure (kg N ha^{-1})	Slurry + Manure (kg N ha^{-1})	Nitrogen Surplus[†] (kg N ha^{-1})
C_2	9	123	132	16
CG	0	38	38	99
G_{2s}	0	0	0	24
G_{2c}	0	0	0	24
G_{2n}	0	0	0	24
G_3	0	43	43	-12

[†]Katayanagi et al. (2008)

Table 4 N_2O and N_2O+N_2 fluxes and $N_2O/(N_2O+N_2)$ ratio measured by denitrification enzyme activity in soils collected from the study site at Shizunai Experimental Livestock Farm just after the soil thawing on 31 March 2005.

Site	N_2O flux[†] (μg N kg^{-1} h^{-1})		N_2O+N_2 flux[†] (μg N kg^{-1} h^{-1})		$N_2O/(N_2O+N_2)$ [†]	P values[‡] $N_2O \times N_2O+N$[‡]
	n	Mean ± S.D.	n	Mean ± S.D.		
C_2	3	0.478 ± 0.122[a]	3	0.519 ± 0.107[a]	0.98 ± 0.41[a]	0.688[ns]
CG	3	0.466 ± 0.106[a]	3	0.229 ± 0.0485[a]	2.0 ± 0.48 [a]	0.0244*
G_{2s}	3	1.60 ± 0.899[a,b]	3	14.0 ± 11.4[a,b]	0.39 ± 0.58 [a]	0.132[ns]
G_{2c}	2	3.84 ± 1.53[a,b]	2	73.6 ± 11.9[c]	0.051 ± 0.013[a]	0.0144*
G_{2n}	3	5.45 ± 4.31[a,b]	3	19.3 ± 32.3[a,b]	7.6 ±8.0 [a]	0.503[ns]
G_3	3	6.63 ± 0.720[b]	3	51.3 ± 15.0[b,c]	0.14 ± 0.046 [a]	0.00681**

[†] The values are means with standard deviation (S.D.). Different letters indicate significant differences among the means for each site by Tukey-Kramer test at $p < 0.05$. There was no significant differences among $N_2O/(N_2O+N_2)$ at all sites.

[‡]The difference between the means of N_2O and N_2O+N_2 was tested by t-test for each site. Levels of significance was as follows: * <0.05, ** <0.01, *** <0.001, ns, not significant.
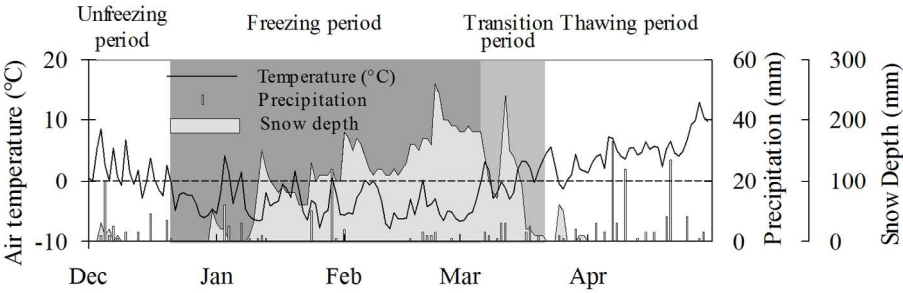
Figure 1. Temporal variability of precipitation observed at the Sasayama Weather Station in the Shizunai Experimental Livestock Farm and air temperature and snow depth observed at the Shizunai Experimental Station from December 2004 to April 2005.
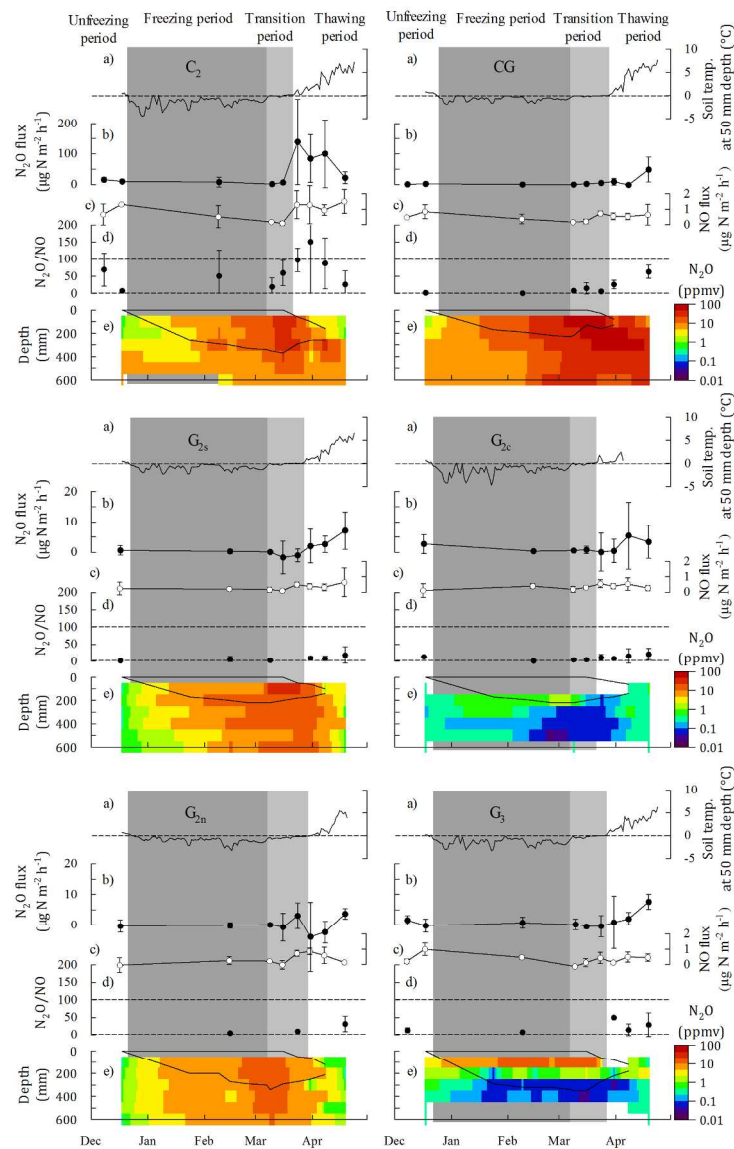144x52mm (300 x 300 DPI)

Figure 2. Temporal variability of a) soil temperature at a 50 mm depth, b) N2O flux, c) NO flux, d) N2O-N/NO-N ratio, and e) N2O concentration in soil with soil freezing depths at C2, CG, G2s, G2c, G2n, and G3 from December 2004 to April 2005. The lines in e) indicate the edge of the frozen soil layers and the soil between the lines froze.
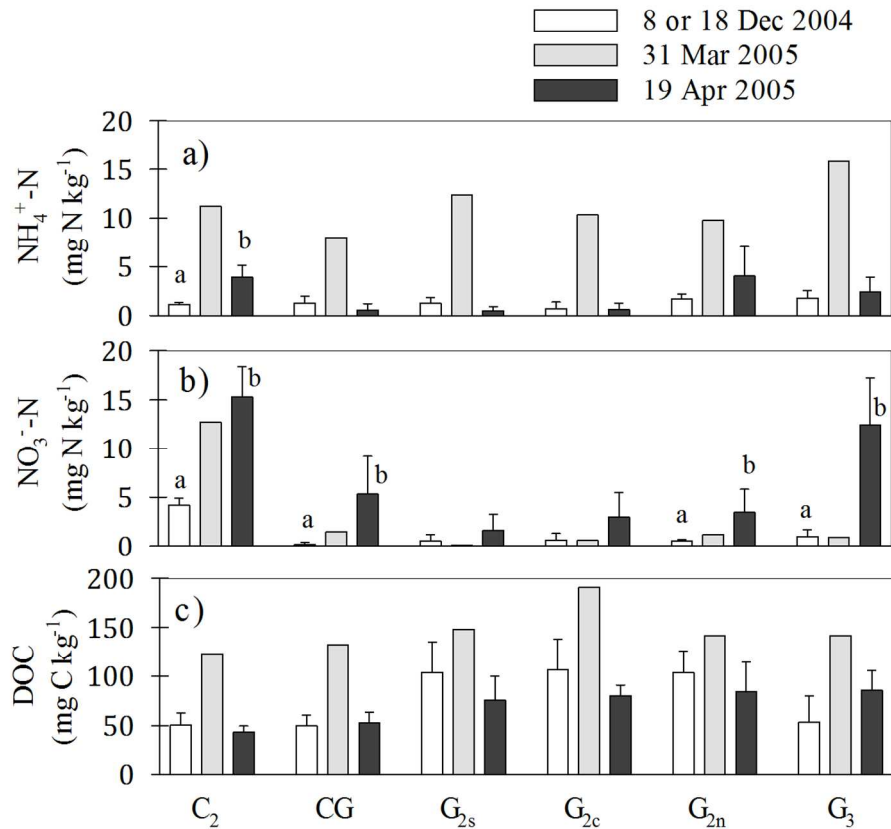170x260mm (300 x 300 DPI)

Figure 3. NH4+-N, NO3--N, and dissolved organic carbon (DOC) concentrations in soil at a 0−50 mm depth at the research sites in the Shizunai Experimental Livestock Farm. Measurements were carried out on 8th (C2, CG and G3) or 18th (G2s, G2c, and G2n) December 2004 before soil freezing, on 31st March 2005 just after the start of soil thawing, and on 19th April 2005 at the end of the thawing period. Paired t-test was used to test for statistically significant differences between the data observed on 8th Dec 2004 and 19th April 2005 for each site. Levels of significance was as follows: * <0.05, ** <0.01, *** <0.001. 121x113mm (300 x 300 DPI)