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4 **Title**

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7 Nitrous oxide emissions and nitrogen cycling in a managed grassland in southern

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17 N_2O emissions and N cycling in grassland

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10 Full-length paper

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For review

1 **Abstract**

2 Nitrous oxide (N₂O) emissions were measured and nitrogen (N) budgets were
 3 estimated for two years in the fertilizer, manure, control and bare plots established in a
 4 reed canary grass (*Phalaris arundinacea L.*) grassland in Southern Hokkaido, Japan. In
 5 the manure plot, beef cattle manure with bark was applied at a rate of 43 - 44 Mg fresh
 6 matter (236 - 310 kg N) ha⁻¹ year⁻¹, and a supplement of chemical fertilizer was also
 7 added to equalize the application rate of mineral N to that in the fertilizer plots (164 -
 8 184 kg N ha⁻¹ year⁻¹). Grass was harvested twice per year. The total mineral N supply
 9 was estimated as the sum of the N deposition, chemical fertilizer application and gross
 10 mineralization of manure (GMm), soil (GMs), and root-litter (GMI). GMm, GMs and
 11 GMI were estimated by dividing the carbon dioxide production derived from the
 12 decomposition of soil organic matter, root-litter and manure by each C:N ratio (11.1 for
 13 soil, 15.5 for root-litter and 23.5 for manure). The N uptake in aboveground biomass for
 14 each growing season was equivalent to or greater than the external mineral N supply,
 15 which is composed of N deposition, chemical fertilizer application and GMm. However,
 16 there was a positive correlation between the N uptake in aboveground biomass and the
 17 total mineral N supply. It was assumed that 58% of the total mineral N supply was taken
 18 up by the grass. The N supply rates from soil and root-litter were estimated to be 331 -

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1 384 kg N ha⁻¹ year⁻¹ and 94 - 165 kg N ha⁻¹ year⁻¹, respectively. These results indicated
2 that the GMs and GMI also were significant inputs in the grassland N budget. The
3 cumulative N₂O flux for each season showed a significant positive correlation with
4 mineral N surplus, which was calculated as the difference between the total mineral N
5 supply and N uptake in aboveground biomass. The emission factor of N₂O to mineral N
6 surplus was estimated to be 1.2%. Furthermore, multiple regression analysis suggested
7 that the N₂O emission factor increased with an increase in precipitation. Consequently,
8 soil and root-litter as well as chemical fertilizer and manure were found to be major
9 sources of mineral N supply in the grassland, and an optimum balance between mineral
10 N supply and N uptake, is required for reducing N₂O emission.

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mineral N

11 **Keywords:** Grassland; Gross mineralization; Manure; Nitrogen surplus; Nitrous oxide

13 1. Introduction

14 Grassland is one of the world's most abundant land cover types accounting for
15 approximately 40.5% of the earth's terrestrial land area, excluding the areas of
16 permanent ice cover (Adams et al. 1990; White et al. 2000). Grassland is an important
17 ecosystem to support the production of herbivorous livestock (Soussana et al. 2007).
18 Application of chemical fertilizer and animal manure to grasslands has increased grass

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4 production, especially in developed countries where grassland-based livestock
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7 production is important (Bouwman et al. 2002). However, Nitrogen (N) application to
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10 grasslands also poses a risk of N loss to the environment in the form of nitrous oxide
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12 (N₂O) emission and nitrate leaching (Bouwman 1996; Jordan et al. 1997).

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14 N₂O is a potent greenhouse gas, which has a global warming potential of 298 times
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16 that of carbon dioxide (CO₂) over a 100-year time horizon (IPCC 2007). Furthermore,
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19 N₂O destroys the stratospheric ozone layer (Crutzen 1974). N₂O concentration in the
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22 atmosphere has increased from about 270 ppbv in the preindustrial era to 319 ppbv in
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25 2005 (IPCC 2007). Global N₂O source have been estimated to be 17.7 Tg N year⁻¹
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28 (IPCC, 2007). N₂O emission from fertilized agricultural fields amounted to 2.8 Tg N
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31 year⁻¹, which is the biggest anthropogenic N₂O source (IPCC2007). Isotopic
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33 measurements made on polar firm air also suggested that the anthropogenic N₂O sources
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36 have been dominated by agricultural emissions (Rahn & Wahlen 2000; Rockmann et al.
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39 2003).

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41 N₂O emissions from soils result principally from microbial nitrification and
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44 denitrification (Bouwman 1990). These processes require mineral N (NH₄⁺ and NO₃⁻) as
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47 a substrate, and are controlled by soil moisture content, temperature, pH and organic
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50 carbon (Bouwman 1990; Maag & Vinther 1996; Mosier 1998; Yamulki et al. 1997). In
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1 managed grassland, the sources of mineral N are considered to be chemical fertilizer,
2 manure, soil organic matter, plant litter, N fixation and atmospheric deposition (Dam
3 Kofoed 1981; Dubeux et al. 2007). Therefore, estimating the supply rate of mineral N
4 from each source and clarifying the factors controlling N₂O emissions can be helpful in
5 establishing environmental-friendly N management.

6 In the grasslands of Southern Hokkaido, Japan, which were used for this study,
7 carbon budgets have been measured using an ecological technique (Shimizu et al. 2009).

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8 We found that the CO₂ fluxes from soil surface including roots in the fertilizer, manure
9 and control plots were higher in spring (beginning of March - end of June) than in other
10 seasons (beginning of July - end of February) at the same soil temperature, while the
11 relationship between CO₂ flux and soil temperature in the root-excluded bare plot was
12 well explained by a single exponential regression throughout the year. This suggests

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13 that there might be an increase in root respiration or heterotrophic respiration of litter
14 produced by fine roots. If the increment in spring was caused by root respiration, the
15 root respiration would not have been counted in the carbon budget. If it was caused by
16 heterotrophic respiration of fine root litter, the heterotrophic respiration was offset by
17 the fine root production because of an unclear yearly change, and had no effect on the
18 estimation of carbon budget. Therefore, we concluded that either root respiration or

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1 heterotrophic respiration had no effect on the carbon budget in our estimations,

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2 However, the distinction between the heterotrophic respiration and root respiration has a

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3 great influence on N budgets. Heterotrophic respiration accompanies N mineralization

4 (Schimel 1986); while an increase in root respiration does not enhance the N

5 mineralization. Also, the processes of nitrification and denitrification after N

Deleted: Therefore, it has been observed that the difference between the heterotrophic respiration and root respiration has a great influence on N budgets.

6 mineralization would have a major influence on N₂O emission (Bouwman, 1990), and

7 the determination of the source of CO₂ emission is an important factor when clarifying

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8 the source of N₂O emission. Actually, a positive relationship between CO₂ and N₂O

9 emissions was reported by several field studies (Toma & Hatano 2007; Chu et al. 2007)

10 and a laboratory incubation (Hashidoko et al. 2007).

11 The objectives of this study were to identify the sources of mineral N, their supply

12 rate in managed grasslands and to clarify the factors controlling N₂O emission from the

13 grassland.

15 2. Methods

16 2.1. Study site

17 This study was conducted for two years from mid-April 2005 to early May 2007 in

18 managed grasslands located on the Shizunai Experimental Livestock Farm, Field

1 Science Center for the Northern Biosphere of Hokkaido University in Southern
 2 Hokkaido, Japan (42°26'N, 142°29'E). The site is characterized by a humid continental
 3 climate with cold winters and cool summers but without apparent wet or dry seasons.
 4 The mean annual precipitation is 1365 mm and the mean annual temperature is 7.9 °C,
 5 with the mean monthly temperature ranging from 20.7 °C in August to -3.9 °C in
 6 January. The site is covered with snow from the end of December to the beginning of
 7 March.

8 The soil is derived from Tarumae (b) volcanic ash, and is classified as Thaptic
 9 Melanudands (Soil Survey Staff 2006; Mollic Andosol (IUSS Working Group, WRB
 10 2006)). A layer of three cm thick root-mat was found on the top, and a 21 cm thick
 11 Ap-layer was found under the root-mat in a survey conducted in August 2004. The C
 12 and N contents in the Ap-layer were 3.7% and 0.33%, respectively, and the C:N ratio
 13 was 11.1.

14 The grassland was established more than 30 years ago and has been continuously
 15 used as grassland. The average of chemical fertilizer application rates from 1984 to
 16 2004 were 133 ± 36 kg N ha⁻¹ year⁻¹. The dominant species of the grassland were reed
 17 canary grass (*Phalaris arundinacea* L.) and meadow foxtail (*Alopecurus pratensis* L.),
 18 and the percentage of legume was less than 2.9% (Table 1). The harvesting was carried

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4 1 out twice a year (21st June and 11th August in 2005 and 27th June and 23rd August in
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7 2 2006) in accordance with the local practice.

8 9 2.2. Study period

10 We defined the crop growing season as the period of time with 7-day moving
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12 average of daily air temperature above 5 °C and the non-growing (NG) season as the
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20 6 rest. The growing season was 215 days in 2005 (From 10th April 2005 to 10th November
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22 7 2005) and 218 days in 2006 (From 15th April 2006 to 18th November 2006), and the NG
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24 8 season was 155 days in 2005 and 151 days in 2006. The growing season was divided
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26 9 into three parts; from the beginning of the growing season to the first crop harvest (G1),
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28 10 from the first harvest to the second harvest (G2), and from the second harvest to the end
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30 11 of the growing season (G3). The divided growing season was 72 days in 2005 and 73
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32 12 days for G1, 51 days in 2005 and 57 days for G2, and 92 days in 2005 and 88 days in
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34 13 2006 for G3. The non growing season was 155days in 2005 and 151 days in 2006.

35 36 37 38 2.3. Treatment

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41 15 Four experimental plots were set up on the study site, one for treatment with
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43 16 chemical fertilizer (fertilizer plot), another with both beef cattle manure and chemical
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45 17 fertilizer (manure plot), a third, with no fertilizer or manure (control plot) and the other
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49 18 with no plants (bare plot). Neither manure nor fertilizer was applied to the bare plot.

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1 The treatments were initiated in the spring of 2005. Eighteen subplots (5 m × 4 m) were
2 established for the fertilizer, manure and control plots with six replications, and six
3 subplots (1 m × 1 m) for the bare plot (Fig. 1). In the bare plot, the plants aboveground
4 and roots were removed by hand, with a shovel and a root-proofing permeable sheet
5 (BKS9812, TOYOBO, Osaka, Japan) was vertically inserted to a depth of 20 cm below
6 the ground surface to inhibit regrowth of roots. The plants grown on the bare plot were
7 removed every couple of weeks.

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fertilizer was applied to the bare plot.

8 Table 2 shows the information on the date of application and the application rates of
9 fertilizer and manure. The fertilizer application rates in the fertilizer plot were at the
10 recommended level for this site on the basis of soil diagnosis, and were 164 kg N ha⁻¹
11 year⁻¹ in 2005 and 184 kg N ha⁻¹ year⁻¹ in 2006 as ammonium sulfate and ammonium
12 phosphate (Table 2). The manure application rates were based on adequate amounts of
13 potassium application to the fields. Beef cattle manure with bedding litter (bark) was
14 applied to the manure plot and the application rates were 44 Mg FM ha⁻¹ (236 kg T-N
15 ha⁻¹ and 5.8 Mg T-C ha⁻¹) in May 2005 and 43 Mg FM ha⁻¹ (310 kg T-N ha⁻¹ and 6.0 Mg
16 T-C ha⁻¹) in May 2006 (Table 2). In the manure plot, the nutrient supply rates from
17 manure were estimated by multiplying the application rate by the mineralization rate
18 (Table 3), and the differences between the supply rates in manure and the application

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4 1 rates in the fertilizer plot were supplied by chemical fertilizer. The N mineralization
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6 2 rates were estimated based on Uchida's model (Shiga et al. 1985) which was developed
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9 3 in Japan and were 13.2% and 7.0%, respectively in the first and second years after
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11 4 application (Table 3). The mineralization rates of phosphorus and potassium from the
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13 5 manure were estimated based on the handbook of animal waste management and
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15 6 utilization in Hokkaido 2004 (Hokkaido Prefectural Experiment Stations and Hokkaido
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19 7 Animal Research Center 2004). The phosphorus mineralization rate was 20% in the first
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22 8 year and 10% in the second year (Table 3). The potassium mineralization rate was 70%
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25 9 in the first year and 10% in the second year (Table 3).

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10 2.4. Nitrogen deposition

11 A rainfall collector (0.9 m length, 0.1 m width) with a tank (20 L) was set up, and
12 the water sample was collected once or twice each month from April to November
13 (Morishita et al. 2004). A bucket (0.3 m diameter, 0.4 m depth) was set up to collect
14 snowfall, and the water sample was collected once a month. Thymol (C₁₀H₁₄O) was
15 used as a biocide in the tank or bucket to prevent biological alteration and utilization of
16 the N species (Gillett & Ayers 1991). All the samples were filtered through a membrane
17 filter (0.2 µm) and kept at 4°C before analysis. NH₄⁺-N content was determined by the
18 indophenol-blue method (UV mini 1240, Shimadzu, Kyoto, Japan), and NO₃⁻-N content

1 was analyzed by ion chromatography (Dionex QIC Analyzer, Dionex Japan, Osaka,
2 Japan). The amount of N deposition was calculated by multiplying the amount of water
3 by NH_4^+ -N and NO_3^- -N concentrations.

4 2.5. Gross N mineralization

5 The soil organic matter, root-litter and manure are supposed to release mineral N
6 during decomposition in the managed grassland, and gross N mineralization (GM) was
7 estimated using the following equation.

$$8 \quad \text{GM} = \text{GM}_s + \text{GM}_l + \text{GM}_m \quad (1)$$

9 where suffixes “s”, “l” and “m” indicate soil organic matter, root-litter and manure,
10 respectively. The GM was estimated by dividing heterotrophic respiration (RH) of soil
11 organic matter, root-litter and manure by each C:N ratio (Luxhoi et al. 2006).

$$12 \quad \text{GM} = \text{RH} / \text{C:N ratio} \quad (2)$$

13 The RH from soil organic matter, root-litter and manure was previously estimated using
14 the static closed chamber method on this study site (Shimizu et al. 2009). These
15 measurements were carried out at the same time with the measurement of N_2O and NO
16 fluxes as described below. RH from the soil organic matter (RHs) was estimated using
17 the exponential regression model for the bare plot driven by the soil temperature at each
18 plot. The RH from manure (RHm) was estimated as the difference in soil respiration,

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1 which is a CO₂ flux from the soil surface composed of RH and root respiration, between
2 manure and fertilizer plots. The RH from root-litter (RHL) was assumed as the following.

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3 Soil respiration in the fertilizer, manure and control plots was higher in spring
4 (beginning of March - end of June) than in the other season, (beginning of July - end of

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5 February) at the same soil temperature (Shimizu et al. 2009). We hypothesized that the
6 increment of a CO₂ flux in the spring was RHL. To estimate the increment of a CO₂ flux
7 in spring, we calculated the cumulative CO₂ flux in spring using the exponential
8 regression equation of the two seasons driven by the soil temperature in spring, and
9 estimated the difference between the cumulative CO₂ fluxes (Shimizu et al. 2009).

10 We used a C:N ratio of soil in the Ap-layer (11.1) to estimate GMs. Roots of less
11 than 2 mm in diameter were obtained four times in 2006 (mid April, late June, mid
12 August and late October), and the C:N ratio was analyzed using an N/C analyzer
13 (SUMIGRAPH NC-1000, Sumika Chemical Analysis Service, Ltd., Osaka, Japan). The
14 average value was 15.5, and we used it for the C:N ratio of root-litter to estimate the
15 GMI. The C:N ratio of manure was 25 for 2005, and was assumed to be 22 for 2006,
16 which was the average of two years, because manure applied in 2005 have remained in
17 2006.

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18 *2.6. N uptake in aboveground biomass*

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4 1 The aboveground biomass, which includes live and dead parts, was measured four
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6 2 times a year; in mid April, late June (before the first crop harvest), mid August (before
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8 3 the second crop harvest) and in late October. The aboveground biomass at the time of
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10 4 the harvest was estimated to be the sum of the harvests and the residue. The harvest was
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12 5 measured by clipping at 5 cm above the ground using the 100 cm × 100 cm quadrates,
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14 6 and the harvest residue and the aboveground biomass in April and October were
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16 7 measured by clipping using the 50 cm × 50 cm quadrates, with six replications
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18 8 conducted in 2005 and eight replications conducted in 2006. All the samples were
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20 9 oven-dried at 70 °C for 72 hours and weighed. Each dried sample was analyzed for the
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22 10 total N content with an N/C analyzer (SUMIGRAPH NC-1000, Sumika Chemical
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24 11 Analysis Service, Ltd., Osaka, Japan).

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33 12 The N uptake in aboveground biomass was estimated as an increment in biomass N
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35 13 during each season. Apparent N recovery (ANR) (kg N (kg N)^{-1}) was estimated using
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37 14 the following equation (Kaffka & Kanneganti 1996):

$$15 \quad \text{ANR} = (\text{CN}_i - \text{CN}_c) / N_i \quad (3)$$

16 16 where CN_i and CN_c are the annual N uptake in aboveground biomass in treated and
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18 17 control plots, and N_i is the total amount of fertilizer and manure N applied to the
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20 18 treatment i .

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4 1 2.7. *N₂O and NO fluxes*
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6 2 N₂O and NO fluxes from the soil to the atmosphere were measured by the static
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9 3 closed chamber method on the fertilizer, manure, control and bare plots (Shimizu et al.
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12 4 2009). The flux measurements were conducted in 2 - 28 day intervals during the crop
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14 5 growing season and 10 - 30 day intervals during the non growing season and between
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17 6 8:00 and 11:00 h each measuring day to minimize the effect of diurnal temperature
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20 7 variation. The stainless steel chambers were 40 cm in diameter and 30 cm high in the
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22 8 fertilizer and manure plots, and 20 cm in diameter and 25 cm high in the control and
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25 9 bare plots. The chambers were placed directly into the soil to a depth of about 3 cm, 12
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28 10 hours before the measurement of each subplot, and contained no aboveground biomass
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31 11 in the fertilizer, manure and control plots. Before closing the chamber, a 250 ml gas
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34 12 sample from the headspace of each chamber was extracted into a Tedlar bag for NO
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37 13 analysis, and a 20 ml gas sample was injected into an evacuated vial (10 ml) for N₂O
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40 14 analysis. This measurement was regarded as time 0 min. After 20 min or 30 min under a
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43 15 closed-chamber condition, 250 ml of the headspace gas sample was extracted from each
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46 16 chamber into a bag, and 20 ml was injected into a vial. From these bag samples, NO gas
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49 17 concentrations were determined in a laboratory within 16 hours using a
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52 18 chemiluminescence nitrogen oxides analyzer (Model 265P, Kimoto Electric, Osaka,
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1 Japan). N₂O gas concentrations were determined in a laboratory within 1 month using a
2 gas chromatograph (Model GC-14B, Shimadzu, Kyoto, Japan) fitted with an electron
3 capture detector.

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4 2.8. Environmental variables

5 Daily precipitation and daily maximum depths of snow were obtained at the closest
6 AMeDAS (Automated Meteorological Data Acquisition System) station by the Japan
7 Meteorological Agency. Air temperature and soil temperature at a 5 cm depth were
8 measured at the same time with the flux measurements using a thermistor thermometer
9 (CT220, CUSTOM, Tokyo, Japan), and soil moisture content at a 0 to 6 cm depth was
10 measured using the Frequency Domain Reflectometry (FDR) method (DIK-311A, Daiki,
11 Saitama, Japan). Soil core samples (14 cm diameter, 13 cm height) were collected in
12 April 2007, and calibration curves were made to calculate water-filled pore space
13 (WFPS) from the FDR device reading ($\text{m}^3 \text{m}^{-3}$) and percent total porosity (Linn &
14 Doran, 1984). The percent total porosity was measured using a 100 ml soil core
15 collected in April 2007 and was regarded as constant throughout the study period
16 because of no tillage.

17 2.9. Statistical analyses

18 Statistical analyses were performed with Prism ver. 4 (GraphPad Software Inc., San

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4 1 Diego, CA, USA) and Excel Statistics version 5.0 (Esumi, Tokyo, Japan). The
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7 2 differences in soil temperature, WFPS and N₂O flux among the plots were analyzed
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10 3 with a repeated-measures one-way analysis of variance (ANOVA) and Bonferroni test.
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12 4 The differences in GMs, GMI, GMm, total mineral N supply, N uptake in aboveground
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14 5 biomass and cumulative N₂O flux among the seasons were analyzed with a
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17 6 repeated-measures analysis of variance (ANOVA) and Bonferroni test. The differences
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20 7 in the annual values between the years and the plots were analyzed with a two-way
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22 8 factorial ANOVA and Bonferroni test.

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25 9 Uncertainties in the N uptake in aboveground biomass and the cumulative N₂O flux
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28 10 were calculated using the following equation:

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$$\text{Uncertainty (\%)} = \{(\text{two-sided 95\% confidence interval}) / 2\} / \text{Means} \times 100 \quad (4)$$

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33 12 The two-sided 95% confidence interval was calculated by using the following equation:

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$$\text{Two-sided 95\% confidence interval} = t(\text{d.f., 0.05}) \times \text{standard error} \times 2 \quad (5)$$

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38 14 where d.f. is the degree of freedom and t (d.f., 0.05) is the t value at 5% significant level
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41 15 with a two-sided alternative.

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46 17 **3. Results**

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48 18 *3.1. Environmental variables*
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1 Daily precipitation and daily maximum depths of snow are shown in Fig. 2a. Annual
2 precipitations were 1152 mm and 1047 mm from mid April 2005 to the beginning of
3 April 2006 and from mid April 2006 to the beginning of April 2007, respectively. These
4 values are smaller than the 12-year average (1365 ± 215 mm), but drought did not
5 occur.

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6 The soil temperature at 5 cm depth increased from April, reaching the maximum
7 temperature from July through August, and then decreased gradually (Fig. 2b). The soil
8 temperature was around 0 °C from December to March (Fig. 2b). There was no
9 difference in soil temperature between the fertilizer and manure plots, but soil
10 temperature was higher in the control plot than in the fertilizer and manure plots ($P <$
11 0.05). Soil temperature in the bare plot was the highest of all plots ($P < 0.01$).

12 The range of WFPS in a 0 to 6 cm depth in 2005 was 67% - 89% in the fertilizer
13 plot, 67% - 90% in the manure plot, 72% - 89% in the control plot and 83% - 94% in
14 the bare plot (Fig. 2c). The WFPS in the fertilizer, manure, control and bare plots
15 decreased to 47%, 48%, 46% and 65%, respectively in mid August 2007, and then
16 increased with an increase in precipitation. There was no difference in the WFPS
17 between the fertilizer and manure plots; however, it was greater in the control plot than
18 in the fertilizer and manure plots ($P < 0.01$). Furthermore, the WFPS in the bare plot

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4 was found to be the greatest of all the plots ($P < 0.01$).
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7 3.2. N deposition

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9 The annual N deposition was $15 \text{ kg N ha}^{-1} \text{ year}^{-1}$ in 2005 and $4 \text{ kg N ha}^{-1} \text{ year}^{-1}$ in
10 2006 (Table 4).
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12 3.3. Gross N mineralization

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14 There was a significant difference in the GMs among the seasons ($P < 0.01$). The
15 GMs in the G2 and G3 seasons ranged from 115 to $161 \text{ kg N ha}^{-1} \text{ period}^{-1}$, and was
16 greater than those in the other seasons (Table 4). There was no significant difference in
17 the GMs between the G2 and G3 seasons. The GMs in the G1 season ranged from 59 to
18 $67 \text{ kg N ha}^{-1} \text{ period}^{-1}$, and the GMs in the NG season ranged from 31 to 37 kg N ha^{-1}
19 period^{-1} (Table 4). The annual GMs in 2005 and 2006 were 367 and $331 \text{ kg N ha}^{-1} \text{ year}^{-1}$
20 for the fertilizer plot, 369 and $336 \text{ kg N ha}^{-1} \text{ year}^{-1}$ for the manure plot, 382 and 348 kg
21 $\text{N ha}^{-1} \text{ year}^{-1}$ for the control plot and 384 and $356 \text{ kg N ha}^{-1} \text{ year}^{-1}$ for the bare plot,
22 respectively (Table 4). The annual GMs value was greater in 2006 than in 2007, and was
23 greater in the bare plot than in the fertilizer plot ($P < 0.01$).
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43 The GMI in the G1 season ranged from 68 to $104 \text{ kg N ha}^{-1} \text{ period}^{-1}$, and was greater
44 than that in other seasons ($P < 0.01$) (Table 4). In the G1 season, the GMI for each plot
45 was greater than the GMs (Table 4). The annual GMI values in 2005 and 2006 were 145
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4 and 126 kg N ha⁻¹ year⁻¹ for the fertilizer plot, 165 and 122 kg N ha⁻¹ year⁻¹ for the
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1 and 126 kg N ha⁻¹ year⁻¹ for the fertilizer plot, 165 and 122 kg N ha⁻¹ year⁻¹ for the
2 manure plot, and 108 and 94 kg N ha⁻¹ year⁻¹ for the control plot, respectively (Table 4).

3 The GMm value was greater in the G2 and G3 seasons than in the NG season (Table
4 4). The annual GMm values in 2005 and 2006 were 88 and 96 kg N ha⁻¹ year⁻¹,
5 respectively (Table 4).

6 3.4. N uptake in aboveground biomass

7 Table 5 shows the N uptake in aboveground biomass. There was a significant
8 difference among the seasons ($P < 0.01$), and the N uptake in aboveground biomass in
9 the G1 season was the greatest, followed by those in the G2, G3 and NG seasons. The
10 negative values for the NG season, which were a result of the decrease in aboveground
11 biomass, suggested that the mineral N could have been supplied to soil by the dead
12 aboveground biomass through the decomposition process. The annual values of N
13 uptake in aboveground biomass in 2005 and 2006 were 249 and 219 kg N ha⁻¹ year⁻¹ for
14 the fertilizer plot, 215 and 201 kg N ha⁻¹ year⁻¹ for the manure plot, and 109 and 134 kg
15 N ha⁻¹ year⁻¹ for the control plot (Table 5). The annual N uptake in aboveground
16 biomass was greater in the fertilizer and manure plots than in the control plot ($P < 0.01$),
17 and there were no significant differences among the years.

18 The ANR in 2005 and 2006 was 85% and 46% for the fertilizer plot, and 29% and

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4 15% for the manure plot, respectively.
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7 3.5. *N₂O* flux
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9 *N₂O* fluxes in the fertilizer and manure plots increased after the application of
10 fertilizer or manure. These remained at a higher level than in the control plot until the
11 beginning of September (Fig. 2). The *N₂O* flux in the fertilizer plot increased after
12 fertilization in May and July to 37 and 314 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in 2005, and to 152 and 211 μg
13 $\text{N m}^{-2} \text{h}^{-1}$ in 2006 (Fig. 2). In the manure plot, however, it reached a high peak after the
14 application of manure and fertilizer in May (276 and 1290 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in 2005 and
15 2006, respectively) (Fig. 2). The *N₂O* flux in the control plot gradually increased with
16 an increase in soil temperature, and reached the maximum values of 50 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in
17 August 2005 and 66 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in July 2006. The *N₂O* flux in the bare plot also
18 increased with an increase in soil temperature, but the maximum value (347 $\mu\text{g N m}^{-2}$
19 h^{-1} in August 2005 and 403 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in July 2006) was higher than that in the
20 control plot. The *N₂O* fluxes in the fertilizer, manure and bare plots were significantly
21 higher than that in the control plot ($P < 0.01$), but there were no significant differences
22 among the fertilizer, manure and bare plots.
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46 The relationship between the *N₂O* fluxes and *N₂O*-N/*NO*-N ratio is shown in Fig. 3.
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48 The *N₂O* flux increased with an increase in the *N₂O*-N/*NO*-N ratio. Bouwman (1990)
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1 summarized the results reported by Anderson and Levine (1986) and Lipschultz et al.
2 (1981), and concluded that the $N_2O-N/NO-N$ ratio was <1 during nitrification and >100
3 during denitrification. Therefore, N_2O could have been produced mainly by
4 denitrification in our study site.

5 The cumulative N_2O flux for all seasons is shown in Table 6. The cumulative N_2O
6 flux was higher in the G2 season than all other seasons except in the manure plot in
7 2006, but there was a significant difference only between the G2 and NG seasons. In
8 ~~each season, the cumulative N_2O flux was compared to the cumulative CO_2 flux (Fig. 4),~~
9 which was reported by Shimizu et al. (2009). The cumulative N_2O flux in each plot had
10 a positive correlation with the cumulative CO_2 flux (Fertilizer plot; Cumulative N_2O
11 flux = $0.36 \times$ cumulative CO_2 flux - 0.23; $R^2 = 0.48$; $P < 0.05$, Manure plot; Cumulative
12 N_2O flux = $0.38 \times$ cumulative CO_2 flux - 0.17; $R^2 = 0.33$; $P = 0.08$, Control plot;
13 Cumulative N_2O flux = $0.08 \times$ cumulative CO_2 flux - 0.05; $R^2 = 0.76$; $P < 0.01$, Bare
14 plot; Cumulative N_2O flux = $1.30 \times$ cumulative CO_2 flux - 0.39; $R^2 = 0.72$; $P < 0.01$)
15 (Fig. 4).

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16 The annual N_2O fluxes in 2005 and 2006 were 2.8 ± 0.7 and 3.0 ± 0.8 $kg\ N\ ha^{-1}\ year^{-1}$
17 for the fertilizer plot, 3.6 ± 1.3 and 4.9 ± 3.0 $kg\ N\ ha^{-1}\ year^{-1}$ for the manure plot, 0.7 ± 0.5
18 and 0.6 ± 0.3 $kg\ N\ ha^{-1}\ year^{-1}$ for the control plot, and 4.2 ± 3.3 and 3.3 ± 2.1 $kg\ N\ ha^{-1}$

1 year⁻¹ for the bare plot (Table 6). There was no significant difference in the annual N₂O
flux among the years, and the annual N₂O flux was significantly higher in the fertilizer,
manure and bare plots than in the control plot (Fertilizer plot: $P < 0.05$, Manure plot: P
< 0.01).

4. Discussion

4.1. Relationship between mineral N supply and N uptake in aboveground biomass

The sources of mineral N supply for plant growth are considered to be N deposition,
chemical and manure fertilizer application, biological N₂ fixation, mineralization from
soil organic matter (GMs), ~~root-litter~~ (GMI) and manure (GMm). Among these
components, N deposition, biological N₂ fixation, chemical fertilizer application and
GMm are all external mineral N supply. In this study, the biological N₂ fixation was
assumed to be negligible for the estimation of total and external mineral N supplies,
because of a low percentage of legumes (Table 1). The plots of the N uptake in
aboveground biomass against the external mineral N supply during ~~each~~ growing season
showed that the N uptake in aboveground biomass was equivalent to or greater than the
external mineral N supply except for the manure plot in the G2 season (Fig. 5a). On the
other hand, the plots of the aboveground N uptake against total mineral N supply

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1 showed that the total mineral N supply exceeded the N uptake in aboveground biomass,
2 and there was a significant linear correlation between them (N uptake in aboveground
3 biomass = $0.58 \times \text{total mineral N supply} - 47$; $R^2 = 0.70$; $P < 0.01$, Eq. (6)). (Fig.5b).
4 This relationship suggests that the rate of plant mineral N uptake to the total mineral N
5 supply would be 58%. This value is comparable to the ANR for the fertilizer plot (85%
6 in 2005 and 46% in 2006). The ANR of this study for the fertilizer plot was consistent
7 with the previously reported values for ryegrass (60%; Dam Kofoed 1981) and orchard
8 grass (69%; Kaffka & Kanneganti 1996). The y-interception of the equation (Eq. (6))
9 was negative value. This can be accounted for by the belowground N uptake and
10 microbial immobilization of N.

11 The annual GMs was 2.2 to 3.6 times larger than the annual GMI. However, the
12 GMI in the G1 season, which accounted for 61 to 83% of the annual GMI, was 1.1 to
13 1.7 times larger than the GMs in the season. This indicates that decomposition of fine
14 roots plays an important role in contributing to the mineral N supply especially for the
15 first crop. Some reports suggest significance of fine roots as a source of belowground
16 litter because of their abundance and rapid turnover (Gholz et al. 1986; Gill & Jackson
17 2000; Gill et al. 2002). Steinaker and Wilson, (2005) reported that fine roots accounted
18 for about 90% of the total litter production in natural grassland in Saskatchewan,

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4 1 Canada. Parton et al. (2007) and Seastedt et al. (1991) showed that mineral N was
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7 2 proportionally released with decomposition of root litters. Fitzhugh et al. (2001)
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10 3 suggested that an increase in the mortality of fine roots due to soil freezing in boreal
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12 4 forests was likely to be an important source of mobile N, based on the observations
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14 5 using mini-rhizotron. They reported that the mortality of fine roots was concentrated in
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17 6 the winter months as concrete frost permeated the rooting zone (Groffman et al. 2001;
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20 7 Ruess et al. 1998; Tierney et al. 2001). This was the same for our study site too, as the
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22 8 soil is frozen up to about 20 centimeters in the winter (Shimizu et al. 2009), and the
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25 9 mortality of fine roots in the winter could be a main source of belowground litter.
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28 10 In this study, GM was estimated by dividing heterotrophic respiration by a C:N ratio
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30 11 (Eq. (3)) and it was important specifically as the source of mineral N supply. GMI was a
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32 12 major source of mineral N supply especially in the spring. In the previous report
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35 13 (Shimizu et al. 2009), although the source of the increment of CO₂ flux in the spring
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38 14 was argued as not only root-litter decomposition but also root respiration, the results of
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41 15 this study revealed that root-litter decomposition could possibly be the main source of
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44 16 an increment of CO₂ fluxes in the spring.
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46 17 4.2. Factors controlling N₂O emission

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48 18 A significant positive correlation between the cumulative N₂O and CO₂ fluxes in the
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1 bare plot (Fig. 4) indicates that the soil organic matter decomposition contributed to the
2 N₂O flux. This relationship was found also in the three other plots of fertilizer, manure
3 and control. The slope of the regression equation for cumulative N₂O and CO₂ fluxes
4 was largest in the bare plot, because CO₂ fluxes in the other plots include root
5 respiration. However, the slope was higher in the fertilizer and manure plots than in the
6 control plot due to an increase in N₂O fluxes with the application of chemical fertilizer
7 and manure (Bouwman 1996).

8 Fig. 6a shows the relationship between the cumulative N₂O flux and the total
9 mineral N supply for each season, which was estimated as the sum of N deposition, the
10 chemical fertilizer application, GMm, GMs and GMI. There was a significant positive
11 correlation between the cumulative N₂O flux and the total mineral N supply
12 (Cumulative N₂O flux = 0.0059 × (total mineral N supply) – 0.103; R² = 0.37; P < 0.01,
13 Eq. (7)). However, the rate of the cumulative N₂O flux to the total mineral N supply was
14 higher in the bare plot than in the other three plots including plant growth (Fig. 6a). This
15 is because of the absence of plant N uptake in the bare plot while the N uptake in
16 aboveground biomass accounted for 58% of the total mineral N supply in the other three
17 plots (Fig. 5b, Eq. (6)). When the cumulative N₂O flux for each season was plotted
18 against the mineral N surplus, which was estimated as the difference between the total

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4 1 mineral N supply and the aboveground N uptake (Fig. 6b), a much better significant
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7 2 positive correlation was found (Cumulative N₂O flux = 0.0120 × (mineral N surplus) –
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10 3 0.537; R² = 0.50; P < 0.01, Eq. (8)). The slope of the regression equation showed that
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12 4 the N₂O emission factor against the mineral N surplus was 1.20%, and a negative value
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15 5 of the y-interception showed immobilization of mineral N (Schimel et al. 1985). Kaiser
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18 6 and Ruser (2000) reported that the annual N₂O emission measured in the arable land
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21 7 soils in Germany had an even stronger correlation with the N balance estimated as the
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24 8 difference between the N fertilization and the crop N uptake than with N fertilization.
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27 9 Katayanagi et al. (2008) also found that N₂O emissions conducted on the same field in
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30 10 this study including cornfields had a positive correlation with the N surplus which was
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33 11 estimated as the difference of all total input (application of fertilizer, manure and slurry,
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36 12 and livestock excreta during grazing) and total output (yield and grazed grass). However,
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39 13 those reports did not take into consideration the contribution of N mineralization to N₂O
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42 14 emission. Kaiser and Ruser (2000) suggested the contribution of gross N mineralization
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45 15 to N₂O emissions from the result, that unfertilized soil produced an N yield of 60 kg N
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48 16 ha⁻¹ year⁻¹ and an N₂O emission of 2 kg N ha⁻¹ year⁻¹. In this study, the sum of GMs and
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51 17 GMI in the fertilizer plot in 2005 and 2006 were 512 and 457 kg N ha⁻¹ year⁻¹,
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54 18 respectively. These values are significantly larger than the application rate of chemical
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1 fertilizer (164 and 183 kg N ha⁻¹ year⁻¹). Mu et al. (2008) reported that the sum of
2 chemical N fertilizer and GMs correlated with the cumulative N₂O fluxes better than
3 only the chemical N fertilizer. Thus, not only amounts of N input and crop N uptake, but
4 also the amount of gross N mineralization is indispensable in predicting the amount of
5 N₂O emission.

6 Despite the significant positive relationship between the cumulative N₂O flux and
7 mineral N surplus, there was variability in the relationship (Fig. 6a). Therefore, we
8 conducted multiple regression analysis to explain the variability. We selected the N₂O
9 emission factor against the mineral N surplus for each season as an objective variable,
10 and mean precipitation, mean daily air temperature and mineral surplus N as
11 explanatory variables. The results showed that the emission factor against mineral N
12 surplus was significantly correlated with the mean precipitation (Cumulative N₂O /
13 mineral N surplus × 100 = 0.195 × mean precipitation - 0.055; R² = 0.21; P < 0.01) (Fig.
14 7). This suggests that an N₂O emission from surplus mineral N increases with an
15 increase in precipitation. In this study, the predominant source of N₂O appeared to be
16 denitrification (Fig. 3), and high precipitation should have enhanced denitrification.
17 Mori et al. (2008) also reported that high precipitation enhanced N₂O production from
18 denitrification. These results indicated that an optimum N input aiming at reducing the

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4 1 N surplus is required to minimize the environmental risk associated with N₂O emission.
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9 3 **5. Conclusion**

10 4 Together with chemical fertilizer and manure, mineralization from soil organic

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15 5 matter and root-litter were the major sources for plant growth and N₂O emission in the

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17 6 managed grassland. Mineral N surplus, which was estimated as the difference between

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20 7 the total mineral N supply and N uptake in aboveground biomass, was a better indicator

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22 8 of N₂O emission than the total mineral N supply. The N₂O emission factor against the

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25 9 mineral N surplus was estimated to be 1.20%, and the emission factor in each season

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28 10 increased with an increase in precipitation. These results also indicated that the

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30 11 reduction in mineral N surplus would be able to mitigate N₂O emission from the

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33 12 grassland.
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1 | **Figure captions**

2 | **Fig. 1.** Location of the study site (a) and the layout of the experimental plots (b)

3 |

4 | **Fig. 2.** Seasonal variations in meteorological variables and the N₂O and NO flux; Daily

5 | precipitation and daily maximum snow depth (a), soil temperature at a 5 cm depth (b),

6 | water-filled pore space (WFPS) (c), N₂O flux (d) and NO flux (e). Data of soil temperature,

7 | WFPS, N₂O flux and NO flux represent means ± S.D. (n = 6). The arrows indicate the timing

8 | of fertilizer or manure application.

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10 | **Fig. 3.** The relationship between N₂O fluxes and the N₂O-N/NO-N rate.

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12 | **Fig. 4.** The cumulative N₂O flux for each seasons (G1, G2, G3 and non growing season

13 | (NG)) compared to the cumulative CO₂ flux. G1 is the period from the beginning of the

14 | growing season to the first crop harvest, G2 is the period from the first harvest to the second

15 | harvest and G3 is the period from the second harvest to the end of the growing season. Data

16 | of the cumulative N₂O flux represent means ± (uncertainties / 100 × means). The cumulative

17 | CO₂ flux was referred to Shimizu et al. (2009).

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19 | **Fig. 5.** N uptake in aboveground biomass during each growing season (G1, G2, G3)

20 | compared to the external mineral N supply (a) and total mineral N supply (b). The external N

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3 1 supply is composed of N deposition, chemical fertilizer application and the gross N
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6 2 mineralization of applied manure. The total mineral N supply is composed of the external N
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8 3 supply, gross N mineralization of soil and gross mineralization of root-litter. G1 is the period
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10 4 from the beginning of the growing season to the first crop harvest, G2 is the period from the
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12 5 first harvest to the second harvest and G3 is the period from the second harvest to the end of
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14 6 the growing season. The data of N uptake in aboveground biomass represent means \pm
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16 7 (uncertainties / $100 \times$ means). The dashed-line indicates a regression model for all values, N
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18 8 uptake in aboveground biomass = $0.58 \times$ total mineral N supply - 47; $R^2 = 0.68$; $P < 0.01$.
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26 10 **Fig. 6.** The cumulative N₂O flux for each season (G1, G2, G3 and non growing season
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28 11 (NG)) compared to the total mineral N supply (a) and the mineral N surplus (b). The total
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30 12 mineral N supply is composed of the external N supply (N deposition, chemical fertilizer
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32 13 application and gross N mineralization of applied manure), gross N mineralization of soil and
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34 14 gross mineralization of root-litter. The mineral N surplus was estimated as the difference
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36 15 between the mineral N pool and N uptake in aboveground biomass. G1 is the period from the
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38 16 beginning of the growing season to the first crop harvest, G2 is the period from the first
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40 17 harvest to the second harvest and G3 is the period from the second harvest to the end of the
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42 18 growing season. The data of cumulative N₂O flux represent means \pm (uncertainties / $100 \times$
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44 19 means). The regression model shown in Fig. 6a is for all values, Cumulative N₂O flux =
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1 0.0059 × (total mineral N supply) – 0.103; $R^2 = 0.37$; $P < 0.01$. The regression model shown
2 in Fig. 6b is for all values, Cumulative N₂O flux = 0.0120 × (mineral N surplus) – 0.537; $R^2 =$
3 0.50; $P < 0.01$.

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5 Fig. 7. The N₂O emission factor to mineral N surplus for each season (G1, G2, G3 and non
6 growing season (NG)) compared to mean precipitation. The mineral N surplus was estimated
7 as the difference between the mineral N pool and N uptake in aboveground biomass. G1 is
8 the period from the beginning of the growing season to the first crop harvest, G2 is the period
9 from the first harvest to the second harvest and G3 is the period from the second harvest to
10 the end of the growing season. The data of N₂O emission factor to mineral N surplus
11 represent means ± (uncertainties / 100 × means). The regression line is for all values,
12 Cumulative N₂O / mineral N surplus × 100 = 0.195 × mean precipitation - 0.055; $R^2 = 0.21$; P
13 ≤ 0.01.

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3 **Tables**

4 **Table 1**

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7 The botanical composition of grassland (dry matter weight percentage) on the study site.

8

	Gramineae			Leguminosae	Broad-leaved weed
	Reed canarygrass	Meadow foxtail	Others	White clover	
Fertilizer plot	77.9	14.4	7.7	0.0	0.0
Manure plot	49.8	48.7	1.2	0.0	0.4
Control plot	54.8	41.9	0.3	2.9	0.1

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Table 2

The applied date and the application rates of chemical fertilizer and manure.

Treatment	Date	Fertilizer type	Application rates (kg ha ⁻¹)			
			T-C	T-N	P ₂ O ₅	K ₂ O
Fertilizer	May 2005	Chemical fertilizer ^a	0	103	23	168
	July 2005	Chemical fertilizer ^a	0	61	23	97
	May 2006	Chemical fertilizer ^a	0	124	50	177
	July 2006	Chemical fertilizer ^a	0	59	18	97
Manure	May 2005	Manure ^b	5833	236	191	266
	July 2005	Chemical fertilizer ^a	0	133	7	70
	May 2006	Manure ^b	5958	310	212	167
	May 2006	Chemical fertilizer ^a	0	71	0	33
	July 2006	Chemical fertilizer ^a	0	59	6	97

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

^b Beef cattle manure with bedding litter (bark) is applied in manure plot.

Table 3

Mineralization rates from the cattle manure.

Year	Mineralization rate ^a from						Estimated supply rate		
	Manure applied in			Manure applied in			(kg ha ⁻¹)		
	2005			2006			T-N	P ₂ O ₅	K ₂ O
2005	13%	20%	70%	-	-	-	31	38	186
2006	7%	10%	10%	13%	20%	70%	57	62	144

^aThe N mineralization rates were estimated based on the Uchida's model (Shiga et al. 1985)

which was developed in Japan and were 13.2% and 7.0% in the first and second years after

application, respectively. The P and K mineralization rates from the manure were estimated

based on the handbook of animal waste management and utilization in Hokkaido 2004

(Hokkaido Prefectural Experiment Stations and Hokkaido Animal Research Center 2004).

Table 4

N deposition, gross N mineralization of soil (GMs), root-litter (GMI) and manure (GMm), and mineral N pool during each growing season (G1, G2 and G3) and non-growing season (NG) (kg N ha⁻¹ period⁻¹). G1 is the period from the beginning of the growing season to the first crop harvest, G2 is the period from the first harvest to the second harvest and G3 is the period from the second harvest to the end of the growing season.

	2005				2006				2005	2006
	G1	G2	G3	NG	G1	G2	G3	NG		
N deposition	12	2	1	1	1	1	2	1	15	4
Gross N mineralization of soil										
Fertilizer plot	60	115	155	37	62	122	115	32	367	331
Manure plot	62	115	155	36	59	129	116	32	369	336
Control plot	64	120	161	37	61	132	121	34	382	348
Bare plot	67	132	150	34	67	153	104	31	384	356
Gross N mineralization of <u>root-litter</u>										
Fertilizer plot	88	44	0	13	104	10	0	13	145	126
Manure plot	103	51	0	11	101	11	0	10	165	122
Control plot	68	27	0	13	75	6	0	13	108	94
Gross N mineralization of manure	13	30	39	6	13	43	34	6	88	96

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Table 5

N uptake in aboveground biomass during each growing season (G1, G2 and G3) and non-growing season (NG) ($\text{kg N ha}^{-1} \text{ period}^{-1}$). G1 is the period from the beginning of the growing season to the first crop harvest, G2 is the period from the first harvest to the second harvest and G3 is the period from the second harvest to the end of the growing season.

	2005				2006				2005	2006
	G1	G2	G3	NG	G1	G2	G3	NG		
Fertilizer plot	138	94	20	-2	137	70	22	-9	249	219
Manure plot	57	133	36	-10	122	52	30	-3	215	201
Control plot	42	55	24	-11	85	41	10	-1	109	134

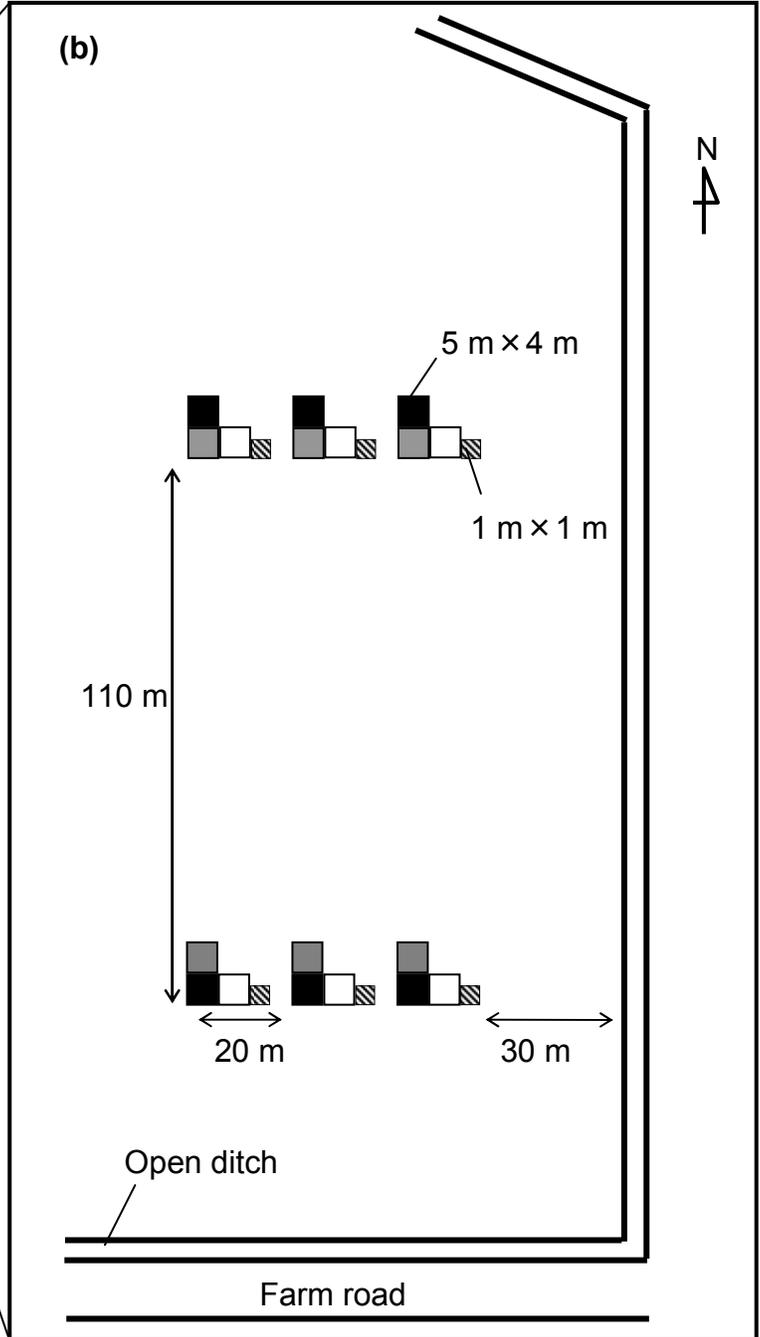
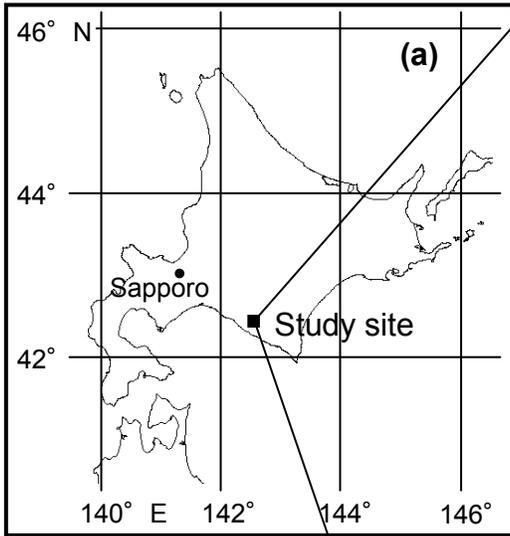
Table 6

The cumulative N₂O flux during each growing season (G1, G2 and G3) and non-growing season (NG) (kg N ha⁻¹ period⁻¹). G1 is the period from the beginning of the growing season to the first crop harvest, G2 is the period from the first harvest to the second harvest and G3 is the period from the second harvest to the end of the growing season.

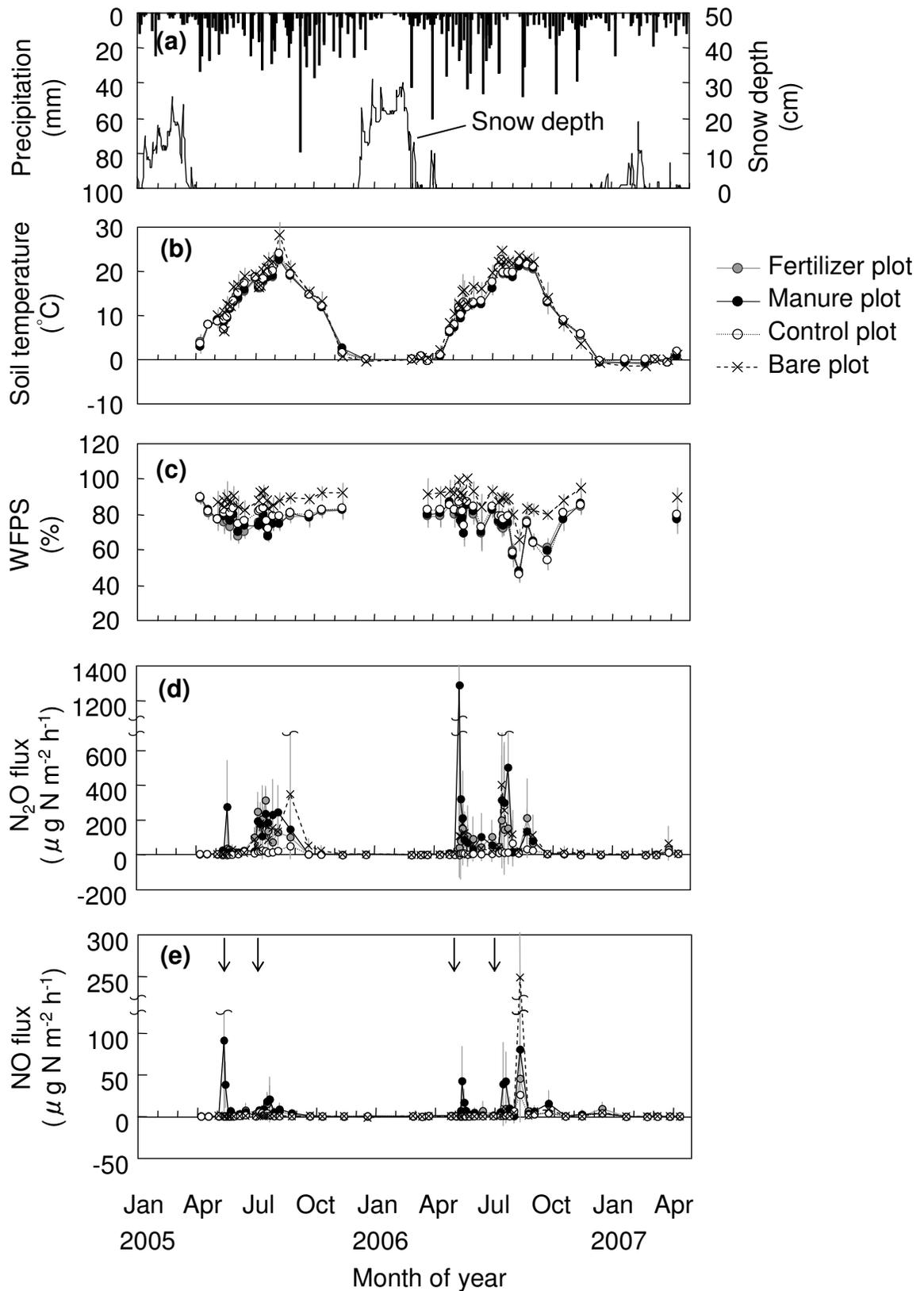
	2005				2006				2005	2006
	G1	G2	G3	NG	G1	G2	G3	NG		
Fertilizer plot	0.2 (0.1)	1.8 (0.4)	0.8 (0.3)	0.1 (0.0)	1.0 (1.0)	1.3 (0.5)	0.6 (0.3)	0.2 (0.1)	2.8 (0.7)	3.0 (0.8)
Manure plot	0.5 (0.3)	1.9 (0.9)	1.2 (0.7)	0.0 (0.0)	2.5 (2.1)	1.9 (1.6)	0.5 (0.3)	0.1 (0.1)	3.6 (1.3)	4.9 (3.0)
Control plot	0.1 (0.0)	0.2 (0.1)	0.3 (0.4)	0.0 (0.0)	0.1 (0.1)	0.3 (0.3)	0.2 (0.1)	0.1 (0.1)	0.7 (0.5)	0.6 (0.3)
Bare plot	0.2 (0.1)	1.4 (0.8)	2.5 (2.9)	0.1 (0.0)	0.8 (0.6)	1.4 (1.8)	0.7 (0.6)	0.4 (0.5)	4.2 (3.3)	3.3 (2.1)

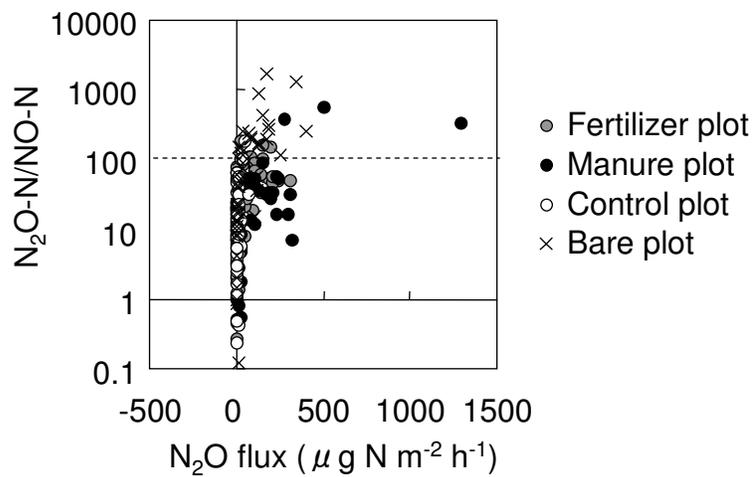
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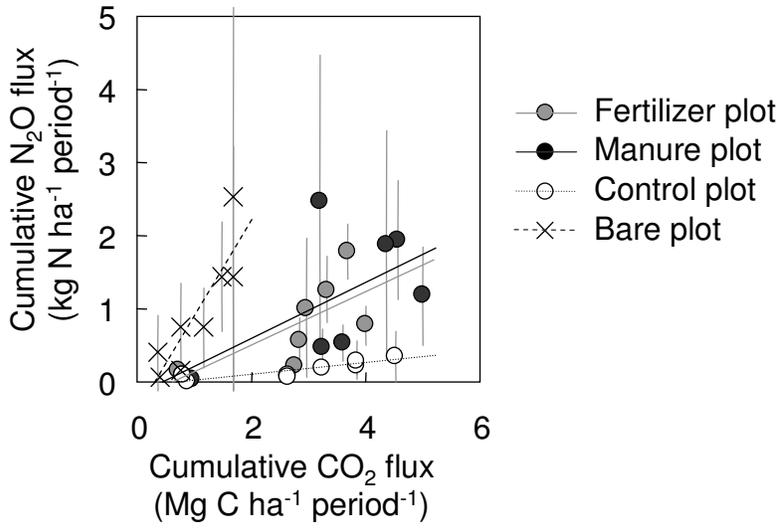
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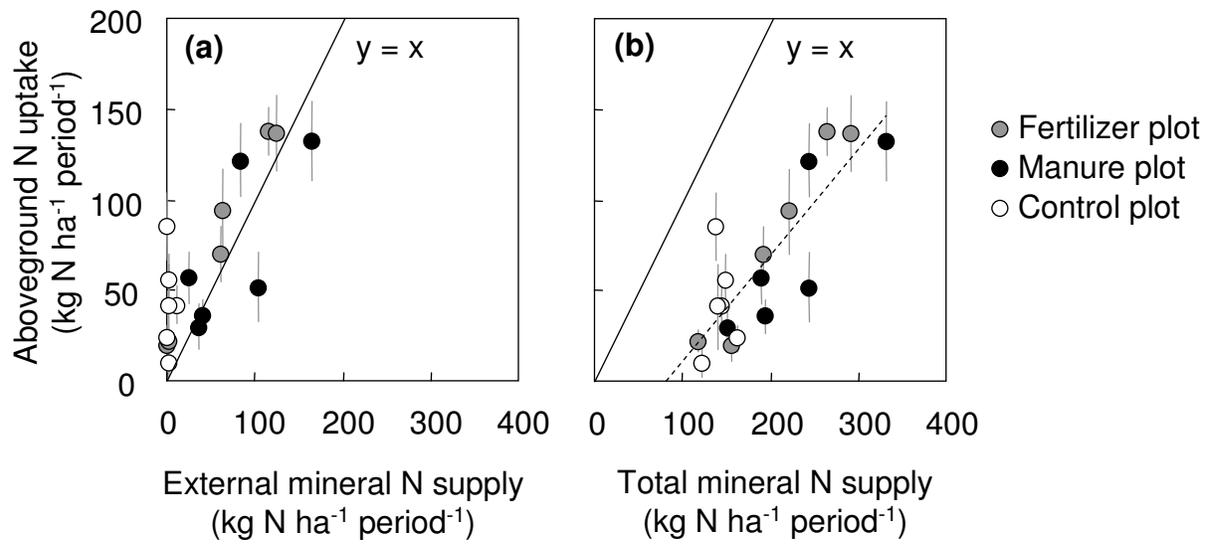
- Fertilizer plot
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- ▨ Bare plot

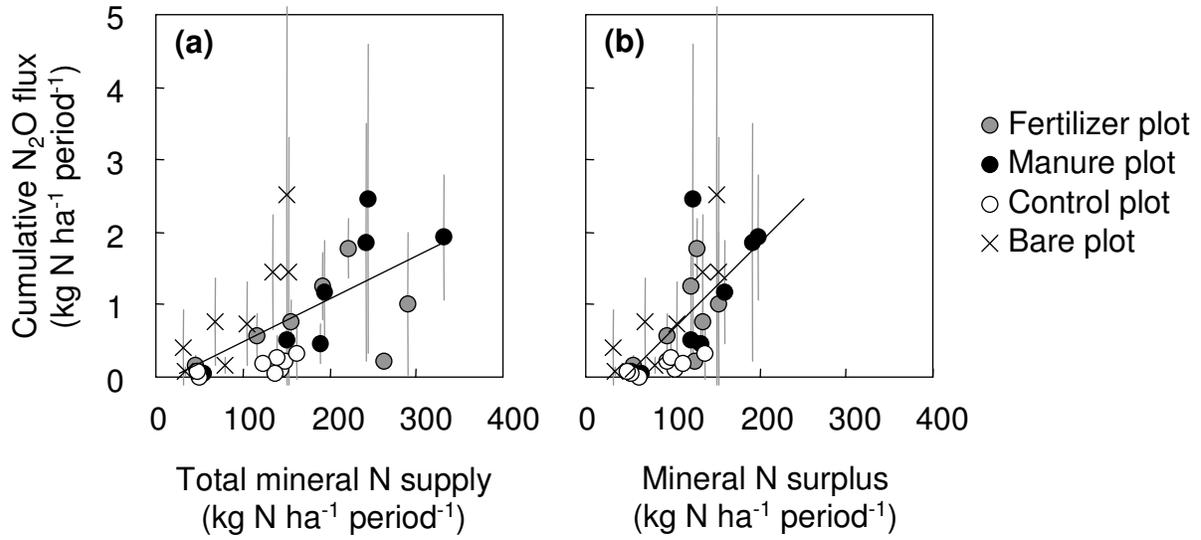


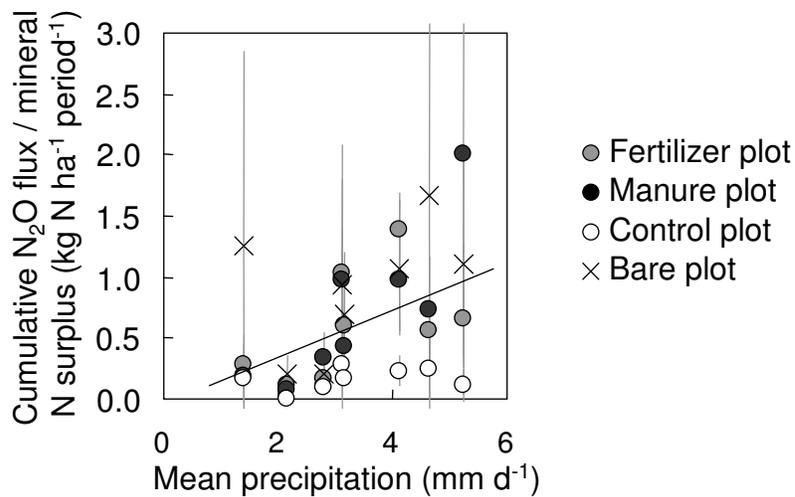




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