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Article

Temporal Dynamics of Nitrous Oxide Emission and Nitrate Leaching in Renovated Grassland with Repeated Application of Manure and/or Chemical Fertilizer

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Abstract: Managed grassland is occasionally renovated to maintain plant productivity by killing old vegetation, ploughing, and reseedling. This study aimed to investigate the combined effect of grassland renovation and long-term manure application on the temporal dynamics of nitrous oxide (N₂O) emission and nitrate nitrogen (NO₃[−]–N) leaching. The study was conducted from September 2013 to September 2016 in a managed grassland renovated in September 2013. In this grassland, two treatments were managed—chemical fertilizer application (F treatment) and the combined application of chemical fertilizer and beef cattle manure (MF treatment)—for eight years before the renovation. The control treatment without fertilization (CT) was newly established in the F treatment. The soil N₂O flux was measured using a closed chamber method. A leachate sample was collected using a tension-free lysimeter that was installed at the bottom of the Ap horizon (25 cm deep), and total NO₃[−]–N leaching was calculated from leachate NO₃[−]–N concentration and drainage volume was estimated by the water balance method. In the first year after renovation, the absence of plant nitrogen uptake triggered NO₃[−]–N leaching following rainfall during renovation and increased drainage water after thawing. NO₃[−]–N movement from topsoil to deeper soil enhanced N₂O production and emission from the soil. N₂O emission in MF treatment was 1.6–2.0 times larger than those of CT and F treatments, and NO₃[−]–N leaching in MF treatment was 2.3–2.6 times larger than those of CT and F treatments in the first year. Mineral nitrogen release derived from long-term manure application increased NO₃[−]–N leaching and N₂O emission. In the second year, N₂O emission and NO₃[−]–N leaching significantly decreased from the first year because of increased plant N uptake and decreased mineral nitrogen surplus, and no significant differences in N₂O emission and NO₃[−]–N leaching were observed among the treatments. In the second and third years, NO₃[−]–N leaching was regulated by plant nitrogen uptake. There were no significant differences in NO₃[−]–N leaching among the treatments, but N₂O emission in MF treatment was significantly smaller than in the F treatment. Long-term manure application could be a possible option to mitigate N₂O emission in permanent grassland; however, the risk of increased NO₃[−]–N leaching and N₂O emission in the renovation year induced by manure nitrogen release should be noted.

Keywords: N₂O emission; NO₃[−]–N leaching; grassland renovation; long-term manure and chemical fertilizer application

1. Introduction

Permanent managed grassland is an important ecosystem for feeding in dairy and beef farming systems. In Hokkaido, Northern Japan, managed grassland is a dominant agricultural land use type, accounting for 44% of the total agricultural land of 11.5×10^3 km² [1]. The farmers continuously use managed grassland for several years and occasionally renovate grassland by ploughing and reseeding to maintain grassland productivity.

Little N uptake in reseeded grassland may increase temporal mineral nitrogen (N) accumulation in soil. Ploughing increases mineral N release from soil and incorporated plant residue [2,3] via the physical disturbance of soil aggregate [4] and soil-residue contact [5], which may increase soil nitrous oxide (N₂O) emission to the atmosphere and nitrate (NO₃[−]–N) leaching to the aquasphere [3]. Davies et al. [2] showed that the net mineral N release after ploughing and reseeding was 85–140 kg N ha^{−1} higher than continued grassland in Scotland. Necpálová et al. [3] showed ploughing and reseeding reduce the total N stock in surface soil by 14% and twofold, respectively, of N₂O emission and NO₃[−]–N leaching in Ireland pasture. Grassland renovation temporally increases N₂O flux after ploughing and fertilization for 1–2 months [6–8] to one year [3]; yet, it often does not affect annual N₂O emission [8]. In addition to little plant N uptake, higher precipitation after renovation stimulate N₂O production by denitrification [6] and larger drainage in the winter increase NO₃[−]–N leaching from accumulated mineral N in soil. The soil freeze-thaw cycle [9] is also reported as an important driver of enhancing N₂O fluxes after grassland renovation. Grassland renovation increases NO₃[−]–N concentration in groundwater for one year [3], and during the winter period, NO₃[−]–N leaching is highly prevalent after grassland renovation [8]. Shepherd et al. [10] found that older grassland has a higher potential for increasing NO₃[−]–N leaching in the first winter of renovation rather than younger grassland, which is likely because of the larger organic N stock in elder grassland.

The mineralized N from manure would be partially lost from soil via N₂O emission and NO₃[−]–N leaching in grassland. The N₂O–N and NO₃[−]–N released from applied manure N were evaluated in field conditions in several studies. Shimizu et al. [11] reported that N₂O–N that was emitted from applied manure N accounted for $0.36 \pm 0.61\%$ of total manure N (76–491 kg N ha^{−1} year^{−1} combined application with 89–233 kg N ha^{−1} year^{−1} of chemical fertilizer) in five Andosol grasslands in Japan. Smith et al. [12] reported that NO₃[−]–N leaching from applied manure N accounted for $9.54 \pm 7.79\%$ of total manure N under 200 kg N ha^{−1} year^{−1} in four grasslands of brown earth soil in England. Maeda et al. [13] found that the repeated application of swine compost (800 kg N ha^{−1} year^{−1}) for more than four years increased total N content in surface soil and also increased NO₃[−]–N leaching to 1 m depth, the same level as chemical fertilizer—the only application (400 kg N ha^{−1}) in Andosol arable cropland in Japan. These results indicate that the mineral N supply from manure was a significant source of N₂O emission and NO₃[−]–N leaching, especially in the field with repeated manure application. However, N₂O emission was smaller in the manure field rather than in the chemical-fertilizer field after the chemical-fertilizer application rate in the manure field was reduced and the mineral N supply from the applied manure was considered [14]. These studies provided empirical evidence of N dynamics under different fertilizer managements in grassland; yet, this evidence is still limited in renovated grassland.

Continuous monitoring is required to understand temporal N dynamics changes, including N₂O emission and NO₃[−]–N leaching associated with grassland renovation. Hence, in this study, we report the result of three years' monitoring of N₂O emission and NO₃[−]–N leaching in a renovated grassland with three different treatment plots for eight years: no fertilizer (CT), chemical fertilizer only (F), and combined manure and chemical fertilizer (MF). The objective was to investigate the effect of renovation on the temporal dynamics of N₂O emission and NO₃[−]–N leaching with long-term repeated manure and/or chemical fertilizer application.

2. Materials and Methods

2.1. Site Description

The study site is located at the Shizunai experimental livestock farm in the Field Science Center for Northern Biosphere of Hokkaido University in Shin-Hidaka City, Southern Hokkaido, Japan (42°26'N, 142°29'E). The site is characterized by a humid continental climate with cold winters and cool summers, but without apparent wet or dry seasons. The mean annual temperature was 8.36 °C and the mean annual precipitation was 1210 mm in the last 10 years. The soil surface is covered with snow from December to March, during which the soil is normally frozen. The soil is derived from Tarumae-b, Usu-c, and Tarumae-c volcanic ash [15] and it is classified as Thaptic Melanudands (Soil Survey Staff, 2006) and Mollic Andosol (IUSS Working Group WRB, 2006). Two adjacent treatments (100 m × 100 m) were established in the study field in 2005, one for chemical fertilizer treatment (F treatment area) and another for chemical fertilizer and composted beef cattle manure treatment (MF treatment area) [16]. These nutrient managements were kept for eight years before this study since 2005 [17] and continued until 2016. Because of long-term nutrient management, the total soil carbon and N concentrations in the top 0–30 cm soil were significantly higher in MF treatment than in F treatment (Table 1).

This new grassland was renovated in August and September 2013 given high weed invasion this year. Herbicide was applied on 13 August and was plowed during 20 September to 24, and 20 kg ha^{−1} of timothy grass (*Phleum pratense* L.) seeds were applied on 24 September.

Table 1. Soil bulk density, total carbon, and nitrogen content in top 0–30 cm soil in chemical fertilizer treatment (F) area and combined manure and chemical fertilizer treatment (MF) area just before renovation. Values are mean ± standard deviation. Same letters besides the values denote no significant difference between F area and MF area ($p < 0.05$).

Treatment	Bulk Density g cm ^{−3}	Total Carbon g C kg soil ^{−1}	Total Nitrogen g N kg soil ^{−1}
F area	0.76 ± 0.06 a	4.73 ± 0.74 b	0.45 ± 0.06 b
MF area	0.75 ± 0.06 a	5.72 ± 0.99 a	0.51 ± 0.07 a

2.2. Experimental Designs and Management Practices

This study was continued for 3 years after grassland renovation from 13 August 2013 (the date of herbicide application), to 26 September 2016 (before the second crop harvest). The control treatment (CT) was additionally established in the F treatment area. We randomly place four replicates of subplots (10 × 10 m in size) of MF treatment in the large MF treatment area (100 × 100 m) and subplots of F and CT in the large F treatment area (100 × 100 m), respectively (Figure S1). We performed the sampling around the center of each subplot to minimize fertilizer contamination from outside the subplot.

Table 2 shows the annual fertilizer and the manure application rate during the study period. The type of chemical fertilizer was a mixture of ammonium sulfate and urea, and the manure was composted beef cattle manure with bedding litter (bark). Chemical fertilizer was applied twice in a year, in spring after snowmelt (April to May), and in early July, in a 2:1 annual application rate, respectively. Manure was applied on 12 September 2013 (between herbicide application and plowing), 18 May 2015, and 19 April 2016. In MF treatment, the N mineralization rate of manure was estimated over 12 years from 2004 to 2016 based on Uchida's model [18] and it was 15.2% in the first year and 2.4–3.1% in the second to twelfth year after application. The chemical fertilizer application rate in MF treatment was designed to roughly fill the gaps between the estimated N mineralization rate from manure in MF treatment and the chemical fertilizer N application rate in F treatment.

Table 2. Annual chemical fertilizer and manure application rate after renovation in chemical fertilizer treatment (F) and combined manure and chemical fertilizer treatment (MF).

Year after Renovation	Treatment	Fertilizer Type	T-C	T-N	P ₂ O ₅	K ₂ O
			kg ha ^{−1}			
First year 2013–2014	F	Chemical fertilizer	0	150	108	212
	MF	Chemical fertilizer	0	47	47	50
		Manure	3591	182	167	115
Second year 2014–2015	F	Chemical fertilizer	0	104	40	141
	MF	Chemical fertilizer	0	57	22	69
		Manure	4193	165	209	144
Third year 2015–2016	F	Chemical fertilizer	0	145	117	190
	MF	Chemical fertilizer	0	86	67	115
		Manure	5110	202	254	175

2.3. Gas Sampling and Analysis

Nitrous oxide (N₂O) and carbon dioxide (CO₂) fluxes were measured using static closed chambers. The four replicate of the gas sampling by this method is acceptable based on spatial variability [19]. The N₂O fluxes were measured in the vegetated area, and the CO₂ fluxes were measured in the bare plot (plant and root-excluded plot) to measure heterotrophic respiration (RH). The bare plot (25 × 50 cm in size) was established within each subplot following a method described by Shimizu et al. [15]. The fertilizer management in the bare plot was the same as that in the vegetation area (Table 2). The chambers were made of stainless steel and were 40 cm in diameter and 30 cm in height in the vegetated area and 20 cm in diameter and 25 cm high in the bare plot. Details of the chambers were as reported by Toma and Hatano [20]. The chambers were placed onto chamber bases just before the sampling during the nonwinter period. The chamber bases were installed permanently at a depth of 5 cm and they could not be used in winter; therefore, the chambers were inserted directly on the snow before measurements. We did not remove the snow during the winter measurements. After each sampling, the chambers were removed from the bases or snow surface. Gas samples were taken between 8:00 a.m. and 12:00 p.m. on each sampling day. The normal sampling frequency was once or twice every fortnight, except in winter, when sampling was conducted once or twice every month. A more intensive sampling regime of every two to five days was carried out after fertilization, rainfall, and harvest events, which were known to stimulate gas flux [21]. Gas samples from the headspace of each chamber were collected using a gas-tight syringe. Twenty mL of the gas sample was injected into a prevacuumed 10 mL vial bottle for N₂O analysis, and 250 mL of the gas sample was collected into a prevacuumed 500 mL Tedlar bag. Chamber headspace gas was taken at 0 min (before closing the chamber cover), 15 min, and 30 min for N₂O, and 0 min and 6 min for CO₂. N₂O and CO₂ concentrations were analyzed using a gas chromatograph that was equipped with an electron capture detector (ECD) (Model GC-14B and Model GC2014; Shimadzu, Kyoto, Japan) and a CO₂ infrared gas analyzer (ZFP9GC11, Fuji Electric, Tokyo, Japan), respectively.

The gas flux from soil was calculated as

$$F = \rho \times \left(\frac{V}{A}\right) \times \left(\frac{\Delta C}{\Delta t}\right) \times \left(\frac{273}{T}\right) \times \alpha \quad (1)$$

where F is the gas flux in $\mu\text{g N m}^{-2} \text{ h}^{-1}$ or $\text{mg C m}^{-2} \text{ h}^{-1}$; ρ is the density of each gas at standard conditions (N₂O = $1.978 \times 10^6 \text{ mg m}^{-3}$ and CO₂ = $1.977 \times 10^6 \text{ mg m}^{-3}$); V is the chamber volume (m³); A is the chamber surface area (m²); $\Delta C / \Delta t$ ($10^{-6} \text{ m}^3 \text{ m}^{-3} \text{ h}^{-1}$) is the ratio of change in gas concentration in the chamber during sampling; T is the air temperature inside the chamber (°C); and, α is the ratio of molar mass of N and C of the molecular weight of each respective gas. Cumulative gas emissions were calculated by linear interpolation between sampling events and the numerical integration of the underlying area using the trapezoid rule. We defined the crop growing season as the seven-day period of the moving average of daily air temperature above 5 °C. The nongrowing season was divided into the winter and thawing periods. The winter period lasts from the end of the growing season to the end of February, when maximum temperatures recorded reach 0 °C, while the thawing period lasts

from when minimum daily temperatures reach 0 °C to when the snow in the soil is completely melted (minimum soil temperatures ~5 °C) [22]. In 2013, we defined the renovation period from when the herbicide was applied (13 August) to the first sampling date after reseeded (7 October).

2.4. Leachate Sampling and Analysis

Gravitational leachate samples were collected with tension-free stainless steel pan lysimeters [23] (25 × 30 cm in size), which were carefully inserted at the bottom of the Ap horizon (25 cm depth) via jacking to minimize soil disturbance. The installation was conducted on 7 June 2013. The drainage water intercepted by the pan sampler was conveyed to a 10 L tank through a plastic tube that was connected to the end of the pan. The lysimeters were temporarily removed from 4 September to 7 October to avoid instrument destruction by plowing during renovation. We put Thymol (C₁₀H₁₄O) as a biocide in the tank to prevent the biological alteration and utilization of the N species [24]. The frequency of leachate sampling was the same as gas sampling. Leachate samples were filtered by a membrane filter (0.2 µm) and were kept at 4 °C until analysis. NO₃[−]-N concentration in leachate water was analyzed by ion chromatography (ICS-1100; Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Soil Sampling and Analysis

Soil samples were collected at 5 cm depth during each sampling day from April to November (nonfreezing period) in all treatments. Soil samples were sieved (2 mm sieve) and extracted in deionized water for pH, NO₃[−]-N, and water extractable organic carbon (WEOC) concentration analysis. Additionally, soil extracts in a 2 mol L^{−1} potassium chloride (KCl) solution were used for ammonium nitrogen (NH₄⁺-N) concentration analysis. Soil extractions were filtered by a membrane filter (0.2 µm) and were kept at 4 °C until analysis. Soil pH was measured using soil water extracts by pH meter (F-8 pH meter; Horiba, Kyoto, Japan) before filtering. WEOC concentration was analyzed by a total organic C (TOC) analyzer (TOC 5000A; Shimadzu, Japan). NH₄⁺-N concentration was determined while using the indophenol-blue method using spectrophotometer (UV mini 1240; Shimadzu, Kyoto, Japan). NO₃[−]-N concentration was analyzed by ion chromatography (ICS-1100; Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Meteorological Data Collection, Drainage, and NO₃[−]-N Leaching Estimation

Daily precipitation data were obtained from the Sasayama station of the Automated Meteorological Data Acquisition System (AMeDAS) of the Japan Meteorological Agency, which is located within 5 km from this study site. Soil freezing depth was measured by a plastic tube filled with methylene blue dye suspended in a PVC outer tube during the no-growing season, and snow depth was measured manually at the same time. Thermocouple thermometers (TR-52; TD, Nagano, Japan) were permanently installed in each treatment to measure soil temperature at 5 cm depth at 30-min intervals.

On each sampling day, the air temperature inside the chamber and soil temperature (5 cm depth) were measured using a handheld thermometer (CT220; CUSTOM, Tokyo, Japan). Soil moisture was measured at 0–6 cm depth using the frequency domain reflectometry (FDR) method (DIK-311A; Daiki, Saitama, Japan). Calibration curves were made to calculate volumetric water content (VWC) from the FDR device reading and to measure VWC using 100 mL of soil core, and water-filled pore space (WFPS) was calculated (VWC divided by percent of total porosity). The soil water stock (mm) of the top 0–30 cm soil was estimated from soil gravimetric water content that was taken by a soil auger (3.5 cm in diameter) and soil dry bulk density.

Cumulative drainages during each sampling date were estimated as

$$D = P - (ET + \Delta S) \quad (2)$$

where D is the cumulative drainage (mm); P is the cumulative precipitation (mm); ET is the cumulative evapotranspiration (mm); and, ΔS is the water stock change (mm) in top 0–30 cm soil. The eddy

covariance technique [16,25] was used to measure ET in this field. Cumulative NO_3^- -N leaching was estimated as the product of NO_3^- -N concentration in leachate sample and cumulative drainage (D) during each sampling date. Instead of setting the drainage volume during the winter period as 0 mm, the calculated cumulative drainage volume during the winter period was set to drain in the start of the thawing period until the first date for water stock measurement in the thawing period.

2.7. Plant N Uptake and Estimation of N Mineralization, Total Mineral N Input, and Mineral N Surplus

Plant biomass was collected four times in a year: in mid-April, in late June (the first crop harvest), in mid-August (the second crop harvest), and in late October. The aboveground biomass during the harvest was estimated to be the sum of the harvest and stubble parts; the harvest part was taken by cutting at 5 cm above the ground; and, the 0–5 cm left from the soil surface was the stubble part. The stubble part and total aboveground biomass in April and October were taken by cutting at the soil surface. All plant samples were taken within 0.5×0.5 m quadrates. Two aboveground samples were collected and averaged for each of the three treatment replicates during each sampling event. The plant samples were oven-dried at 70°C for more than 72 h and weighed. Each dried sample was analyzed for total C and N contents with an N/C analyzer (SUMIGRAPH NC-1000; Sumika Chemical Analysis Service Ltd., Osaka, Japan). The N uptake in aboveground biomass was estimated as an increment in biomass N during each season.

Heterotrophic respiration (RH), as measured by the closed chamber in the CT and F treatments, was regarded as heterotrophic respiration from soil organic matter decomposition (RH_{soil}), while RH in MF treatment included RH_{soil} and heterotrophic respiration from manure decomposition (RH_{manure}). Therefore, RH_{manure} in MF treatment was estimated by subtracting the RH from the F treatment. Gross N mineralization (GM) from soil (GM_{soil}) and manure (GM_{manure}) were estimated by dividing RH_{soil} and RH_{manure} by a C:N ratio of soil and manure, respectively [17,22], as follows:

$$GM_{(\text{soil}, \text{manure})} = \frac{RH_{(\text{soil}, \text{manure})}}{C : N \text{ ratio}_{(\text{soil}, \text{manure})}} \quad (3)$$

The total mineral N input was calculated as the sum of chemical fertilizer input, GM_{soil} , and GM_{manure} , as follows:

$$\text{Total mineral N input} = \text{Chemical fertilizer N} + GM_{\text{soil}} + GM_{\text{manure}} \quad (4)$$

Additionally, N surplus was calculated as the difference between total mineral input and plant N uptake, as follows:

$$\text{Mineral N surplus} = \text{Total mineral N input} - \text{Plant N uptake} \quad (5)$$

2.8. Statistical Analysis

The daily change of soil temperature, moisture, and chemical properties were analyzed by repeated measurements in a two-way analysis of variance (ANOVA) to test for differences between treatment years. Spearman's rank correlation coefficient was calculated among N_2O fluxes, leachate NO_3^- -N concentration, and daily meteorological and soil chemical properties since N_2O fluxes and leachate NO_3^- -N concentrations were not fitted with normal distribution by the Shapiro–Wilk test.

The annual N_2O emission, NO_3^- -N leaching, RH, and plant N uptake were analyzed by two-way ANOVA to test for differences between the treatment years. The Tukey–Kramer test ($\alpha = 0.05$) was used for multiple comparisons for annual N_2O emission, NO_3^- -N leaching, RH, and plant N uptake. Annual N_2O emission and NO_3^- -N leaching were fitted to the lognormal distribution before ANOVA. Simple linear regression analysis was used to explain the relationship between N_2O emissions and NO_3^- -N leaching and total chemical fertilizer N input, total mineral N input, plant N uptake, and mineral N surplus. All statistical analyses were done using R software (version 3.5.0) [26].

3. Results

3.1. Weather and Soil Variables

Figure 1a shows the daily precipitation and air and soil temperature at 5 cm depth. The mean annual temperatures were similar across three years ($8.5\text{--}8.7\text{ }^{\circ}\text{C}$), and they are similar to the mean value of the last 10 years ($8.4 \pm 0.4\text{ }^{\circ}\text{C}$). Annual precipitation in the first and second years (1331 and 1062 mm, respectively) were similar to the mean value of the last 10 years ($1210 \pm 194\text{ mm}$), while the third year was wetter (1711 mm) because of heavy rain in June to August 2016.

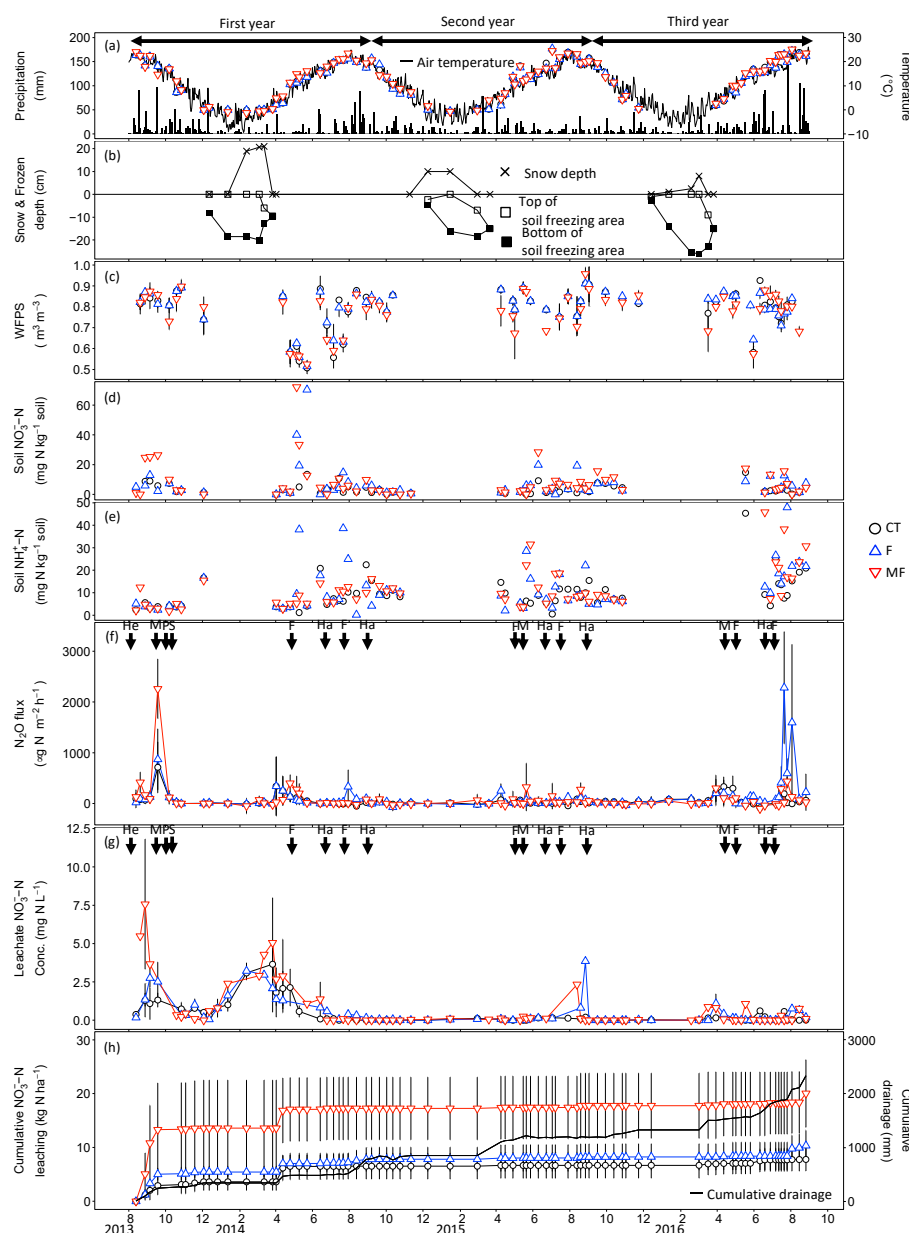


Figure 1. Daily change of precipitation, air and soil temperature depth (a); snow and soil freezing depth (b); water-filled pore space (WFPS) (c); soil $\text{NO}_3^- \text{-N}$ concentration (d); soil $\text{NO}_3^- \text{-N}$ concentration (e); N_2O flux (f); leachate $\text{NO}_3^- \text{-N}$ concentration (g) and cumulative drainage volume and $\text{NO}_3^- \text{-N}$ leaching (h). Error bar show standard deviation. CT is unfertilized control plots (○); F is chemical fertilizer plots (△); MF is combined manure and chemical fertilizer plots (▽). Black arrows for letters indicates the timing of management as He: Herbicide application, P: Plowing, S: Seeding, F: Chemical fertilizer application, M: Manure application, Ha: Harvest.

Soil temperature at 5 cm depth varied from -0.5 to 25.5 °C, and no significant differences were observed among the years and treatments. This field was covered with snow from December to March, and soil freezing reached a 20–25 cm depth in early March before it completely melted in late March (Figure 1b).

WFPS at 0–6 cm soil varied from 0.51 to 0.96 $\text{m}^3 \text{m}^{-3}$. The annual mean WFPS was not significantly different among the treatments but was significantly higher in the second and third years after renovation (0.80 and 0.81 $\text{m}^3 \text{m}^{-3}$, respectively) than in the first year (0.75 $\text{m}^3 \text{m}^{-3}$) ($p < 0.001$) (Figure 1c).

Soil pH varied from 4.96 to 7.08 and it was significantly different among the treatments ($p < 0.001$) and years ($p < 0.001$) (Figure S2). Soil pH in MF treatment was higher than in CT and F treatment, and the pH in the F treatment was the lowest. Soil pH was the lowest in the first year after renovation, followed by the third year, and was the highest in the second year.

Soil WEOC concentration varied from 26.5 to 259.8 mg C soil^{-1} (Figure S2) and it was significantly higher in MF treatment than in CT and F treatments ($p < 0.001$) and also significantly higher in the second and third years than the first year ($p < 0.001$).

3.2. Soil and Leachate Mineral N

Soil NO_3^- -N concentration increased to 24 – 26 mg N kg soil^{-1} in MF treatment for 21 days after herbicide application, and soil NO_3^- -N concentration in CT and F treatment was 2 – 12 mg N kg soil^{-1} in the same period. Soil NO_3^- -N concentration increased to more than 60 mg N kg soil^{-1} in F and MF treatments for a few days after the first chemical fertilization in May 2014 and immediately decreased to less than 20 mg N kg soil^{-1} (Figure 1d). Significant differences were observed in soil NO_3^- concentrations across the treatments ($p < 0.05$) and years ($p < 0.05$). Soil NO_3^- -N concentration was the highest in MF treatment, followed by F, and the lowest in CT in the average of three years, and it was highest in the first year after renovation, followed by the third year, and the lowest in the second year.

Soil NH_4^+ -N concentration varied from 0.2 to 47.9 mg N kg soil^{-1} and it showed short peaks after chemical fertilization (Figure 1e). Although no significant difference was found in NH_4^+ -N concentration among the treatments, soil NH_4^+ -N concentration was significantly higher in the third year after renovation than the other two years ($p < 0.001$).

Figure 1g shows the temporal change in leachate NO_3^- -N concentration, which increased after herbicide application until manure application during the renovation period was higher in MF (3.6 – 7.6 mg N L^{-1}) treatment than in CT and F treatment (0.1 – 2.7 mg N L^{-1}), and then decreased to less than 1 mg N L^{-1} . Leachate NO_3^- -N concentration increased again during the winter period under soil freezing and it reached about 4 mg N L^{-1} on 27 March 2013, and then decreased after thawing. NO_3^- -N concentrations were always lower than 0.3 mg N L^{-1} from September 2014 to March 2016, except short-term peaks in MF (2.3 mg N L^{-1}) treatment and F (3.9 mg N L^{-1}) treatment in August 2015, which were observed after the application of chemical fertilizer in each treatment. Several small peaks of leachate NO_3^- -N concentration of about 1 mg N L^{-1} were observed in the thawing period (F and MF treatments) after the application of chemical fertilizer in May (MF treatment) and in July (F and MF treatments) in 2016. Leachate NH_4^+ -N was always traced in all treatments.

3.3. N_2O Flux

Figure 1f shows the temporal change of N_2O fluxes. A large peak of N_2O flux was observed on 18 September 2013 (35 after herbicide application, six days after manure application, and two days before for plowing), in the renovation period of the first year in all treatments (713.8 , 871.0 , and 2260.9 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in CT, F, and MF, respectively). The largest and third largest N_2O fluxes in this study period were observed in F treatment on 21 July (2283.3 $\mu\text{g N m}^{-2} \text{h}^{-1}$) and August 3 (1596.3 $\mu\text{g N m}^{-2} \text{h}^{-1}$) in 2016, nine and 22 days after chemical fertilizer application, respectively, while the N_2O fluxes in MF treatment ranged from 18.7 to 473.3 $\mu\text{g N m}^{-2} \text{h}^{-1}$ in the same date. Increased N_2O fluxes during the thawing period were observed in all treatments in April 2014 and

2016. The N_2O flux in the thawing period varied from 200 to $400 \mu\text{g N m}^{-2} \text{h}^{-1}$ and continued for 15–25 days.

N_2O flux had a significant positive correlation with soil temperature, soil NO_3^- -N content, and leachate NO_3^- -N concentration and it had a significant negative correlation with soil pH in the combined three years (Table 3). For individual years, a significant positive correlation between N_2O fluxes and leachate NO_3^- -N concentrations were showed in the first and third years, and N_2O fluxes in the second year had a significant positive correlation with soil NH_4^+ -N and the sum of soil NH_4^+ -N and NO_3^- -N instead.

Table 3. Spearman's correlation coefficient describing the relationship between N_2O flux and soil and soil environmental and mineral N variables after renovation (except for winter season).

Soil and Leachate Variables	N_2O Flux ($\mu\text{g N m}^{-2} \text{h}^{-1}$)			
	First Year (2013–2014)	Second Year (2014–2015)	Third Year (2015–2016)	Combined Year (2013–2016)
soil temperature	0.29 **	0.39 ***	0.13 n.s.	0.17 *
WFPS	−0.16 n.s.	−0.13 n.s.	−0.19 n.s.	−0.11 n.s.
soil pH	−0.43 ***	−0.09 n.s.	−0.08 n.s.	−0.27 ***
soil NO_3^- -N	0.19 n.s.	0.24 n.s.	0.08 n.s.	0.23 **
soil NH_4^+ -N	−0.49 ***	0.26 *	0.11 n.s.	−0.1 n.s.
soil NO_3^- -N + NH_4^+ -N	−0.14 n.s.	0.33 *	0.13 n.s.	0.09 n.s.
soil WEOC	−0.05 n.s.	0.10 n.s.	0.08 n.s.	−0.1 n.s.
Leachate NO_3^- -N conc.	0.28 *	−0.02 n.s.	0.43 ***	0.26 ***

***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, n.s.: not significant; WFPS: water filled pore space; WEOC: water extractable organic carbon.

3.4. Cumulative N_2O Emission and NO_3^- -N Leaching

Annual N_2O emission was significantly different among the treatments ($p = 0.025$) and years ($p < 0.001$), and an interaction was observed between treatments and years ($p = 0.003$) (Table 4). N_2O emissions in the first and third years were significantly higher than in the second year. In the first year, N_2O emissions in MF were higher than CT and F; however, in the third year, they were the highest in F, followed by CT, and the lowest in MF. No significant differences in N_2O emission were found among treatments in the second year. N_2O emission during renovation had a high contribution to cumulative emission, accounting for 54.5–80.7% of the total in the first year and 18.3–55.3% of the sum of three years. N_2O emission from the F treatment in summer 2016 (after chemical fertilizer application) also had a high contribution to the annual emission in the third year (46.0% total) and it accounted for 20.1% of the sum of three years of N_2O emission (Table S1).

Table 4. Annual N_2O emission and annual NO_3^- -N leaching after renovation in the unfertilized control treatment (CT), chemical fertilizer treatment (F), and combined manure and chemical fertilizer treatment (MF). Values are mean $\pm$ standard deviation. Same letters besides the values denote no significant difference ($p < 0.05$).

Year	Treatment	N_2O Emission $\text{kg N}_2\text{O-N ha}^{-1} \text{year}^{-1}$			Nitrate Leaching $\text{kg NO}_3^- \text{-N ha}^{-1} \text{year}^{-1}$		
First year (2013–2014)	CT	6.51 ± 3.26 ab			6.52 ± 2.41 a		
	F	7.98 ± 3.91 ab			7.55 ± 1.95 a		
	MF	13.12 ± 2.72 a			17.26 ± 5.82 a		
Second year (2014–2015)	CT	0.99 ± 0.18 b			0.18 ± 0.31 c		
	F	2.30 ± 0.55 bc			0.68 ± 0.60 bc		
	MF	2.51 ± 0.89 bc			0.50 ± 0.70 bc		
Third year (2015–2016)	CT	5.82 ± 0.82 ab			1.09 ± 0.34 ab		
	F	13.61 ± 7.24 a			2.13 ± 2.61 ab		
	MF	3.22 ± 2.23 bc			2.27 ± 2.13 ab		
ANOVA		df	f value	p-value	df	f value	p-value
Treatment (T)		2	4.42	0.025	2	1.86	0.176
Year (Y)		2	32.74	< 0.001	2	32.19	< 0.001
T $\times$ Y		4	5.50	0.003	4	0.67	0.674

Annual NO_3^- -N leaching was significantly different among the years ($p < 0.001$) (Table 4). The annual NO_3^- -N leaching was the highest in the first year, followed by the third year and the second year. Although the statistical significance was weak for the difference among treatments, NO_3^- -N leaching in MF in the first year was the largest, followed by F and CT in the first year. However, in the second and third years, NO_3^- -N leaching in MF was similar to F, but that in CT was always lower than in MF and F. The renovation, growing, and thawing periods in the first year were the main terms of NO_3^- -N leaching in this study. They contributed 26.1–54.0% during renovation, 13.8–20.4% during the growing season after renovation in the first year, and 17.4–36.7% during thawing in the first year of the total of cumulative leaching in three years (Table S1).

A significant positive correlation was observed between annual N_2O emissions and annual NO_3^- -N leaching (Figure 2). Annual N_2O emission against annual NO_3^- -N leaching in the F treatment in the third year was higher than in other treatments and years (Figure 2).

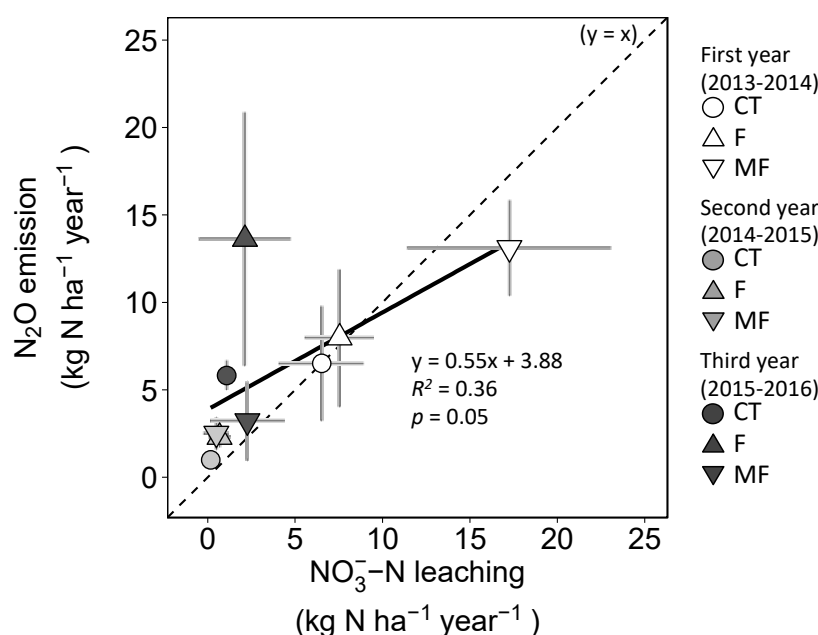


Figure 2. Relationship between annual N_2O emission and NO_3^- -N leaching. CT is unfertilized control plots ($\circ$); F is chemical fertilizer plots ($\triangle$); MF is combined manure and chemical fertilizer plots (∇). Error bar represents standard deviation. The black solid line indicates a simple linear regression model for all values. The dashed line indicates the line of $y = x$.

3.5. Plant N Uptake, Heterotrophic Respiration (RH), N Mineralization and Mineral N Surplus

Plant N uptake was significantly different among treatments ($p < 0.001$) and years ($p < 0.001$), and an interaction was observed ($p < 0.01$) (Table 5). Plant N uptake was highest in the second year, followed by the third and first years. Plant N uptake in CT was always lower than in F and MF, and that in F was higher than in MF in the first and second years, while they were similar in the third year.

Annual heterotrophic respiration (RH) was significantly different among the treatments ($p < 0.001$) and years ($p < 0.001$) (Table S2). RH in MF was always higher than in CT and F, and no significant difference was found between CT and F. RH in the third year was the highest, followed by the first and second years.

Surplus mineral N was higher in the first and third years than the second year, basically because of higher RH and lower plant N uptake in these two years in all treatments. In addition, higher chemical fertilizer N input may also contribute to higher surplus mineral in these two years in F treatment (Table 5).

Table 5. Mineral nitrogen input, plant N uptake, and mineral N surplus after renovation in unfertilized control treatment (CT), chemical fertilizer treatment (F), and combined manure and chemical fertilizer treatment (MF). Values are mean $\pm$ standard deviation.

Year	Treatment	Chemical Fertilizer N Input	GM_{soil}	GM_{manure}	Plant N Uptake	Mineral N Surplus
		kg N ha ⁻¹ year ⁻¹				
First year	CT	0	526 $\pm$ 72	0	61 $\pm$ 9	465 $\pm$ 76
	F	150	547 $\pm$ 64	0	147 $\pm$ 45	551 $\pm$ 82
	MF	47	492 $\pm$ 67	151 $\pm$ 14	101 $\pm$ 7	589 $\pm$ 59
Second year	CT	0	440 $\pm$ 60	0	75 $\pm$ 17	365 $\pm$ 71
	F	104	476 $\pm$ 40	0	240 $\pm$ 25	340 $\pm$ 35
	MF	57	411 $\pm$ 56	80 $\pm$ 40	173 $\pm$ 5	375 $\pm$ 60
Third year	CT	0	682 $\pm$ 73	0	46 $\pm$ 11	635 $\pm$ 70
	F	145	720 $\pm$ 133	0	164 $\pm$ 30	701 $\pm$ 103
	MF	86	637 $\pm$ 68	64 $\pm$ 14	158 $\pm$ 7	629 $\pm$ 37

GM_{soil} and GM_{manure} are gross mineralized N from the soil and the manure, respectively. Total mineral N input is the sum of chemical fertilizer N input, GM_{soil} , and GM_{manure} . Mineral N surplus is the reminder of subtraction plant N uptake from total mineral N input.

Annual N₂O emission had a positive linear correlation with mineral N surpluses ($p < 0.05$) (Figure 3), while not with chemical fertilizer N input, total mineral N input, and plant N uptake. Annual NO₃⁻-N leaching had no significant correlation with these N balance variables, although mineral N surplus showed the strongest correlation values (Figure 3) ($r = 0.29$, $p = 0.45$) among these variables.

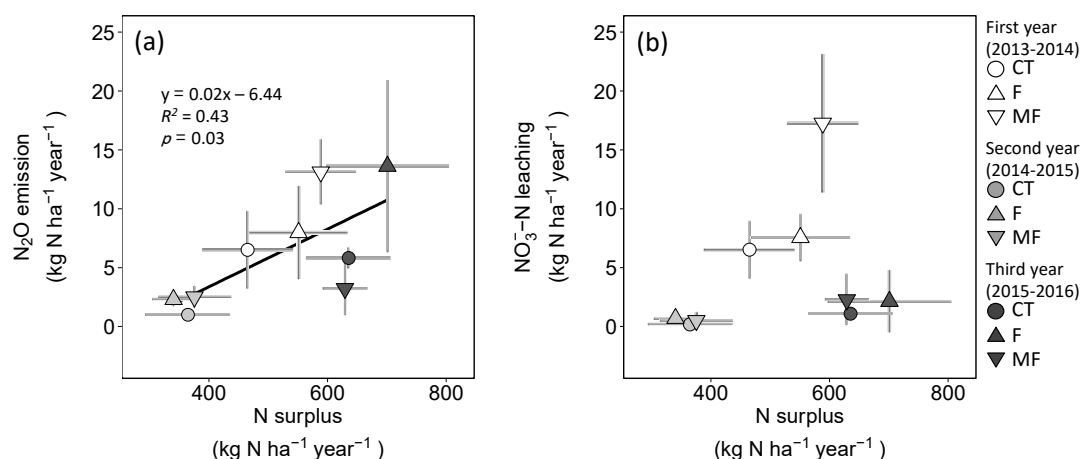


Figure 3. Relationship between annual N₂O emission (a), annual NO₃⁻-N leaching (b) and nitrogen surplus (N surplus). CT is unfertilized control plots (○); F is chemical fertilizer plots (△); MF is combined manure and chemical fertilizer plots (▽). Error bar represents standard deviation. The black solid line indicates a simple linear regression model.

4. Discussion

4.1. Temporal Nitrogen Dynamics Changes under Grassland Renovation and Permanent Management

In the first year, large peaks of N₂O flux and leachate NO₃⁻-N concentration were observed after herbicide application and during thawing (Figure 1f,g), and a significant positive correlation was observed between N₂O fluxes and leachate NO₃⁻-N concentrations (Table 3). In the Andosol, which has fewer cracks and in which rainwater moves mainly in a matrix flow [27], it is reported that most of the N₂O that is emitted to the atmosphere is produced in the top 30 cm soil [28], associated with the infiltration of rainwater and NO₃⁻-N in the micropores. Applying herbicide and killing the previous vegetation induced a large amount of accumulation of excess NO₃⁻-N produced by N mineralization and nitrification in soil. The movement of excess NO₃⁻-N to a relatively anaerobic deeper soil layer

with drainage water (Figure 1h) might enhance N_2O production by denitrification [29,30] and caused N_2O flux peaks during renovation (Figure 1f). Limin et al. [25] showed the significant contribution of root litter production during summer to winter on carbon stock in permanent managed grassland in the same region as this study. This suggests significant soil N uptake and accumulation into living root and root litter from summer to winter in permanent managed grassland.

The leachate NO_3^- -N concentration increased during the freezing period in the first year (Figure 1g) and decreased after thawing, followed by increased N_2O fluxes for about one month. A high contribution of N_2O to annual N_2O emission in the freezing-thawing period was reported in the grassland-corn rotation [13] and renovated grassland [9]. The physical release and/or new production of N_2O are known as measured pathways of N_2O burst in the thawing period [31]. N_2O production and accumulation in the freezing period [32] and immediate N_2O diffusion in the thawing period were shown [22] on Andosols in Hokkaido, Japan. Müller et al. [33] found that ^{15}N -fertilizer was initially immobilized, but $^{15}\text{NO}_3^-$ -N concentration was increased during freezing and then decreased with the increasing of $^{15}\text{N}_2\text{O}$ fluxes during the thawing period in Germany grassland soil. Immobilized $^{15}\text{NO}_3^-$ -N became available through the death of microbes and the leakage of intracellular materials [34,35]. The patterns of temporal NO_3^- -N concentration and N_2O flux change during freezing and thawing (Figure 1f,g) are similar to the results of Müller et al. [33]. This indicates that newly produced N_2O from increased NO_3^- -N after thawing might have contributed largely to one month of continued high N_2O fluxes (Figure 1g) in our study. In both the renovation and thawing period, NO_3^- -N leaching was initially increased, followed by N_2O flux peaks. This indicates that N_2O production in subsoil induced by NO_3^- -N leaching from topsoil might also have an important contribution to N_2O emissions that are associated with grassland renovation and thawing in this study.

In the second year, N_2O fluxes had a significant positive correlation with soil temperature, soil NH_4^+ -N concentration, and the sum of soil NH_4^+ -N and NO_3^- -N concentration, but had no correlation with leachate NO_3^- -N concentration (Table 3). This suggests that only surface soil mineral N concentration is a factor inducing increased N_2O fluxes through nitrification and denitrification, with little contribution of N_2O production to deeper soil linking with NO_3^- -N leaching, as shown in the first year. In the third year, a significant positive correlation was observed only between N_2O flux and leachate NO_3^- -N concentration (Table 3) because of increased N_2O flux and leachate NO_3^- -N concentration during thawing and after chemical fertilizer application in July (Figure 1f,g). In the growing season in the third year, a large N_2O flux peak in F treatment was observed on 21 July 2016, while a leachate NO_3^- -N concentration peak in F was observed on 3 August 2016. These dates were nine and 22 days after chemical fertilization (12 July 2016). These results indicate that the large N_2O flux in F in the third year was induced by N_2O production in topsoil before NO_3^- -N leaching [28]. Gradual infiltration by matrix flow in topsoil [27] could be explained by the 13-day gap between the peaks the N_2O flux and leachate NO_3^- -N concentration. Cumulative precipitation in the first and second harvest periods in the third year (1138 mm for 90 days and 831 mm for 56 days, respectively) was larger than in the second year (388 mm for 60 days and 73 mm for 47 days, respectively) (Figure 1a,h). Heavy rain in the third year might have enhanced N_2O production that is associated with NO_3^- -N infiltration in topsoil in the third year rather than the second year.

4.2. Annual N_2O Emissions and Nitrate Leaching in Renovated Grassland

Annual N_2O emissions in the first year were 3.47–6.58 times larger than that in the second year (Table 4). Our results of N_2O emissions in the first year were 3.8–7.3 times higher than the mean values of N_2O emission in the Hokkaido region, while our results in the second year were almost the same as the mean values in this region [11] (Figure 4). Several previous studies comparing N_2O emissions in renovated and no-renovated treatments at the same time reported 1.3–12.1 times larger N_2O emissions in renovated treatment than no-renovated treatment in annual measurements [3,7,8], and similarly, 1.3–12.0 times higher in renovated treatment than no-renovated treatment in short-term (7–24 weeks) measurements [2,6,36].

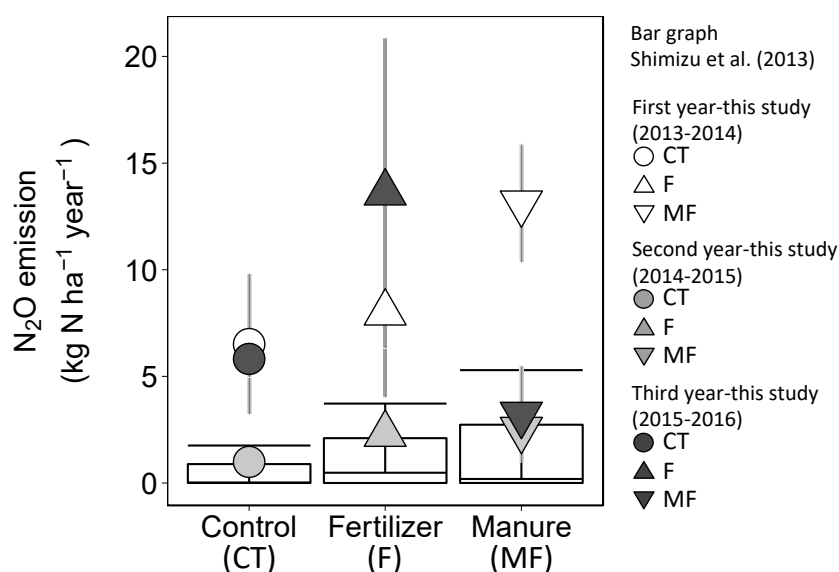


Figure 4. Comparison of annual N_2O emission in this study and Shimizu et al. [11]. CT is unfertilized control plots ($\circ$); F is chemical fertilizer plots ($\triangle$); MF is combined manure and chemical fertilizer plots (∇). Bar graph shows mean value of annual N_2O emissions from across three permanent managed grasslands on different Andosols in Hokkaido region, Japan, reported by Shimizu et al. [11]. The management method of treatments in this study (CT, F, and MF) is following Shimizu et al. [11] (Control, Fertilizer, and Manure, respectively). Error bar shows standard deviation.

Annual NO_3^- -N leaching in the first year was 11.1–36.2 times larger than that in the second year (Table 4). NO_3^- -N leaching in renovated and no-renovated treatments were compared in several studies using lysimeters [37,38] and porous ceramic suction cups [2,10]. They reported that the cumulative NO_3^- -N leaching in renovated treatment was 0.9–63 times larger than no-renovated treatment in the first year after renovation [2,10,37,38]. In the second year, the difference of NO_3^- -N leaching between renovated and no-renovated treatments became smaller (0.2–2.0 times larger in renovated treatments) [2,10]. Although a possibility of difference exists on NO_3^- -N concentration in drainage water among instruments and depth for correcting samples [23], increased cumulative NO_3^- -N leaching that is associated with grassland renovation in our study is in the similar range of that in previous studies [2,10,37,38].

As discussed in 4.1, excess NO_3^- -N is associated with herbicide application and thawing and might enhance N_2O production in deeper soil in the first year, while N_2O was mostly produced within the topsoil in the third year associated with the chemical fertilizer application and rainfall. This can be seen in the relationship between cumulative N_2O mission and NO_3^- -N leaching. The positive correlation between N_2O emission and NO_3^- -N leaching (Figure 2) primarily reflect that both of N_2O and NO_3^- -N leaching were from the same size of mineral N pool in each treatment in each year. Additionally, it also might indicate that the amount of NO_3^- -N that is leached from topsoil to subsoil regulate the amount of N_2O production in the anaerobic spot during infiltration. However, annual N_2O emission against annual NO_3^- -N leaching in the F treatment in the third year was higher than in other treatments and years, because N_2O was mostly produced within the topsoil.

In the first year after renovation, the absence of plant N uptake led the temporal mineral N accumulation in surface soil, and some parts of them were lose by N_2O emission and NO_3^- -N leaching from soil. In addition to mineral N release from soil, the amount of mineral N release from manure was strongly increased the surplus mineral N (Table 5), N_2O emission, and NO_3^- -N leaching in MF treatment (Figure 3). In this study field, MF treatment has a significantly higher N content in soil than F treatment due to eight years repeated manure application before this study (Table 1). Therefore, not only currently applied manure N but also accumulated N in soil that is caused by historical repeated manure application might contribute to increase surplus mineral N, N_2O emission,

and NO_3^- -N leaching in MF treatment. In contrast, increased above ground plant N uptakes and smaller GM_{soil} might reduce surplus mineral N, N_2O emission, and NO_3^- -N leaching in the second year (Figure 3).

N_2O emission in the third year was 1.28–5.91 larger than in the second year (Table 4). N_2O emission in the third year might also be induced by larger mineral N surplus in the third year than other years (Figure 3) given small plant N uptakes in the third year (Table 5). Furthermore, annual precipitation in the third year (1711 mm) was larger than in the first and second years (1331 and 1062 mm, respectively) because of heavy rain in June to August 2016. Both the large mineral N surplus and heavy precipitation in the third year might increase N_2O emission in the Andosol [11,39].

In the third year, annual NO_3^- -N leaching was not different among the treatments, but annual N_2O emission in F was 2.34 times larger than in CT and 4.23 times larger than in MF treatment (Table 4). The clear chemical fertilizer that induced N_2O emission was seen in F treatment in the third year, and it might have been enhanced under heavier rain conditions [11] in the third year. In MF treatment, the amount of chemical fertilizer application was reduced (Table 2) by taking N mineralization from both the current and previously repeated manure application into account. The reduction of chemical fertilizer N in MF treatment could have reduced chemical fertilizer-induced N_2O emission in MF treatment as compared with F treatment in grassland [14].

5. Conclusions

The mineral N release from manure increased the N_2O emission and NO_3^- -N leaching in the first year after grassland renovation. In the second year, there were no differences in N_2O emission and NO_3^- -N leaching depending on fertilizer management. In the third year, there were also no differences in NO_3^- -N leaching depending on fertilizer management, while N_2O emission in F treatment was higher than in MF treatment. The little plant N uptake after renovation enhanced N_2O emission and NO_3^- -N leaching from manure mineralized N. N_2O production in anaerobic deeper soil following with NO_3^- -N leaching might be a significant source of N_2O emission during grassland renovation. While, the infiltration of chemical fertilizer N into the topsoil might stimulate N_2O emission in the third year after renovation.

Long-term manure application to the grassland could possibly mitigate N_2O emission after two years from grassland renovation; however, the risk of increased NO_3^- -N leaching and N_2O emission induced by manure N release should be noted.

Supplementary Materials: The followings are available online at <http://www.mdpi.com/2073-4433/9/12/485/s1>.

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