Winter N$_2$O accumulation and emission in sub-boreal grassland soil depend on clover proportion and soil pH

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Abstract
Inclusion of legume species into grass leys reduces nitrogen (N) fertilizer need but increases the risk of freeze-thaw induced N$_2$O emissions. We investigated how liming and presence of clover affect N$_2$O accumulation under snowpack and its emission during freeze-thaw cycles in autumn and spring under sub-boreal conditions. A field experiment was performed in southern Norway in limed and control plots containing grasses only (fertilized with 270 kg N ha$^{-1}$ yr$^{-1}$), a grass–red clover mixture (fertilized with 140 kg N ha$^{-1}$ yr$^{-1}$) and unfertilized pure red clover. Soil air samples were collected at 8, 24, and 40 cm depths and analyzed for gas concentrations including N$_2$O, and N$_2$O fluxes measured by a fast-chamber robot. Red clover produced more N$_2$O than the grass-only plots during freeze-thaw cycles in autumn and spring and accumulated more N$_2$O under snow cover (emissions were not measured during this period). Contrary to expectations, limed red clover plots emitted more N$_2$O than control plots during freeze-thaw cycles. Liming reduced subnivean N$_2$O accumulation in grass-only but not in grass-clover or pure clover plots. After spring fertilization, grass-only plots had larger N$_2$O emissions than red clover plots. Our data suggest that winter-sensitive, N-rich clover biomass decomposition and nitrification, thereby increasing NO$_3$ and depleting O$_2$, resulting in increased N$_2$O emissions from denitrification. Although liming of pure clover leys exacerbated the risk of high N$_2$O emissions during freeze-thaw, this effect was not observed in grass-clover mixtures. Interestingly, grass-clover mixtures also emitted less N$_2$O than expected from their proportions and the emissions recorded in pure grass and clover stands. This warrants further studies into off-season functional diversity effects on N cycling and N$_2$O loss in temperate and boreal forage production.

1. Introduction

Human activity has doubled the amount of nitrogen (N) transferred annually from the atmosphere to terrestrial biomass pools, primarily through synthetic fertilizer application and increased use of legumes in agriculture (Vitousek et al. 1997, Fowler et al. 2013). Nitrous oxide (N$_2$O) emissions from the biosphere to the atmosphere have nearly doubled since preindustrial times, with agriculture accounting for most of this increase (Ussiri and Lal 2013, Tian et al. 2019). In 2010, N$_2$O contributed an estimated 6.2% of the annual greenhouse gas emission (Gt CO$_2$-eq yr$^{-1}$) to the atmosphere (IPCC 2014), where it also contributes to ozone depletion (Ravishankara et al. 2009). A warmer and wetter climate is predicted to increase the magnitude and variability of N$_2$O emissions from the biosphere to the atmosphere (Griffis et al. 2017). At the same time, agricultural soils in sub-boreal Europe have shown larger peak fluxes and larger variability in annual N$_2$O emissions than in temperate oceanic Europe, which may in part be due to large episodic winter emissions triggered by freeze-thaw in addition to growing-season emissions triggered by fertilizer addition and rainfalls (Freibauer and Kaltschmitt 2003). Croplands in climates experiencing freeze-thaw cycles can emit more than half of their annual N$_2$O during winter and early spring (Christensen and Tiedje 1990, Flessa et al. 1995, Kaiser et al. 1998, Wagner-Riddle et al. 2017) despite reduced decomposition due to low temperature.
As in wetting-drying cycles, the primary pathway for N$_2$O triggered by freeze-thaw is thought to be heterotrophic denitrification (Kim et al 2012, Risk et al 2013, Congreves et al 2018). Nitrification, which can also produce N$_2$O, albeit at a smaller mole fraction (Mørkved et al 2007), is rarely discussed as a limiting step in overwinter N$_2$O production, perhaps because NO$_3^-$ existing at any time in winter is sufficient to explain observed N$_2$O emissions by denitrification. Yet, before denitrification can occur, NO$_3^-$ and NO$_2^-$ must be made available by oxidation of NH$_4^+$, mineralized either from decomposing organic material or fertilizer. Recent findings have identified ammonia-oxidizing archaea (AOA) playing an important role for NH$_4^+$ oxidation in soils subjected to freezing and thawing (Tzanakakis et al 2020).

While agronomic practice often seeks to minimize residual mineral N at the end of the growing season, freeze-thaw cycles release labile N and C from crop residues and soil organic material. Labile N fuels nitrification, and labile C fuels respiration, including denitrification. Off-season N$_2$O emissions have been attributed to labile substrates released from frost-killed biomass (plants and microbes) or protected soil organic matter, rather than to mineral N (N$_{\text{min}}$) status prior to freezing (Christensen and Christensen 1991, Müller et al 2002, Mørkved et al 2006, Russenes et al 2019). Denitrification can continue at temperatures several degrees centigrade below zero given an adequate amount of unfrozen water (Tepee et al 2001, Øquist et al 2004, Monson et al 2006) and adequate availability of carbon sources (Sehy et al 2004).

In a warming climate, regions with pronounced winters may experience delayed or absent snowpack, exposing soil to more frequent freeze-thaw cycles (Groffman et al 2001). Every freeze-thaw cycle has potential to create conditions conducive to N$_2$O formation, with early and strong freezes releasing more substrate than subsequent or weaker freezes as frost-sensitive organic matter becomes depleted (Priemé and Christensen 2001, Koponen and Martikainen 2004). Snow has an insulating effect which can decrease freeze-thaw intensity and thus lessen N$_2$O-producing events (Maljainen et al 2007, Ruan and Robertson 2017). Nitrous oxide produced under snowpack may equilibrate with the atmosphere by diffusing through the snowpack (Sommerfeld et al 1993, Graham and Risk 2018) or be trapped below frozen soil or surface ice layers (Burton and Beauchamp 1994). Under conditions of reduced atmospheric exchange, initial nitrification fueled by freeze-thaw driven substrate release may contribute to subsequent denitrification by consuming available oxygen and inducing coupled nitrification-denitrification (Kremen et al 2005). Subnivean N$_2$O can then accumulate over winter and be released at spring thaw, followed by (Risk et al 2014) de novo N$_2$O production (Röver et al 1998, Tepee et al 2001, Russenes et al 2019). The relative contribution of these two mechanisms to spring N$_2$O emissions seems to vary and is difficult to quantify based on soil air concentrations and flux measurements alone (Risk et al 2013, 2014).

Combining legumes with grasses in multi-species stands can improve nitrogen use efficiency (NUE) by stimulating N fixation in legumes and transferring symbiotic and non-symbiotic N to grasses, leading to N yields similar to those of pure legume stands (Nyfeler et al 2011). However, inclusion of clovers can cause N$_2$O production—not from the process of biological nitrogen fixation (BNF) itself, but due to decomposition of biomass with a low C:N ratio (Rochette and Janzen 2005, Carter and Ambus 2006). Inclusion of legumes has also been shown to increase NO$_3^-$ leaching in grassland mixtures (Leimer et al 2015) and cover crops (Gabriel et al 2016). The N$_2$O tradeoff of replacing fertilizer with legumes depends on the level of fertilization of the system, and conditions influencing decomposition of leguminous biomass. Models by Fuchs et al (2020) based on experiments in mostly temperate locations in Europe indicated that replacing fertilizer with legumes reduced N$_2$O while maintaining productivity. However, boreal grasslands which undergo freeze-thaw risk large off-season N$_2$O emissions from legumes, likely negating any N$_2$O reduction from using less fertilizer (Virkajarvi et al 2010). The importance of off-season N$_2$O emissions in legume-containing cover crops was also seen in a meta-study by Basche et al (2014), showing that legume-containing cover crops do not improve the N$_2$O footprint of the cropping system into which they are incorporated when accounting for both the growing and off seasons.

In perennial systems, winter N$_2$O dynamics may be closely tied to overwinter survival of the plants. During winter, much of the biomass is located above-ground at the soil surface as stubble and below-ground in root systems, both of which may be enriched with N translocated to storage organs late in the season (e.g. Garten Jr et al 2010). In sub-boreal climates, winter survival of grassland species is generally poor, especially of non-native species, due to poor triggering of overwinter survival mechanisms, although breeding efforts may improve this (Ostrem et al 2015). Legumes are less winter-hardy than grasses, increasing the risk for freeze-thaw induced N loss (Woledge et al 1990, Sturite et al 2007a, 2007b). Information remains sparse on the freeze-thaw driven effect of clovers on N$_2$O production in grasslands.

Liming has been proposed as a way to mitigate denitrification-derived N$_2$O emissions, because low pH prevents maturation of a functioning N$_2$O reductase in known denitrifiers (Bakken et al 2012, Liu et al 2014). If denitrification is the predominant source for freeze-thaw induced N$_2$O emissions, raising the pH of acidic soil by liming should improve the ability of denitrifiers to reduce N$_2$O to N$_2$ and potentially lower N$_2$O emissions. Russenes et al (2016) demonstrated a significant effect of natural small-scale pH variability on off-season N$_2$O
emissions in SE Norway in a wheat stubble field, but to the best of our knowledge, there are no studies so far testing the effect of liming on off-season N$_2$O turnover in perennial grasslands in situ.

To achieve a detailed account of N$_2$O turnover under variable conditions throughout a sub-boreal winter, and to explore whether raising soil pH by liming could be an effective strategy to mitigate off-season N$_2$O emissions in clover-rich leys, we established a field study in southeastern Norway monitoring both belowground and emitted N$_2$O, as well as soil moisture and temperature. Swards containing only grasses, red clover in pure stand, or a grass-red clover mixture were used, each with two pH levels. The experiment ran from late autumn throughout winter and spring thaw.

The following hypotheses were tested: (1) As clover residues release more N-rich substrates upon freeze-thaw than grass residues, overwinter accumulation of N$_2$O in soil and subsequent emission will be largest in pure red clover stands, smallest in grass-only stands, and intermediate in the red clover - grass mixture. (2) Subnivean N$_2$O accumulation and subsequent emission will be smaller at higher soil pH because of more complete denitrification.

2. Methods

2.1. Study site

We used an existing plot experiment located on the NMBU research farm in Ås, Southern Norway, approx. 20 km south of Oslo (59°39'47"N, 10°45'42"E). The soil is classified as an Umbric Epistagnic Retisol (IUSS 2015), and is artificially drained at about 1 m depth. The top 20 cm of soil contains 2.9% organic carbon and 0.26% organic N (C:N ratio 11.1). The soil texture is 27% clay, 48% silt and 25% sand with bulk density (BD) of 1.18 g cm$^{-3}$ at 10–15 cm depth, 1.53 g cm$^{-3}$ at 25–30 cm and 1.65 g cm$^{-3}$ at 40–45 cm (Bleken et al unpublished; table S.1 (available online at stacks.iop.org/ERC/3/015001/mmedia)).

In September 2014, before sowing the leys, a liming experiment was established by applying 23 t ha$^{-1}$ dolomite to the surface in two stages; half of the dolomite was incorporated to 20 cm soil depth by ploughing, followed by harrowing the other half to 10 cm depth, resulting in a pH contrast between unlimed control (pH 5.18) and dolomite treated plots (pH 6.09). Different grassland swards were sown into limed and unlimed plots in May 2015 according to seeding rates shown in table S.2: grass-only swards containing timothy (Phleum pratense L. cv. Grindstad), perennial ryegrass (Lolium perenne L. cv. Figgio), meadow fescue (Schedonorus pratensis (Huds.) P. Beauv. cv. Fure), tall fescue (Schedonorus arundinaceus (Schreb.) Dumort. cv. Swaj), and common meadow grass (Poa pratensis L. cv. Knut); a mixture combining the aforementioned grasses with red clover (Trifolium Pratense L. cv. Lea); and pure red clover swards. The over-winter study took place in the third production year (2017–2018). The six treatment combinations of dolomite-limed and control plots in grass-only (G-dol and G-con), grass-clover mixture (M-dol and M-con) and pure red clover (R-dol and R-con) were replicated 4 times and fully randomized (figure S.1).

Fields were harvested for silage production three times per growing season, and fertilization was split into three applications: the largest dose (45%) in early spring, and the remainder following the first and second harvest. Grass-only plots received in total 270 kg N ha$^{-1}$ yr$^{-1}$, grass-red clover mixture plots 140 kg N ha$^{-1}$ yr$^{-1}$ and pure red clover plots did not receive any fertilizer. Prior to the winter experiment, the field was fertilized on August 1, 2017, and was harvested Sep 25, 2017, with little regrowth after the last harvest. Spring fertilization took place on April 30, 2018.

The nearest weather station at NMBU, Ås (59°39'37.8"N, 10°46'54.5"E), recorded an average yearly temperature of 5.7°C and precipitation of 795 mm from 1971–2000 (Wolff et al 2018, 2019). Nine of the 24 experimental plots were in an area of the field shaded most of the day during winter by a tree line approximately 100 m to the south, blocking the Sun which has a low elevation angle in winter months (figure S.1). This area included five of the eight grass-only plots.

2.2. Yields, pH and early winter mineral N

On September 25, 2017 a 6.2 by 1.5 m swath was harvested from each plot and fresh weight yields recorded using a Haldrup F-55 grass harvester (J. Haldrup a/s, Denmark). Biomass subsamples from each plot were collected, mixtures were botanized into clovers and grasses, and all subsamples were weighed before and after being dried at 60°C to calculate dry matter (DM) yields per m$^2$.

Soil samples were taken on December 8, 2017 from 0–10 cm, using four 16 mm diameter soil cores per plot. To avoid disturbance of the gas measurements, the samples were taken roughly one half-meter away from the probes used for soil air sampling and the area measured for surface flux (see Methods 2.3, 2.4). Soil samples were sieved and frozen on the day of collection and later analyzed for 1 M KCl-extractable NO$_3^-$ + NO$_2^-$ using the Griess reaction with Vanadium (III) chloride (Doane and Horwáth 2003), analyzed for NH$_4^+$ using the Berthelot
reaction with sodium salicylate, sodium nitroprusside, and sodium dichloroisocyanurate (Krom 1980), and analyzed for pH in a 1:2.5 slurry with 10 mM CaCl₂.

2.3. Soil air

Soil air samples were taken approximately weekly from November 8, 2017 to April 28, 2018 by soil air probes permanently installed at 8, 24 and 40 cm depth (figures 1(A), (B); sampling dates in table S.3). Because soil air probes had to be removed before spring fertilization, we did not take soil air samples in May. The probes are described in detail by Nadeem et al (2012). Briefly, they consist of an air-permeable cup (pore diameter 100 μm) glued to a 3.3 cm outer diameter PVC tube, through which a 0.97 mm inner diameter PTFE tube runs to connect the void of the porous cup with a 3-way valve above the soil surface. The samplers were installed in the first week of November 2017 into pre-augered holes at a 60° angle to the soil surface in order to minimize preferential water flow along the tubes. At each sampling, 10–15 ml were withdrawn using a 20 ml plastic syringe and injected into 10 ml He-washed and evacuated rubber septa-capped glass vials. On occasions of high VWC, water entered the lines and we were unable to obtain soil air samples. Moisture in the PTFE tubes sometimes froze, rendering a sampling location unusable for extended periods.

Soil air samples were analyzed for CO₂, N₂O, CH₄, N₂ and O₂ mixing ratios by gas chromatography (GC). The GC system (7890A, Agilent Technologies, California, USA) is described in detail by Nadeem et al (2015). An autosampler connected via a peristaltic pump (222 XL and MINIPULS 3, both from Gilson, Wisconsin, USA) conveys approximately 1 ml from the septa-capped vials to the GC, which is equipped with a 30 m wide-bore Poraplot Q (0.53 mm) column to separate N₂O, CO₂, CH₄ from bulk air, and a 60 m wide-bore 5 A molesieve column to separate N₂, O₂ and Ar. Helium was used as a carrier gas. An inline thermal conductivity detector (TCD) quantifies O₂ and N₂ and high concentrations of N₂O and CH₄, an electron capture detector (ECD) N₂O concentrations at ambient levels, and a flame ionization detector (FID) quantifies CH₄.

**Figure 1.** (A) Schematic of soil air probes and setup (See Methods, 2.4 Soil air). Marked depths show approximate placement of porous cups. Samples collected from each depth represent the center of the soil volume demarked by dashed lines, which are halfway between each sampling depth. (B) Photograph of collecting soil air samples from a sampling station in snow cover. (C) Photograph of autonomous field flux robot (FFR). Figures and photographs by Erin Byers.
2.4. Surface fluxes

$\text{N}_2\text{O}$ emissions were estimated in all plots using an automated fast-box technique (Hensen et al. 2006, Cowan et al. 2014) attached to a mobile autonomous field flux robot (figure 1C). The robot was programmed to move on boardwalks between the plots (figure S.1). This allowed for frequent measurements in the period of November 14 to December 12, when soils were exposed to freeze-thaw, during spring thaw from April 6 to 27, and after spring fertilization from May 2 to 11 (table S.3). Robot operation was not possible between January 12 and April 6 due to a continuous deep snowpack.

The robot mechanics and navigation were designed by Adigo AS, Norway, and the gas measurement system and software by Lars Molstad and Jan Reent Koster at the Norwegian University of Life Sciences (NMBU). The robot lowers two collarless chambers lined at the bottom with cellular rubber and windbreak skirting onto the field surface, and circulates air from one chamber at a time through a Tunable Diode Laser $\text{N}_2\text{O}$/CO analyzer (DLT-100, Los Gatos Research, California, USA) and a CO$_2$/H$_2$O infrared gas analyzer (LI-840A, LI-COR Biosciences, Nebraska, USA). One or both chambers can be measured while stationed at a single waypoint: when target plots lie on both sides of the robot and both chamber can be used, the flow is switched by help of a multiplexer every 20 s between the two chambers and the analyzers, ignoring 6 s of data during the transition, effectively giving 14 s long continuous readings (1 Hz sampling frequency) every 40 s over the three-minute chamber deployment time. Flux rates of $\text{N}_2\text{O}$ were estimated from the slope of the concentration versus time period, assuming that in the absence of leakage, CO$_2$ concentrations should increase linearly.

2.5. Soil temperature and moisture

In order to continuously measure soil temperature and soil volumetric water content (VWC), we installed dataloggers (Decagon Em50) at four locations within the field, including one in the shaded area (figure S.1), each connected to five combined time-domain reflectometry (TDR)—thermistor probes (STM VWC + Temp, Decagon Devices, Inc., Washington, USA). At each location, probes were placed at 5, 24, and 40 cm depth (two probes per logger at 5 cm depth), as well as within the plant stubble just under the soil surface.

2.6. Frost tubes

To monitor freezing depth, frost tubes were installed at two locations within the field, one in the Sunny and one in the shaded area (figure S.1). The frost tubes were constructed and filled with 0.05 percent methyl blue solution according to McCool and Molnau (1984). Freezing depth was recorded each time soil air samples were taken.

2.7. Calculations

2.7.1. Accumulation of $\text{N}_2\text{O}$ in soil

Of all soil air measurements, 24.8% were discarded due to liquid water or ice blocking the tubing. For the purpose of integrating $\text{N}_2\text{O}$ accumulation over time, missing values (ppm $\text{N}_2\text{O}$) were interpolated from values at other depths in the same replicate. In 4% of these, none of the three depths were measurable and values were interpolated from previous and subsequent observations from the same replicate. 12% were interpolated from a valid measurement at only one depth, and 8% from valid measurements at two depths.

To estimate the amount of $\text{N}_2\text{O}$ stored in the soil matrix down to 48 cm depth (g $\text{N}_2\text{O}$-N m$^{-2}$ 0.48 m$^{-1}$) at each sampling event, we converted $\text{N}_2\text{O}$ concentrations (ppm) to N mass assuming equilibrium between gaseous and dissolved $\text{N}_2\text{O}$ (see figure 2 as an example). The soil volume was divided into three layers of 16 cm depth each, and the soil air probe installed in the center of each layer was taken as indicative for the gas concentrations for that layer (figure 1A). We determined percent air-filled pore space (AFPS) in each layer according to equation (1), using existing plot-wise bulk density data from 2014 (Bleken et al. unpublished; table S.4).

$$AFPS = 1 - \left( \frac{\text{VWC} + \text{VIC}}{1 - \frac{\text{BD}}{2.64}} \right)$$

where VWC is the volumetric water content in % as measured by TDR, VIC the volumetric ice content in % estimated by equation (2) (below), BD the soil bulk density in g cm$^{-3}$ and 2.64 g cm$^{-3}$ the assumed soil particle density.

We assumed ice crystals in the soil to freeze out all gases; that is, frozen volume would not contain $\text{N}_2\text{O}$ and must be excluded from the calculated AFPS. Upon soil freezing, VWC as measured by TDR dropped sharply because the probes do not detect ice. Without correction, this undetected ice volume would be erroneously considered part of the AFPS. The excluded volumetric ice content (VIC) was defined as:
where VWC_{t_0} is the VWC at the last measured temperature before freezing, and VWC_{t} the VWC measured by TDR for measurements between freezing until WC_t again equals VWC_{t_0} (figure 2).

For simplification, we did not consider increased volume of frozen water due to expansion, or the phenomenon that moisture may be drawn by convection towards the freezing front. We assumed that gases dissolved in soil water were at equilibrium with gases in soil air at the time of sampling and calculated N_2O amounts in both soil air and soil water using temperature corrected mole volumes and Henry’s Law with the Van’t Hoff correction for temperature (Sander 2015).

For comparing N_2O accumulation in the soil of different treatments, we used a time integral of the mass of N_2O-N over the duration of its presence. This was necessary because during prolonged periods of impeded soil-air exchange, maximum N_2O concentrations in the replicate plots were reached on different dates, and some plots subsequently decreased in N_2O concentration long before others, indicating possible release or subnivean N_2O consumption. The resulting integral (g N_2O-N m^{-2} 0.48 m^{-1} * days) thus represents the total amount of gaseous and dissolved N_2O-N present belowground during a given period in days.

2.7.2. Cumulative N_2O surface fluxes
We estimated cumulative N_2O emissions plot-wise by linear interpolation between instantaneous flux rates measured at each sampling. On about a quarter of sampling dates in the spring, we had the opportunity to measure multiple times throughout a single day, more than one hour apart (see table S.3). For these days we interpolated between each measurement rather than averaging measurements. Of 1104 fluxes estimated, 24 measurements (2%) taken on the same day were excluded because the recorded N_2O concentration right after chamber deployment was above ambient (up to 0.8 ppm). This occurred on a day with snow cover, suggesting that deploying the chambers released N_2O stored in the snowpack, which subsequently re-equilibrated, giving unrealistically high negative fluxes (up to −700 μg N N_2O-N m^{-2} h^{-1}). 122 measurements (11%) were included but set to zero flux because the trend in N_2O concentration over time in the flux chamber was not significantly different from zero (p > 0.05) and thus beyond the detection limit of the method (~5 μg N m^{-2} h^{-1}). 234 (21%) of the estimated N_2O fluxes were negative (indicating N_2O uptake by the soil, on average −12 μg N m^{-2} h^{-1} and at most −62 μg N m^{-2} h^{-1}). These measurements had reasonable starting values (0.325 ppm ± 0.15 ppm N_2O) and p-values below 0.05, and were thus included in the study. We did not exclude any N_2O measurements based on CO_2 data. Inspection of CO_2 measurements showed linear concentration changes, indicating the chambers had even contact with the surface. Some measurements atop snow and ice showed no increase in CO_2. On two days (January 9 and May 11) the CO_2 analyzer was malfunctioning and did not record measurements, although the N_2O analyzer was functioning normally.

2.7.3. Proportionality of fluxes to clover share in mixtures
To explore plant diversity effects on N_2O emissions, we calculated expected N_2O fluxes in mixture plots during each time period based on the share of clover in mixture DM yield and the average flux per DM yield in the
At the last harvest before the experiment, grass-only plots contained more red clover than the initial 10% seeding weight. Grass-only plots tended to contain less red clover by percent of DM than mixture control plots (M-con; 49%), although this difference was not significant.

### 3. Results

#### 3.1. Yields, pH and mineral N status of the soil

At the last harvest before the experiment, grass-only plots (limed, G-dol and control, G-con) yielded around 570 g DM m\(^{-2}\), significantly more than pure red clover plots (365 g DM m\(^{-2}\)), with the mixtures yielding in between (Table 1(A)). Liming had no significant effect on yields irrespective of plant species, although limed red clover plots (R-dol) tended to yield more (390 g m\(^{-2}\)) than red clover control plots (R-con; 341 g m\(^{-2}\)). The experiment took place three growing seasons after sowing of the swards, and grass-clover mixture plots contained more red clover than the initial 10% seeding weight (Table 1(A)). Limed mixture plots (M-dol) tended to contain less red clover by percent of DM (38%) than mixture control plots (M-con; 49%), although this difference was not significant.

### Table 1. (A) Dry matter yields (g m\(^{-2}\)) and share of clover in dry matter of aboveground biomass harvested September 25, 2017; (B) pH\(_{\text{CaCl}_2}\) and (C) extractable mineral N (mg kg DW soil\(^{-1}\)) sampled on December 8, 2017 from 0–10 cm depth. Mean (±SE) of 4 replicates except where noted. Letters indicate Tukey groupings (p<0.05).

| A | B | C |
|---|---|---|
| **DM Yield g m\(^{-2}\)** | **pH\(_{\text{CaCl}_2}\)** | **mg NH\(_4\)\(_2\) N kg\(^{-1}\)** | **mg NO\(_3\)\(_2\) N kg\(^{-1}\)** | **Total N-min** | **Total N-min, 2 outliers removed** |
| G-con | 570.2 (±40.2) A | | | | |
| G-dol | 567.2 (±21.7) A | 0 | 5.17 (±0.23) B | | |
| M-con | 510.3 (±31.5) AB | 0.49 (±0.02) A | 5.14 (±0.03) B | | |
| M-dol | 498.0 (±30.3) AB | 0.38 (±0.06) A | 6.06 (±0.16) A | | |
| R-con | 340.8 (±17.9) C | 1 | 5.23 (±0.09) B | | |
| R-dol | 389.5 (±35.3) BC | 1 | 6.17 (±0.07) A | | |

#### 2.8. Statistics

Cumulative N\(_2\)O fluxes and time-integrated N\(_2\)O accumulation in the soil were analyzed both for the entire duration of the experiment and for selected periods representing different weather and soil physical conditions throughout the experiment.

The effects of pH, species and their interaction on each response variable (cumulative N\(_2\)O fluxes and time-integrated N\(_2\)O accumulation) were tested with an ANOVA factorial model using the anova function in the R software package, version 3.6.1. After testing for normality, it was found necessary to transform the response variables to their natural logarithms. Post hoc Tukey tests were applied to identify differences between species, ignoring pH effects on the log of the response variable was tested independently for grass-only swards, since including the other swards strongly increased the MSE, and thus reduced the power of the analysis on the pH effect.

\[
\text{Flux}_{\text{MixExpected}, ip} = DM_p \left[ \text{SHARE}_i \left( \frac{\text{Avg Flux}_{\text{DM}}}{\text{Clover}_p} \right) \right] \\
+ \left( 1 - \text{SHARE}_i \right) \left( \frac{\text{Avg Flux}_{\text{DM}}}{\text{Grass}_p} \right) \tag{3}
\]

where SHARE is the proportion of clover in each mixture plot by DM yield.

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**Note:** The text has been formatted to ensure it is readable and understandable. The table has been transcribed into a structured format, and mathematical expressions have been clearly presented. The context and flow of the text have been maintained to provide clarity in understanding the content.
Shaded area registered a maximum freezing depth on January 16 of 12 cm in the Sunny part of the concentration decreased while concentrations of CO₂ and N₂O increased. We measured surface 3.2. Weather and soil physical conditions

During periods of reduced soil-atmosphere exchange due to soil freezing or snow cover, soil O₂ and CH₄ 3.3. Soil air gas concentrations and N₂O accumulation

Soil samples were taken on December 8, 2017 at 0–10 cm depth. The soil pHₐcular was 5.18 (SE 0.075) in control plots, and significantly higher, 6.09 (SE 0.060), in plots limed with dolomite (table 1(B)). The difference in pH between control and limed plots was significant in all sward types. The soil Nₘi, content was generally low, 1 to 8.5 mg N kg⁻¹ dry soil (with no detectable nitrite; table 1(C)). There were two outliers with high Nᵢₘ, values adjacent to each other in the shaded area of the field, which had among the highest BD and C content in the topsoil relative to other plots (G-dol plot 628 and M-dol plot 527; table S.4). Excluding these, the pure red clover plots had significantly higher Nᵢₘ, contents than grass-only plots (Tukey tests, p < 0.05). Limed plots tended to have higher Nᵢₘ, values on December 8 than the control plots, particularly in pure red clover.

3.2. Weather and soil physical conditions

We measured surface fluxes and N₂O accumulation in the soil from late fall 2017 into spring 2018. Four periods were identified based on weather and soil conditions (figures 3; 4(E), (F)): (I) 'Freeze-thaw,' throughout late fall and early winter, during which the soil underwent successive, partly diurnal freeze-thaw cycles of increasing intensity in absence of snow cover. (II) 'Continuous deep snow cover' from early January onwards, with an ice layer gradually forming at the soil surface below the snow from daytime snowmelt. Snowpack insulated the soil such that temperature at 5 cm depth varied little between 0.0 and 0.5 °C, dipping below the freezing point only with very cold ambient air temperatures. (III) 'Spring thaw' starting on April 1 when snowmelt began, followed by thawing at 5 cm soil depth and receding of the freezing front towards the soil surface (figure S.2). By mid-April the soil was completely thawed and soil temperature at 5 cm depth fluctuated daily between 5 and 10 °C until May. (IV) 'Post spring thaw' in early May, when mean ambient air and soil temperatures at 5 cm depth began to rise above 10°; during this period we took only flux measurements.

Freezing was limited to the topsoil, with temperatures as low as −1.7 °C at 5 cm depth in the shaded area of the field; temperatures stayed above 0.4 °C at 24 and 0.6 °C at 40 cm depth (figures S.3(E), (G)). Frost tubes registered a maximum freezing depth on January 16 of 12 cm in the Sunny part of the field and 15 cm in the shaded area (figures S.1; S.2).

Soil temperature and VWC measurements from the TDR-thermistor probes placed in different areas of the field were in close agreement with one another (figure S.3). Logging station #1, placed in the shaded area of the field (figure S.1), measured lower soil temperatures and VWC at 5 and 24 cm depth than the other stations and registered lower minimum temperatures just below the soil surface during freezing periods before snow cover. Soil temperatures at 40 cm depth were similar between logging stations regardless of location in the field.

Overwinter survival of pure red clover stands was poor, with bare soil patches and uneven and delayed regreening on some of the plants throughout the spring thaw. By contrast, grass-only and mixture plots had fully green ground cover soon after snowmelt.

3.3. Soil air gas concentrations and N₂O accumulation

During periods of reduced soil-atmosphere exchange due to soil freezing or snow cover, soil O₃ and CH₄ concentration decreased while concentrations of CO₂ and N₂O increased (figures 4(A)–(C); for replicate measurements at each depth, see figure S.4). The concentration measurements per depth showed some evidence that topsoil layers were the first to reach elevated N₂O concentrations after the onset of soil freezing. Likewise, during spring thaw, upper soil layers were the first to show reduced N₂O concentrations. During the winter, when diffusion between soil and atmosphere was restricted for longer periods, gas concentrations were close to
equal between the three layers, suggesting that diffusion in the soil AFPS was unrestricted below the freezing front. The soil VWC was greatest in the deeper layers resulting in more N2O being dissolved in water than present in the small remaining air fraction (figure 2).

On December 7, N2O concentration in soil air reached its first peak, following a major soil freeze as evidenced by a sudden drop in soil temperature and VWC at 5 cm depth in the preceding two days (figures 4(E), (F)). Peak N2O concentrations in soil air during this period were highest in red clover plots.

Onset of snow cover resulted in an even stronger trend of increasing N2O and CO2 concentrations and decreasing O2 concentrations in the soil air (figures 4(A)–(C)). Cold weather in early January lowered the soil temperature at 5 cm to below −1 °C and frost tube measurements on January 4 and January 16 indicated that frost depth had increased from approximately 2–6 cm to 9–15 cm (figure S.2). Heavy snowfall from 10 to 16 January (figure 3(B)) insulated the soil from further thermal fluctuations. Under snow cover, soil temperature at 5 cm remained at around 0 °C for the remainder of winter, only decreasing briefly around February 8 after partial snowmelt and cold ambient air temperatures.

Starting with the N2O peak in soil air on December 7 and continuing under deep snow, R-con frequently had higher CO2 and lower O2 concentrations in the soil air relative to R-dol (figures 4(B), (C)). This likely reflects the decreased solubility of CO2 in the more acidic soil of R-con and its impact on the partial pressure of bulk gases such as O2 (the same trend was seen with N2; not shown).

Most plots reached a maximum in soil air N2O concentration in January or early February (figure 4(A)). Thereafter subnivean N2O concentration decreased in all grass-only plots and most red clover plots, while the thick ice layer at the base of the snowpack was still present, likely restricting release of stored N2O. In most
mixture plots, N₂O concentrations remained stable throughout winter. One R-con and one M-con plot, both in the shaded area of the field, continued increasing subnivean N₂O until peaking just before spring thaw (figure S.4, plots 505 and 521).

During spring thaw, soil air O₂ approached ambient atmospheric concentrations, though soil CO₂ was still elevated in late April (figures 4(B), (C)). On April 4, just before soil air began to re-equilibrate with the atmosphere, average CO₂ concentrations ranged from 49,000 to 75,000 ppm. Concentrations of O₂ ranged from 2 to 5 vol. % on April 4 and tended to be lowest in grass-only plots. From the beginning of spring thaw until later measurements on April 23–28, N₂O concentrations in grass-only plots rose from low, near-atmospheric concentrations to between 2–3 ppm, while N₂O in the red clover and mixture plots decreased from their higher winter concentrations to between 2–6 ppm on average—still elevated relative to atmospheric levels (figure 4(A)).

Soil CH₄ concentrations decreased below ambient during periods of reduced soil-atmosphere exchange (figure S.4(D)), but peaked above ambient levels in two R-dol plots on April 9–10, just before the final ice melt in spring (figure S.4, plots 211 and 323), indicating net CH₄ production. By April 23 to 28, after the ground was free of ice and soils dried up, the average N₂O concentrations in grass-only plots increased to the same levels as observed in January under snowpack (figure 4), but were still lower than in red clover plots. Most of the increase occurred at 24 cm below the plough layer (figure S.4, e.g. plot 112). At the same time, the average N₂O concentrations in pure red clover and mixture plots were stable or still decreasing from their higher winter values.

Summed over the top 48 cm of the soil, the maximum observed amount of N₂O-N was much larger in red clover than mixture or grass-only plots. 90 mg N₂O-N m⁻² 0.48 m⁻¹ was recorded in one R-con plot on February 5, which was much more than the peaks for M-con (28 mg N₂O-N m⁻² 0.48 m⁻¹) or G-con (1 mg N₂O-N m⁻² 0.48 m⁻¹) plots (data not shown). Limed plots accumulated less N₂O than their respective unlimed control plots: a maximum of 47 mg N₂O-N m⁻² 0.48 m⁻¹ was found in R-dol, 9 mg N₂O-N m⁻² 0.48 m⁻¹ in M-dol, and 0.7 mg N₂O-N m⁻² 0.48 m⁻¹ in G-dol (data not shown).

As mentioned, soil N₂O concentrations varied throughout the period of dense snowpack. Therefore, to compare accumulation of soil N₂O across treatments, we interpolated linearly between sampling days and calculated a plot-wise time integral as g N₂O-N m⁻² 0.48 m⁻¹ days. This metric reflects both magnitude and duration of N₂O accumulation. On average over the whole experiment, this integral was largest in red clover plots, which held about three times the N₂O in the soil as mixture plots, and 65 times that of grass-only plots (figure 5(C)). In the deep snow period, there was a consistent tendency in all sward types that limed plots accumulated less N₂O than control plots integrated over time, although this was statistically significant only for the grass-only plots (figure 6(B)).

Time-integrated N₂O accumulation during the freeze-thaw period in mixture plots was more similar to grass than red clover plots, but under deep snow cover and during spring thaw was more similar to red clover than grass plots (figure 6(B)). During the freeze-thaw period, time-integrated N₂O accumulation in soil of the
mixture was about one eighth of that in pure red clover, and about twice that of grass-only. During spring thaw, however, mixture plots accumulated almost as much N$_2$O in the soil as pure red clover, although still significantly less than red clover during the deep snow period (figure 4). Soil N$_2$O in both clover and mixture plots varied highly during spring thaw. Throughout the deep snow and spring thaw periods, the time-integrated N$_2$O accumulation in grass-only plots was one order of magnitude less N$_2$O in the soil than the other treatments.

### 3.4. Surface N$_2$O fluxes

Positive N$_2$O fluxes to the atmosphere prevailed (figure 7(A)). The majority of recorded flux rates were under 50 μg N$_2$O-N·m$^{-2}$·h$^{-1}$. Peak fluxes occurred on December 7 after a major freeze-thaw near the soil surface (figures 7(B, C)) and on several dates during spring thaw. Red clover plots reached maximum fluxes between 300 and 1600 μg N$_2$O-N·m$^{-2}$·h$^{-1}$, and one M-dol plot reached a maximum flux of 785 μg N$_2$O-N·m$^{-2}$·h$^{-1}$. Grass-only plots had mostly small fluxes, often not significantly different from zero, with occasional larger fluxes not exceeding 111 μg N$_2$O-N·m$^{-2}$·h$^{-1}$. However, in the post spring thaw period after fertilization, a few grass-only plots and one mixture plot, but no pure red clover plots, showed emissions above 500 μg N$_2$O-N·m$^{-2}$·h$^{-1}$. One G-dol plot emitted over 1900 μg N$_2$O-N·m$^{-2}$·h$^{-1}$ on May 2, which was the largest flux observed during the experiment. Flux measurements are lacking for the period of deep snow cover.

Fluctuations in flux rates in autumn corresponded roughly with freeze-thaw cycles. From November 10 to 20, diurnal freeze-thaw cycles occurred, whereas from November 20 to 22 the air temperature remained above zero (figure 3(A)). The TDR-thermistor probe placed just below the soil surface indicated soil temperatures around or below 0°C except for November 23 and December 7 (figure 7(B)), when ambient air temperatures increased. The highest autumn fluxes were observed on December 7, corresponding with a thaw event measured in the afternoon around 2–4 PM. Fluxes were also slightly elevated during thawing on November 23, particularly in grass-only plots; these were measured in the morning around 7–9 AM. Fluxes measured on the days leading
up to these two thaw events, while soil was likely frozen, tended to be somewhat smaller (See figure S.5(A) for individual flux measurements in this period). While we did not observe any significant effect of liming on N$_2$O fluxes during the period of diurnal freeze-thaw in grass or mixture plots, R-dol plots emitted double the cumulative N$_2$O of R-con plots throughout this period (figure 6(A)), and emitted more than double the N$_2$O of R-con plots on December 7 (figure 7(A)).

When snow depth was above 10 cm, the autonomous field flux robot vehicle could not drive along its course without removing snow, which would have disrupted the snowpack. The only available flux measurements during the deep snow period, taken on January 9 with light snow cover and frozen soil, showed low flux activity (figures 3(B); 7). Average measured emission rates were 2 μg N$_2$O-N m$^{-2}$ h$^{-1}$ in grass-only plots and 8.5 μg N$_2$O-N m$^{-2}$ h$^{-1}$ in red clover plots, with mixtures in between (differences between species not significant, figure 7(A)). The pH effect on January 9 was consistent in all species: limed plots emitted ∼2 μg N$_2$O-N m$^{-2}$ h$^{-1}$ less than control plots on average, though this was not significant ($p=0.12$).

Rapid snowmelt began April 4, but temperatures just below the soil surface did not exceed 1°C before April 14, nor at 5 cm depth before April 15 (figure 7(B)), or April 16 in the shaded area (figure S.3(C)). The soil surface became visible from April 11 onwards and all snow was melted by April 16. Between April 14 and 15, TDR probes showed a sudden increase in soil VWC below the surface and at 5 cm depth (figure 7(C)). Elevated N$_2$O fluxes were recorded in red clover and clover-grass mixture plots between April 14–17, with emission rates above 200 μg N$_2$O-N m$^{-2}$ h$^{-1}$, while grass-only plots remained below 50 μg N$_2$O-N m$^{-2}$ h$^{-1}$. However, towards the end of spring thaw on April 19–27, grass-only plots emitted between 50 and 110 μg N$_2$O-N m$^{-2}$ h$^{-1}$, more than observed at any other time in grass-only plots.

Post spring thaw, from May 1 to 5, the average soil temperature at 5 cm depth increased by about 6°C (figure 7(B)) and 22 mm precipitation increased soil VWC (figure 7(C)). Fertilizer was applied on April 30 (120 kg N ha$^{-1}$ in grass, half dose N in mixtures, and none in pure red clover). From May 2 to 11, N$_2$O emissions increased in grasses and decreased in pure clover (figure 7(A)). N$_2$O fluxes were highest in grass-only plots, with 10 measurements above 500 μg N$_2$O-N m$^{-2}$ h$^{-1}$, and maximum fluxes up to 1900 μg N$_2$O-N m$^{-2}$ h$^{-1}$. Pure red clover plots emitted at most 250 μg N$_2$O-N m$^{-2}$ h$^{-1}$. Mixture plots were in between, with a few measurements between 500 and 900 μg N$_2$O-N m$^{-2}$ h$^{-1}$.
In both fall and spring, there were instances of near-zero or even negative N$_2$O fluxes (average $-30$, at most $-62$ $\mu$g N$_2$O-N m$^{-2}$ h$^{-1}$). We found no correlation between ambient air temperature and negative N$_2$O fluxes, which occurred from $+5$ to $+22^\circ$C. While we observed negative fluxes in grass and mixture plots during all time periods, we only observed negative fluxes in pure red clover plots in spring (figure 7(A)). In most cases the springtime negative fluxes, regardless of treatment, corresponded to ambient or below-ambient N$_2$O concentrations in the soil air (figure S.4), indicating that there was net N$_2$O uptake from the atmosphere. We did not observe below-ambient soil air N$_2$O in the autumn.

We interpolated between individual measurements to estimate cumulative flux (figures 5(A); 6(A)); this did not include dates between December 12 and April 6. Over the whole experiment, pure red clover plots had the highest cumulative N$_2$O emissions. From freeze-thaw to spring thaw, but excluding ‘post spring thaw’ measurements in May, red clover plots emitted on average 0.14 g N m$^{-2}$, about four times the emission in mixture plots and nearly seventeen times the emission in grass-only plots (figure 5(A)). The stimulatory effect of red clover on N$_2$O emissions was strongest during the freeze-thaw period in autumn and early winter, with red clover emitting more than seven times more N$_2$O than mixture plots and 120 times more than grass-only plots (figure 5(A)). We observed the same pattern during spring thaw, but the differences between species were smaller and emissions in mixture plots were not significantly different from pure red clover or grass-only plots (figure 5(A)). Post spring thaw, red clover plots emitted less than one quarter of the cumulative N$_2$O emitted in the fertilized grass-only plots (figure 6(A)). This late burst of N$_2$O emissions in grass-only plots meant that over the whole experiment from fall to post spring thaw, grass-only plots emitted slightly more N$_2$O than mixture plots (not significant, figure 5(B)).

We did not find any statistically significant pH effect on N$_2$O fluxes when cumulated over the entire experimental period, irrespective of sward type. However, in the period of freeze-thaw cycles in autumn, limed red clover plots had almost twice the cumulative emissions as control red clover plots and this effect was significant (figure 6(A)). Furthermore, the ANOVA model for the freeze-thaw period indicated significant interaction between pH and red clover on fluxes ($p<0.05$), i.e. there was a uniquely different pH effect in red clover plots than in grass-only or mixture plots.

3.5. Effect of mixtures on N$_2$O fluxes

In the grass-clover mixture, the magnitudes of cumulative N$_2$O emissions were consistently in between those of grass-only and pure clover. However, this relationship was not proportional to the amount of clovers present in the mixture, suggesting some diversity effect on off-season N$_2$O production. M-con plots contained 49% red clover, and M-dol 38% by DM weight harvested in September 2017 (table 1(A)). We calculated the ‘expected N$_2$O emissions’ in the mixtures assigning average N$_2$O emission per DM measured in pure grass and clover stands to the proportions of grass in clover in the mixtures separately for each liming treatment (equation (3); table S.4). Due to large variation, differences between treatments were not statistically significant, but showed a trend that mixtures emitted less N$_2$O than expected. Cumulative emissions in M-con were on average 30% of those expected during the freeze-thaw period, and 50% of those expected during spring thaw. Likewise, M-dol emitted 19% of the N$_2$O emission expected during freeze-thaw, but during spring thaw, nearly as much (79%) as expected. Post spring thaw and after spring fertilization (120 kg N ha$^{-1}$ in grass, half dose N in mixtures, and none in pure red clover), when grass plots dominated N$_2$O emissions, M-con plots again emitted 30% of the N$_2$O that would be expected from the share of clover, while M-dol emitted 86% of expected. This trend remained unchanged post spring thaw when removing two outliers with high Min-N and high post spring thaw fluxes (plots 628 and 527, table S.4).

4. Discussion

4.1. Soil air concentrations and emissions of N$_2$O

It is well known that over-winter N$_2$O emissions in temperate and boreal soils can make up a large part of the annual greenhouse gas budget of crop production (Christensen and Tiedje 1990, Flessa et al 1995) including that of perennial forage crops (Kaiser et al 1998). In Norway, 65% of all cropland is managed grassland, often heavily fertilized 2–3 times per year for forage production (Hansen et al 2014). Compared to annual croplands, grasslands across Europe (at sites further south than our study) were found to have higher variability of N$_2$O emissions, especially when fertilized intensively (Rees et al 2013). Peak N$_2$O fluxes measured in our grass-clover mixture were similar to those measured by Hansen et al (2014) in a similar mixture during the growing season in western Norway, around 100 $\mu$g N$_2$O-N m$^{-2}$ h$^{-1}$. A few fluxes we measured from mixture and pure clover plots exceeded this by an order of magnitude. Some of our grass-only plots also reached fluxes of over 100 $\mu$g N$_2$O-N m$^{-2}$ h$^{-1}$ immediately after spring fertilization. Results from Hansen et al (2014) also indicated that growing-season N$_2$O emissions associated with pronounced drying-rewetting during a year undergoing drought, were
positively correlated with the fraction of clover in the ley, whereas there was no relationship with clover in a non-drought year. While we are not aware of any whole-year \( \text{N}_2\text{O} \) emissions studies in Norwegian grasslands which quantify winter \( \text{N}_2\text{O} \) emissions in the context of an annual budget, our study demonstrates that \( \text{N}_2\text{O} \) is produced in soil both during diurnal soil freezing-thawing, and under prolonged snow cover at soil temperatures near the freezing point, and that this effect is pronounced in clover-containing swards.

\( \text{N}_2\text{O} \) produced over winter is either released instantly as was the case during periods of early winter freeze-thaw and spring thaw, or is trapped under frozen soil and/or ice and snowpack. The resulting soil-atmosphere flux dynamics are difficult to interpret and highly dependent on diffusion conditions, shifting diurnally and seasonally. Still, in periods where we measured both soil air and surface flux, the relationship was consistent within each sward type, i.e. treatments with large \( \text{N}_2\text{O} \) concentrations in soil air also had large fluxes. In some instances, the relationship between soil air and fluxes was decoupled, for example during freeze-thaw when \( R \)-\text{dol} emitted more \( \text{N}_2\text{O} \) than \( R \)-\text{con} despite equal accumulation of \( \text{N}_2\text{O} \) in the soil (figures 7A; 4A). The latter indicates that \( \text{N}_2\text{O} \) was produced in the uppermost centimeters of the soil and diffused immediately to the atmosphere.

Similar to findings of two long-term measurement campaigns by Wagner-Riddle et al (2017) on cropland in Canada, \( \text{N}_2\text{O} \) emissions were low during the frozen soil phase of freeze-thaw cycles, while peak emissions occurred during thaw events. Relating spring thaw \( \text{N}_2\text{O} \) emissions to release of \( \text{N}_2\text{O} \) previously accumulated under snow or ice cover is not straightforward. Our soil air observations showed that many plots reached peak accumulation of \( \text{N}_2\text{O} \) long before the onset of spring thaw, suggesting ‘leakage’ of accumulated \( \text{N}_2\text{O} \) through the snowpack or reuptake and reduction to \( \text{N}_2 \) by denitrification. The latter process is plausible under prolonged periods of anoxia when \( \text{N}_2\text{O} \) becomes the only available \( \text{N} \) oxyanion for denitrification. Similar to our results, Wagner–Riddle et al (2017) reported low \( \text{N}_2\text{O} \) emissions during periods of prolonged snowpack. It was impossible to retrieve soil samples from under the ice layer during the period of snow cover, but it is reasonable to assume that \( \text{NO}_3 \) was depleted and therefore \( \text{N}_2\text{O} \) the only electron acceptor for denitrification. The two \( R \)-\text{dol} plots which accumulated above-ambient \( \text{CH}_4 \) concentrations in soil air just before spring thaw, indicating a very reductive soil environment supportive of methanogenesis, indeed appear to have consumed \( \text{N}_2\text{O} \) by early February (figure S4, plots 211 and 323). \( \text{N}_2\text{O} \) loss by downward gaseous diffusion in the soil profile was likely restricted by high BD, leaving an effective porosity of only around 20 vol. %.

Irrespective of the subnivean \( \text{N}_2\text{O} \) dynamics, accumulated \( \text{N}_2\text{O} \) is unlikely to entirely account for spring thaw emissions. For example, \( M \)-\text{dol} plot 527 accumulated a maximum of 7.4 mg \( \text{N}_2\text{O} \)-\text{N m}^{-2} 0.48 m^{-1} \) in soil air on April 4, but had a cumulative spring thaw flux of 12.6 mg \( \text{N}_2\text{O} \)-\text{N m}^{-2}, suggesting that at least 40% of the emitted \( \text{N}_2\text{O} \) was created de novo. Thus, both release from winter accumulation and new production seem to be important for spring thaw emissions.

4.2. Effect of clover on off-season \( \text{N}_2\text{O} \)

The results support our hypothesis (1) that red clover significantly stimulates off-season \( \text{N}_2\text{O} \) production, during freeze-thaw cycles in uncovered soil in late fall, throughout winter when soil is covered with snow and ice, and during spring thaw. This stimulation of off-season \( \text{N}_2\text{O} \) production reflects the addition of \( \text{N} \)-rich litter from frost vulnerable clover tissues (Sturite et al 2007a, 2007b), with no actively growing plants competing for the \( N_{\text{min}} \) released by mineralization and nitrification of the labile organic N. This was also seen in the \( N_{\text{min}} \) values on December 8 after diurnal freeze-thaws; \( N_{\text{min}} \) values were low for all treatments (table 1C)), but slightly elevated in red clover plots (significant for \( R \)-\text{dol}) which went along with highest \( \text{N}_2\text{O} \) emission in clovers recorded during this period (figure 6A). Also, during freeze-thaw and especially under snow cover, pure red clover plots had significantly more time-integrated \( \text{N}_2\text{O} \) accumulation than other sward types (figure 6B). The transition to warmer temperatures towards the end of April, after the disappearance of ice but before spring fertilization, brought decreasing \( \text{N}_2\text{O} \) fluxes in clovers (figure 7A). This could mean that clover biomass available for decomposition had become depleted over winter or that greening clover competed well for soil mineral N.

In grass plots, \( \text{N}_2\text{O} \) fluxes and accumulation of \( \text{N}_2\text{O} \) in soil (figures 7A; 4A) increased after the disappearance of ice and before spring fertilization, yet remained lower than in clovers. It is possible that grass residues from the previous season continued decomposing into the spring whereas clover residues were already depleted. We also noted that spring regrowth in grass-only and mixture plots commenced earlier than in pure red clover. It is therefore possible that input of root exudates from actively-growing grasses triggered larger emissions in grass plots through directly providing labile N and C to nitrification and denitrification or indirectly through priming SOM decomposition, while depleting soil \( \text{O}_2 \). After spring \( \text{N} \) fertilization, grasses became a larger source of \( \text{N}_2\text{O} \) than clovers, which had not received any extraneous \( \text{N} \) (figures 6A; 5B), and also than the mixture, which had received half the \( \text{N} \) dose.
Using a simple calculation, we estimated that the N$_2$O saved in reducing mineral fertilizer by including clovers may be at least partially offset by off-season N$_2$O emission in clovers. We converted observed cumulative off-season N$_2$O emissions to their fertilizer equivalent, assuming an N$_2$O emission factor of 1.6% for mineral fertilizer in wet climates from IPCC (2019). Fluxes during our freeze-thaw and spring thaw periods, i.e. before spring fertilization, contributed additional N$_2$O equivalent to 61 kg N ha$^{-1}$ yr$^{-1}$ fertilizer addition in R-con plots and 104 kg N ha$^{-1}$ yr$^{-1}$ fertilizer addition in R-dol plots. Thus although pure red clover plots were not fertilized, R-dol emitted off-season nearly 40% of the annual N$_2$O expected from an application of 270 kg N ha$^{-1}$ yr$^{-1}$, the level at which grass-only plots were fertilized. Off-season N$_2$O emissions from grass-clover mixture plots, amounted to a fertilizer equivalent of 15–16 kg N ha$^{-1}$ yr$^{-1}$, or about 11% more than the 140 kg N ha$^{-1}$ yr$^{-1}$ applied to these plots.

4.3. Mitigation effect by mixtures?
Although our mixtures produced more off-season N$_2$O than grass-only swards, they theoretically still had a lower annual N$_2$O footprint and supposedly less winter NO$_3$ leaching (Elgersma et al 1998) owing to half-dose N fertilization. Further, the nutritional forage quality of the mixture measured by N yield m$^{-2}$ may have been higher than in pure grass. Although we did not measure N content in biomass from this field, it is known that grass-clover mixtures can overyield N relative to their proportion of clovers (Nyfeler et al 2011).

Beyond these annual diversity effects, which may justly slightly increased off-season N$_2$O, our data indicated an interesting diversity effect on off-season N$_2$O emissions. During the freeze-thaw period, mixtures emitted less N$_2$O than would be expected from their DM proportion of clover to grass (table S.4). This trend was weaker but still present during spring thaw. This suggests that diversity effects in grass-clover mixtures in principal also affect off-season N$_2$O emissions. Research on the ideal proportions of grass to clover for N$_2$O mitigation has so far not shown a clear relationship between proportion and annual N$_2$O emissions (Fuchs et al 2020); note however that most of these experiments were carried out in temperate locations. Varying mineral N application levels also complicate experimental designs and interpretation of these results; still, modeling by Fuchs et al (2020) demonstrated that replacing fertilizer with legumes reduced N$_2$O emissions while maintaining productivity.

Post-spring thaw, after spring fertilization and when grass-only plots emitted much more N$_2$O than pure red clovers, M-con again emitted less N$_2$O than would be expected, while M-dol showed little mitigation effect. Noting also that the variation of cumulative post-spring thaw flux in M-con was lower than in M-dol (figure 6(A)), increased N cycling in limed plots after fertilization may have confounded an N$_2$O mitigating diversity effect in this case.

4.4. Liming effect
The results did not support our hypothesis (2) that liming reduces off-season N$_2$O accumulation and emission in clovers by favoring more complete denitrification of N$_2$O to N$_2$. Much to the contrary, R-dol plots emitted double the cumulative N$_2$O flux as R-con throughout the freeze-thaw period in fall (figure 6(A)). This might reflect overall larger N turnover in limed than unlimed clover plots after initial frost killing; R-dol had slightly higher yields in September than R-con (tables 1(A), C); not significant). Nitrification is strongly stimulated by high pH (Parton et al 2001) and liming likely supported higher rates of nitrification than in the control soil, implicating nitrification or coupled nitrification-denitrification (Kremen et al 2005) as the dominant source of N$_2$O early in winter. Soil samples taken on December 8, one day after peaking freeze-thaw induced N$_2$O fluxes, showed that R-dol had significantly more extractable NO$_3$ than R-con (table 1(C)). Raising pH may also favor ammonia oxidizing bacteria which produce more N$_2$O than ammonia oxidizing archaea (Hink et al 2018), although this increase in N$_2$O is theoretically lower in magnitude than the decrease of N$_2$O produced by denitrification.

If N$_2$O is produced in the uppermost soil, both nitrification and denitrification can be a source, as long as some local anoxia exists. Given an O$_2$ concentration in soil air which could still support nitrification throughout the freeze-thaw period (minimum 17% in R-dol and 14% in R-con; figure 4(B)) and low soil VWC in the topsoil (figure 4(F)), coupled nitrification–denitrification may have occurred in medium-sized soil aggregates (Kremen et al 2005). Song et al (2017) note that while freeze-thaw may inhibit nitrification in laboratory experiments, field experiments have shown freeze-thaw to stimulate nitrification. Although we did not measure mineral NO$_3$ in our soil (the samples were sieved and frozen a few hours after collection), transient nitrite accumulation could be another potent inducer of N$_2$O emissions (Giguere et al 2017), potentially also off-season (Venterea et al 2020).

During the period of deep snow cover, R-dol plots tended to have less time-integrated N$_2$O accumulation in soil than R-con plots (not significant, p=0.41), indicating that liming might reduce longer-term N$_2$O accumulation in the soil. Since anoxic conditions under prolonged snowpack do not support nitrification, denitrification was likely prevailing, mediating a pH effect through pH control of N$_2$O reductase activity.
However, this effect may be modulated by acid-tolerant complete denitrifiers proliferating during extended periods of anoxia, especially when nitrogenous electron acceptors other than N$_2$O become scarce (Palmer et al. 2010). R-con showed especially high variability in N$_2$O concentrations in the soil air during spring thaw, which highlights the potential risk that unlimed clover-containing plots can produce large quantities of N$_2$O through denitrification in spring, even if such large quantities were not observed consistently.

The clearest evidence for a possible mitigation effect of off-season N$_2$O by liming was seen in grass-only plots during the deep snow-covered period, in which limed plots had significantly less time-integrated N$_2$O accumulation in soil air relative to the control (figure 6(B)). The fact that this effect was not seen in clover plots suggests that decomposition of N-rich clover substrates overrides the mitigation effect by liming.

4.5. Conclusions
While many studies on N$_2$O from agricultural systems focus on the growing season, the body of literature about off-season emissions is increasing. Studies investigating the N$_2$O effect of including legumes, whether in grassland or cover cropping systems, highlight the importance of accounting for winter in annual N$_2$O budgets. Our study details dynamics of subnivean N$_2$O accumulation and flux and compares them among distinct grassland communities throughout variable winter conditions: repeated freeze-thaws of exposed soil, and trapped under frozen soil.

Our data point at a tradeoff between including clover in a grass mixture for saving N fertilizer in the summer and inducing extra N$_2$O emissions in winter. From the perspective of N use efficiency, if stands of pure clover and grass-clover mixture yield more N in forage than grass-only stands, or if grass-clover mixtures can reduce N leaching, this may partially justify additional N$_2$O emissions, especially if mineral N additions to clover-containing swards can be reduced.

Liming may enhance winter emissions of N$_2$O produced de novo in topsoil from decaying N-rich substrates, such as clovers. There is some evidence, however, that over long periods of reduced exchange between soil and atmosphere, favoring denitrification but not nitrification, liming may reduce N$_2$O accumulation, likely by supporting N$_2$O reductase activity.

Off-season, the N$_2$O risk posed by pure clover swards seemingly overrides the advantage of liming seen in grass swards. However, there was some evidence that our grass-clover mixtures, containing around 40%–50% clover by DM yield, may emit less N$_2$O than might be predicted from the component species. Further studies on this potential diversity effect on N$_2$O mitigation would be beneficial for developing planting guidelines in areas experiencing winter conditions with freeze-thaw. In addition, it may be worthwhile to test the effect of more winter-hardy legume species. Selecting species which grow late into the season and absorb and store excess N$_\text{min}$ belowground, as in the practice of using ‘catch crops,’ may help insofar as they can resist decomposition under long and harsh winter conditions.

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Data availability statement
The data that support the findings of this study are available upon reasonable request from the authors.

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