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Dinitrogen (N_2) pulse emissions during freeze-thaw cycles from montane grassland soil

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Abstract

Short-lived pulses of soil nitrous oxide (N_2O) emissions during freeze-thaw periods can dominate annual cumulative N_2O fluxes from temperate managed and natural soils. However, the effects of freeze thaw cycles (FTCs) on dinitrogen (N_2) emissions, i.e., the dominant terminal product of the denitrification process, and ratios of N_2/N_2O emissions have remained largely unknown because methodological difficulties were so far hampering detailed studies. Here, we quantified both N_2 and N_2O emissions of montane grassland soils exposed to three subsequent FTCs under two different soil moisture levels (40 and 80% WFPS) and under manure addition at 80% WFPS. In addition, we also quantified abundance and expression of functional genes involved in nitrification and denitrification to better understand microbial drivers of gaseous N losses. Our study shows that each freeze thaw cycle was associated with pulse emissions of both N_2O and N_2, with soil N_2 emissions exceeding N_2O emissions by a factor of 5–30. Increasing soil moisture from 40 to 80% WFPS and addition of cow slurry increased the cumulative FTC N_2 emissions by 102% and 77%, respectively. For N_2O, increasing soil moisture from 40 to 80% WFPS and addition of slurry increased the cumulative emissions by 147% and 42%, respectively. Denitrification gene *cnorB* and *nosZ* clade I transcript levels showed high explanatory power for N_2O and N_2 emissions, thereby reflecting both N gas flux dynamics due to FTC and effects of different water availability and fertilizer addition. In agreement with several other studies for various ecosystems, we show here for mountainous grassland soils that pulse emissions of N_2O were observed during freeze-thaw. More importantly, this study shows that the freeze-thaw N_2 pulse emissions strongly exceeded those of N_2O in magnitude, which indicates that N_2 emissions during FTCs could represent an important N loss pathway within the grassland N mass balances. However, their actual significance needs to be assessed under field conditions using intact plant-soil systems.

Keywords Freeze-thaw cycles · Dinitrogen · Nitrous oxide · Denitrification · Functional genes · Soil moisture · Fertilization

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Introduction

In the last century, human activities have more than doubled the amount of reactive nitrogen (Nr) in the biosphere, mainly through fossil fuel combustion and fertilization (Fowler et al. 2013). Among the multiple nitrogen (N) transformation pathways, microbial denitrification, the reduction of nitrogen oxides (NO_3^- and NO_2^-) to NO and N_2O, and ultimately N_2 gases under anaerobic conditions, is the most important mechanism responsible for the permanent removal of Nr in terrestrial ecosystems (Kulkarni et al. 2008; Groffman 2012; Butterbach-Bahl et al. 2013). Moreover, it can be considered as a significant source or sink process for the potent atmospheric greenhouse gas N_2O (Chen et al. 2015). Despite its importance, denitrification is poorly understood at all scales ranging from soil micropores to global (Galloway et al. 2008; Butterbach-Bahl et al. 2013), mainly due to its notoriously high spatiotemporal variability and the fundamental difficulty in measuring soil N_2 emissions (Kulkarni et al. 2008; Sgouridis and Ullah 2015). Therefore, a reliable estimate of the denitrification rates as well as of the ratios of the different N gases produced during denitrification is essential to improve our ability to fully account for the N fluxes and pools in different ecosystems at multiple scales (Groffman et al. 2006; Qu et al. 2014; Sgouridis and Ullah 2015).

Soil freeze-thaw cycles (FTCs) occur regularly in ecosystems at mid- to high latitude as well as high altitude. Changing climate conditions are likely to decrease the duration of the snow period and the thickness of snowpack (Henry 2008), which in turn could lead to an increase in the frequency of FTCs (Matzner and Borken 2008). Freeze-thaw cycles were shown to trigger pulse N_2O emissions both in laboratory- (e.g., Nyborg et al. 1997; Goldberg et al. 2008; Wagner-Riddle et al. 2008) and field experiments (e.g., Wolf et al. 2010; Németh et al. 2014; Morse et al. 2015). The underlying mechanisms include a strong stimulation of denitrification due to liberation of C and N substrates in combination with anaerobic conditions, or a physical entrapment and release of previously produced gases when ice or snow cover thaw (Mørkved et al. 2006; de Bruijn et al. 2009; Kim et al. 2012; Risk et al. 2013; Wu et al. 2014; Wertz et al. 2016). However, due to the methodological difficulties in the quantification of N_2 that further rise under conditions of frozen soil, very little is known on effects of soil FTCs on N_2 fluxes, the N_2/N_2O emission ratio and total denitrification losses (Nyborg et al. 1997; Wagner-Riddle et al. 2008). Currently, available approaches for quantifying the gaseous end-products ($N_2 + N_2O$) of denitrification are limited and can be classified into (1) the acetylene blockage method, which exploits acetylene inhibition of the reduction of N_2O to N_2 (Groffman et al. 2006; Felber et al. 2012), (2) the ^{15}N gas flux technique, which requires $^{15}NO_3^-$ application to soil followed by the measuring of $^{15}N_2O$ and/or $^{15}N_2$ in the gaseous end-products (Kulkarni et al. 2014; Sgouridis and Ullah 2015), and (3) the gas-flow soil core (GFSC) method, which allows the direct simultaneous measurement of N_2O and N_2 fluxes from soil cores when the soil atmosphere is replaced by a mixture of He/O_2 (Butterbach-Bahl et al. 2002; Wang et al. 2011). Among these approaches, the GFSC method has the advantage that it does not require addition of isotopically labeled substrates or inhibitors to the soil (Chen et al. 2015; Wen et al. 2016).

Soil moisture has been identified as one of the crucial drivers of temporal variability of soil-atmosphere N gas fluxes through the regulation of oxygen availability and the soil microbes' respiration chain (Wu et al. 2010; Friedl et al. 2016). While the stimulating effects of soil moisture on denitrification and associated N trace gas fluxes for unfrozen soils have been very well documented, there is less information on the effects of different soil moisture levels on pulse N_2O emissions during freeze-thaw periods (Teepe et al. 2004; Kim et al. 2012). Wu et al. (2014) indicated that a threshold value of soil moisture could exist that needs to be surpassed to trigger N_2O peaks during thawing. On the other hand, non-significant N_2O emissions during thawing periods under very moist conditions might be partly due to the further reduction of N_2O to N_2 by denitrification because of complete denitrification under strictly anaerobic conditions (de Bruijn et al. 2009; Wu et al. 2014). Based on a meta-analysis, Gao et al. (2018) found that the increase of soil moisture during FTCs can lead to increasing losses of N_2O due to the enhanced substrate release from the ice-induced physical soil disturbances. Another key driver for soil N gas fluxes is the availability of N, as in most studies, N fertilizer input has been shown to stimulate nitrification and denitrification as well as gaseous N emissions (Qu et al. 2014; Kuang et al. 2019).

Montane grasslands, receiving high N inputs in the form of urine and animal manure, are widespread in Central Europe and store large amounts of soil organic N (Wiesmeier et al. 2013). In these ecosystems, manuring is often taking place in autumn, winter, and early spring as the storage capacity for slurry is limited and requires field application of slurry also during the non-growing season. However, during these periods several FTCs may occur. So far, however, no study directly quantified both N_2 and N_2O losses from montane grasslands, which prevents the closing of N balances on the ecosystem scale and hampers the development of sound management strategies targeted to improve slurry N use efficiency.

Besides the direct quantification of N gas emission rates, also the abundance of nitrifiers and denitrifiers as well as the expression of genes driving both processes can provide insights into the regulation of N_2O production rates and its reduction to N_2 (Chen et al. 2015; Pan et al. 2018). A growing number of studies has been pointing out that N_2O fluxes are linked to the expression rates of genes triggering nitrification and denitrification, but the reported results are not consistent (Henderson et al. 2010; Németh et al. 2014; Chen et al. 2015).

For example, Henderson et al. (2010) did not detect significant relationships between the abundance of denitrifiers and the expression levels of the related functional genes on N_2O emissions in agricultural soils. Chen et al. (2015) found that *nos*Z gene expression was closely related to N_2 rather than N_2O emission in montane grassland soils. The effects of cold temperatures on the abundance of nitrifiers and denitrifiers as well as the expression of the related genes have recently been examined in different soils with, however, no parallel measurements of both N_2O and N_2 fluxes (Jung et al. 2011; Nemeth et al. 2014; Wertz et al. 2016). Németh et al. (2014) reported that N_2O emissions were inversely related to the *nos*Z transcript levels (i.e., the genes involved in N_2O reduction to N_2 in denitrification) in agricultural soils during FTCs. Generally, difficulties in bridging spatial scales between gene analyses in small soil samples and N gas flux measurements at larger scales impair our understanding of relationships between expression of functional nitrifier and denitrifier genes and N_2O and N_2 fluxes.

Therefore, the main objectives of this study were to (1) quantify FTC N_2O and N_2 emissions and (2) to explore the effects of soil moisture and fertilization on the abundance and activity of nitrifier and denitrifier communities and the associated N_2O and N_2 fluxes during soil FTCs. We hypothesized that FTCs would cause peak emissions of both N_2O and N_2 mainly triggered by denitrification with N_2 losses exceeding N_2O losses. Furthermore, we expected that slurry application increases FTC N gas pulse emissions and that at higher soil moisture, the importance of N_2 would increase over the importance of N_2O. Additionally, we hypothesized that N gas dynamics would be closely linked to the expression pattern of genes triggering denitrification. To test these hypotheses, we conducted a laboratory incubation experiment exposing a montane grassland soil to FTC with measurements of N_2/N_2O emissions by use of the He soil core technique accompanied by measurements of abundance and activity of nitrifers/denitrifers, as well as supporting parameters such as soil dissolved organic and inorganic C and N concentrations.

Materials and methods

Study site and soil sampling

The study site, Graswang, is a typical pre-alpine grassland ecosystem located in Southern Bavaria, Germany (11.03° E, 47.57° N) at an elevation of 870 m above sea level. This site is a part of the TERENO preAlpine long-term ecosystem observatory (Wolf et al. 2017) and has been extensively managed (Wang et al. 2016). The mean annual air temperature and precipitation were 6.9 °C and 1347 mm, respectively, from 2014 to 2017 (Kiese et al. 2018). The investigated soil is a C- and N-rich Haplic Cambisol (IUSS Working Group WRB 2006) with a pH of 7.1 and a bulk density of 0.9 g cm^{-3}. The topsoil (Ah horizon, 0–20 cm) consists of 10% sand, 68% silt, and 23% clay with a soil organic carbon (SOC) of 89 mg g^{-1} and total nitrogen (TN) of 8.0 mg g^{-1} (Unteregelsbacher et al. 2013). Further information can be found in Unteregelsbacher et al. (2013) and Wang et al. (2016).

For the experiments, approximately 50 kg surface soils (0–20 cm) were collected in October 2014 at six sampling spots. The soil samples were homogenized and sieved (5 mm mesh) and stored at 4.0 °C for 3 weeks until the start of the freeze-thaw experiments to allow an adaptation of the soil microbiome to cold temperatures. To investigate the effects of soil moisture and fertilization, soil samples were equally separated into three treatments. For the first and second treatment, the soil moisture was adjusted to 40% and 80% water-filled pore space (40% WFPS and 80% WFPS), respectively, whereas the third group (80% WFPS + F) was fertilized with cow slurry (20 mg N kg^{-1} dry soil), representing a typical autumn slurry-N addition of 36 kg N ha^{-1}, and then adjusted to 80% WFPS. The TN contents of the cow slurry were 48.1 g N kg^{-1} dry mass and 2.5 g N kg^{-1} fresh mass with NH_4^+-N contributing 55% to total N. The slurry had a C/N ratio of 8.2:1 and a pH of 7.4 (i.e., only slightly higher than soil pH of 7.1). The incorporation procedure mimicked a modern slurry injection application, which is increasingly applied to reduce N losses, rather than traditional surface application. In Germany, the fertilizer ordinance law requires that surface slurry application is entirely replaced by incorporation techniques by the year 2025. Soils from each treatment were split in two parts. One part of the soil was placed in glass bottles (159 samples for each treatment with three replicates, 50 g soil per bottle) for destructive soil chemical and microbial analyses, whereas the remaining soil (ca. 6 kg of each treatment) was packed in four soil cores (15 cm height, 12.5 cm diameter) and compacted to the original bulk density of 0.9 g cm^{-3}. Finally, cores were inserted in the helium-soil-core incubation system for automated direct measurements of N_2O and N_2 production (Butterbach-Bahl et al. 2002; Wang et al. 2011).

Soil incubation experiments

Bottles with soil samples for destructive analysis were placed in incubators (Thermo Electron LED BK 700, GmbH, Germany) to maintain constant temperature levels and to simulate FTCs. The air within the incubator was exchanged with outside air by a pump to avoid gas accumulation and temperature heterogeneities. Three FTCs (during approx. 16 weeks) were used to measure the effects of sequential FTCs on the abundance and activity of nitrifiers and denitrifiers and the associated N_2O and N_2 fluxes. The temperature in the first FTC was set to − 5 °C for 2 weeks and then to + 5 °C for 2 weeks. Topsoil temperatures of − 5 °C are observed at the

field site under winter conditions when no or little snow is covering the soil, but in some years are prevented by thick snow cover. The second and third freezing cycles (− 5 °C) lasted for 4 weeks, followed by thawing at + 5 °C for 2 weeks. To investigate the effect of temperature near the freezing point during thawing on N_2O and N_2 fluxes and the abundance and activity of nitrifiers and denitrifiers, the temperature during the third thawing was set to 0 °C for 3 days after 4 weeks at − 5 °C followed by + 5 °C for 1 week (Fig. 1).

The abundance (DNA) and activity (mRNA) of nitrifiers and denitrifiers, the concentrations of soil exchangeable NH_4^+, NO_3^-, dissolved organic carbon (DOC) and N (DON), as well as microbial biomass carbon (MBC) and N (MBN), were determined by destructive harvesting of triplicate bottles at several time points during each FTC to identify controls of N gas fluxes. The soil exchangeable NH_4^+, NO_3^-, DOC, and DON concentrations and MBC and MBN were measured with different temporal resolution during the first two FTCs: (1) once per week or bi-weekly during the soil freezing periods, (2) twice per day (9:00 in the morning and 16:00 in the afternoon) during the first week after thawing, and (3) daily during the second week after thawing. During the third FTC, these parameters were quantified every 2 days after thawing (Fig. 1). The abundance and activity of nitrifiers and

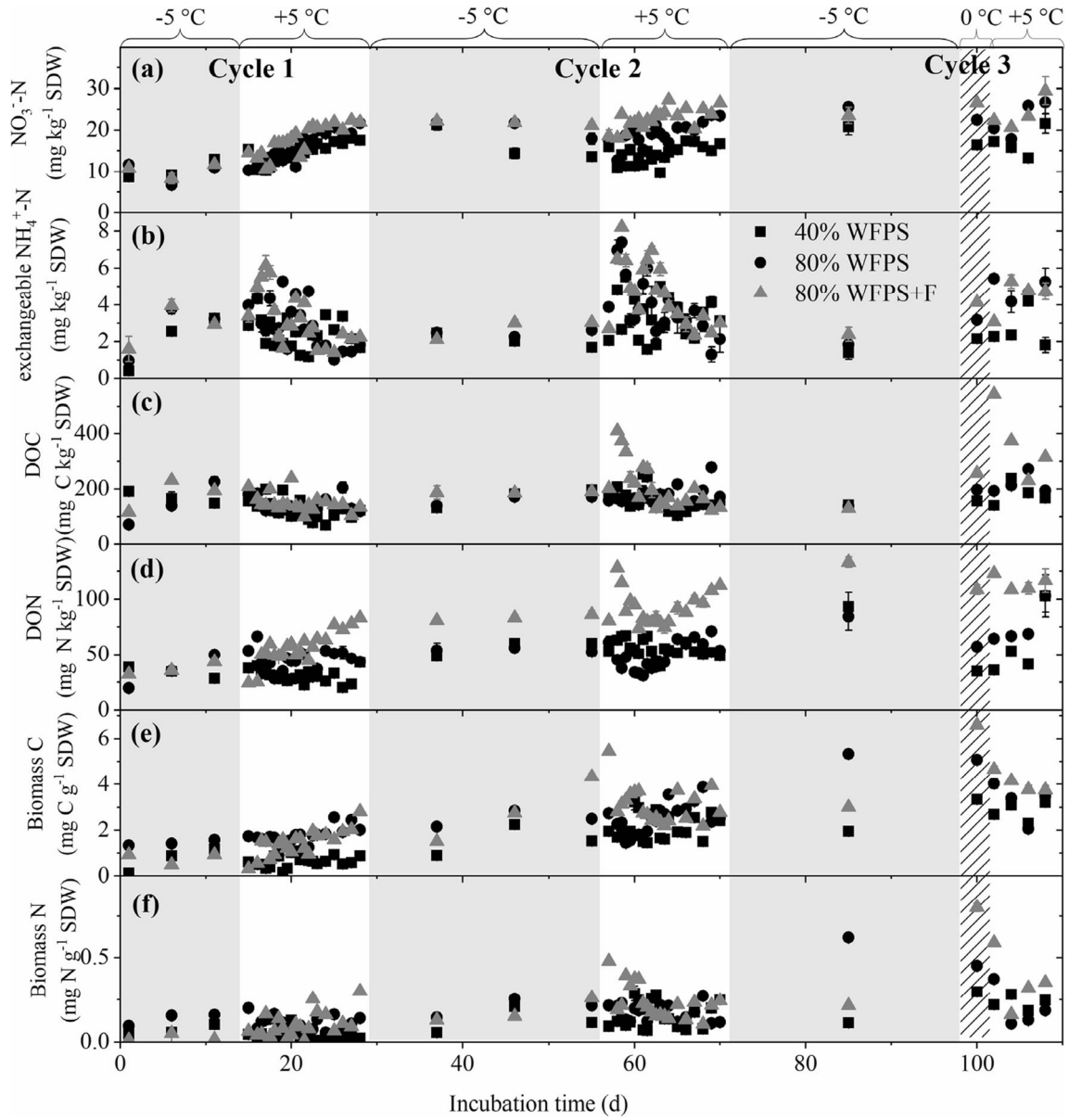

Fig. 1 Dynamics of soil NO_3^- (**a**), exchangeable NH_4^+ (**b**), DOC (**c**), and DON (**d**) contents and microbial biomass C (**e**), and N (**f**) during the freeze-thaw cycles of different treatments. WFPS: water filled pore space; F: fertilization with cow slurry; SDW: soil dry weight. Gray color indicates freezing periods, the shaded area is indicating incubation at 0 °C

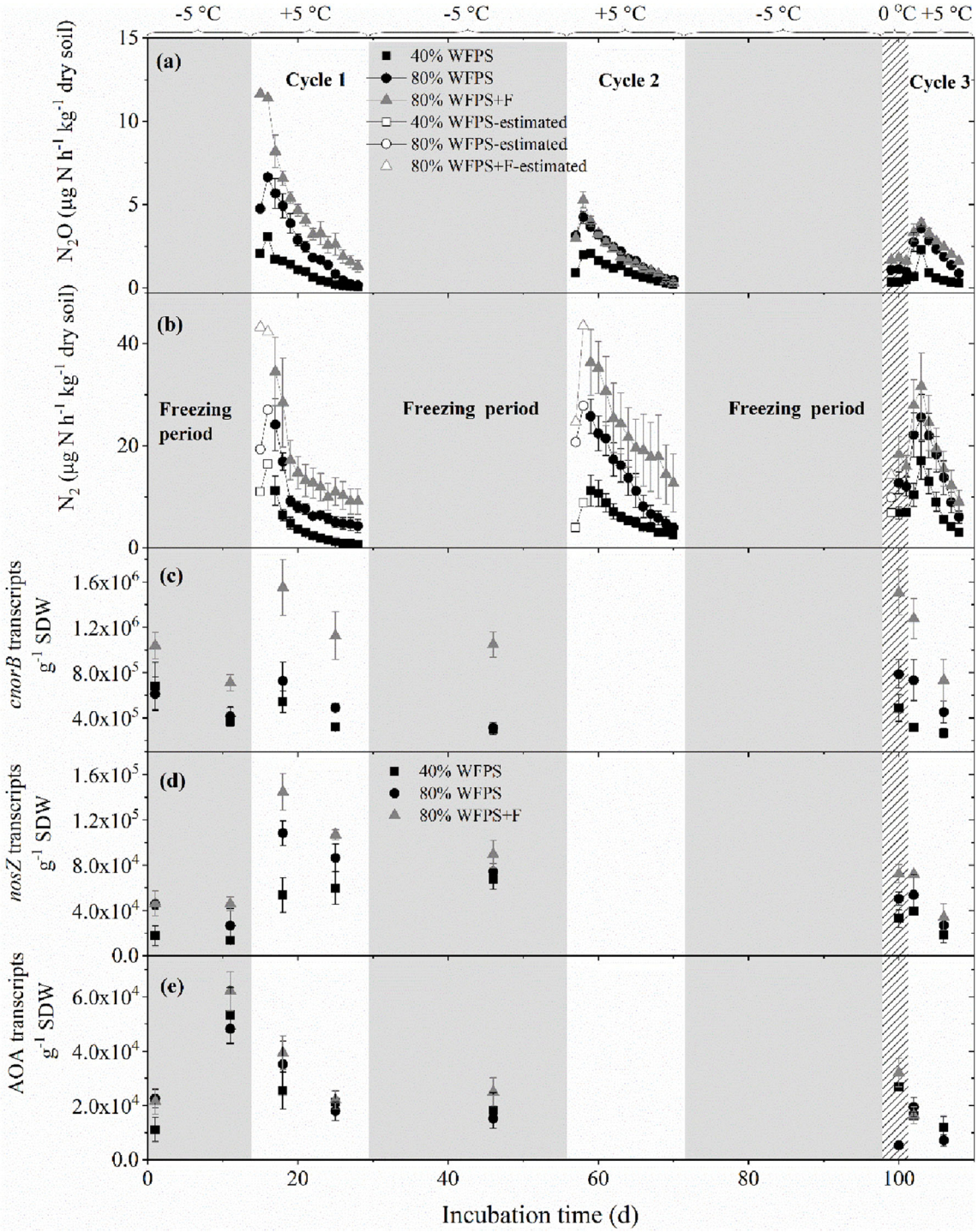

Fig. 2 Dynamics of N_2O (**a**) fluxes, N_2 (**b**) fluxes ($N = 4$), gene expression of *cnorB* (**c**), *nosZ* clade I (**d**), and AOA (**e**) (RNA level, $N = 3$) during the freeze-thaw cycles of different treatments. WFPS: water filled pore space; F: fertilization with cow slurry; SDW: soil dry weight. Gray color indicates freezing periods, the shaded area is indicating incubation at 0 °C

denitrifiers were detected at eight defined time intervals across the entire incubation period (Fig. 2).

The same incubation scheme was applied in parallel for the soil cores used for N_2 and N_2O gas flux measurements. However, because we experienced problems running the helium soil core system at − 5 °C, the soil cores were stored during the freezing periods in freezers and then were transferred in frozen form into the He system. Direct measurements of N_2O and N_2 fluxes were thus started with daily temporal resolution after introducing frozen soil to the He system for subsequent N gas flux measurements at 5 °C (first two cycles) or 0 °C (third cycle). Consequently, no measurements are

available for the freeze phase, where emissions are assumed to be low. As only four vessels were available for simultaneous measurements by the helium soil core system, an alternating incubation scheme was used to fully use the capacities of the system (see Fig. S1).

N_2O and N_2 flux analysis

The N_2O and N_2 fluxes were measured during each thawing period over the three FTCs using an automated helium-soil-core incubation system (Butterbach-Bahl et al. 2002; Wang et al. 2011). The basic measurement principle is the exchange of N_2 gas by helium in an extremely gastight soil-headspace system so that biological N_2 production then can be precisely measured without the disturbing atmospheric N_2 background. For this purpose, the vessels containing the soil are purged with He-based gas mixture until full N_2 removal. After purging, the system switches to a static chamber mode to detect N_2 and N_2O concentration increases in the constant gas volume of the headspace and thus to measure soil N_2 and N_2O emissions. The system contains four vessels for soil cores of 12.5 cm diameter and 15 cm height, in which both soil and headspace air at approximately background composition are replaced by a synthetic atmosphere of 20% O_2 (purity grade of 5.5), 80% He (purity grade of 5.0), N_2O (400 ppbv), and N_2 (25 ppmv). The small N_2 background of 25 ppmv is artificially established at the end of the exchange procedure in order to start N_2 measurements with well detectable concentrations. Quantitative exchange of atmospheric background in soil and headspace with synthetic background was achieved by repeated pressure reduction to 700 mbar in the vessels containing the soil, followed by purging with the He gas mixture from the bottom of the soil cores to the headspace above the soil cores. The extreme tightness of the system against intrusion of atmospheric N_2 is ensured by using double He-purged sealings, by submerging vessels and all tubing connections under water and by placing all valves, sample loops, and system control units in a He-flushed gastight box. In this study, the background exchange period started with 48 evacuation/purging cycles within the first 24 h, followed by 24 h of pure purging from bottom to top of soil cores without evacuation. The successful removal of N_2 from the soil core with this procedure was confirmed by a conservative calculation of the remaining N_2 concentration within the soil cores, which is described in the Supplementary Materials & Methods.

During the entire incubation period, temperature levels were controlled by a water/glycol bath that contains the incubation vessels. The measurement of N_2O fluxes started 1 h after closing the vessels during thawing, whereas the measurements of N_2 fluxes began 48 h after closing the vessels because, as outlined above, this was the time required to completely exchange the atmospheric background (especially N_2) with the synthetic background. Thus, the missing values of N_2 flux during the initial stages after thawing were estimated using the measured N_2O flux values and the most closely available N_2/N_2O ratios (Fig. 2). An electron capture detector (ECD, Shimadzu, Germany) and a pulse discharge He ionization detector (PDHID, Vici AG, Switzerland) were used for the detection of N_2O and N_2, respectively. Further information about the setup of the system, replacement of soil atmosphere, and the calculation of fluxes can be found in previous publications (Butterbach-Bahl et al. 2002; Wang et al. 2011; Chen et al. 2015; Wen et al. 2016).

Analysis of abundance and activity of nitrifers and denitrifers

Soils for molecular analyses were immediately frozen at − 80 °C after sampling. DNA and RNA were co-extracted from 0.4 g homogenized dry soil using the method described by Lueders et al. (2004) and the Precellys 24 Instrument (Peqlab, Erlangen, Germany). The quality and quantity of the nucleic acids were assessed using a spectrophotometer (Nanodrop; PeqLab, Germany) and agarose (1.5% (*w*/*v*)) gel electrophoresis. Afterwards, the extract was divided into two subsamples. One was used for DNA analysis and the other subsample was used to prepare RNA by digestion and purification with DNase Max kit (MO BIO Laboratories, Carlsbad, CA, USA). The absence of DNA in the RNA samples was confirmed by performing a 16S PCR reaction, using the universal eubacterial primers 968f (5′-aac gcg aag aac ctt ac-3′) and 1401r (5′-cgg tgt gta caa gac cc-3′). The cDNA was synthesized with the "High capacity cDNA reverse transcription kit" (Life Technologies, Darmstadt, Germany) according to the instructions. The success of cDNA synthesis was confirmed by performing PCR targeting the 16S rRNA gene as described above. Both DNA and cDNA extracts were stored at − 20 °C until use.

Quantitative real-time PCR (qPCR) was carried out on a 7300 real-time PCR system (Life Technologies, Darmstadt, Germany) using SYBR green as a fluorescent dye. Primer sets and plasmids containing the targeted gene fragments and PCR reaction mixtures, and thermal profiles for each gene are shown in supplementary Table S1. Dilution series of the different DNA extracts were tested in a pre-experiment with randomly picked soils to avoid inhibition of PCR. Dilutions of the DNA extracts of 1: 80 turned out to be most suitable and were used for all qPCRs. The standard curves for all the detected genes were created using 10-fold dilution series (10^1~10^7 copies) of the respective plasmids containing the targeted gene fragments. All PCR runs started with an initial enzyme activation step performed at 95 °C for 10 min followed by gene specific thermal profiles (Table S1), and a melting curve, consisting of 95 °C for 15 s, 60 °C for 30 s, and a subsequent temperature increase up to 95 °C with a ramp rate of 0.03 °C s^{-1}. Specificity of the amplified products was

checked by the observation of a single melting peak and the presence of a unique band of the expected size in a 2% agarose gel. PCR efficiencies (Eff) were calculated from the standard curve by the formula $Eff = [10^{(-1/slope)} - 1] \times 100\%$ and accounted for 91 to 94% for 16SrRNA gene, 99% for *nirK* gene, 91% for *nirS* gene, 87% for *cnorB* gene, 91 to 96% for *nosZ* clade I gene, 90% for *amoA* AOA gene, and 91% for *amoA* AOB gene. The N_2O reductase, encoded by the *nosZ* gene, has two major clades: clades I and II (Jones et al. 2013). As an earlier study using the same soil found very high explanatory power of *nosZ* clade I transcripts for N_2 emissions, (Chen et al. 2015) and because this study focused on nitrifiers and denitrifiers, only *nosZ* clade I genes were considered. Clade II harbors many non-denitrifying N_2O reducers (Hallin et al. 2018).

Soil analyses

Gravimetric soil water content was determined by drying the soil samples at 105 °C until constant weight. Soil WFPS was calculated from bulk density and volumetric soil moisture content using a particle density of 2.65 g cm^{-3}. Approximately 20 g of soil was extracted with 50 ml 0.5 M K_2SO_4 for measuring mineral N (exchangeable NH_4^+-N and NO_3^--N), DOC, and dissolved TN at each sampling time point as described by Dannenmann et al. (2009). Quantification of DOC was based on an infrared detector (Multi N/C 3100 TOC/TN-Analysator, Analytik Jena, Jena, Germany) (Dannenmann et al. 2009). The concentrations of exchangeable NH_4^+ and NO_3^- were determined using continuous flow injection colorimetry (Skalar San plus system, Skalar Analytical B.V., Breda, Netherland) in a commercial laboratory (Landwirtschaftliches Labor Dr. Janssen, Gillersheim, Germany). Moreover, MBC and MBN contents in soil samples were measured using the chloroform fumigation-extraction method (Dannenmann et al. 2009; Wu et al. 2014).

Statistical analyses

Statistical analyses were carried out using SPSS 15.0 (SPSS Inc., Chicago, USA) and Microcal Origin 9.0 software (Origin Lab Inc., Northampton, USA). Data were checked for normal distribution using the Kolmorov-Smirnov test and for homogeneity of variances using the Levene test; data were log-transformed when necessary. Analyses of significant differences in destructively analyzed soil parameters between treatments and frost versus thaw periods were carried out using ANOVA (with LSD method) while respective analyses for N gas fluxes were based on repeated measures ANOVA. Linear regression analysis was used to investigate the relationships between the N gas fluxes and soil characteristics and associated gene abundance/expression. Differences were considered significant at $P < 0.05$.

Results

Chemical soil characteristics

The dynamics of exchangeable NH_4^+, NO_3^-, DOC, DON, MBC, and MBN during three FTCs are shown in Fig. 1. Generally, we found no significant differences in NO_3^-, DON, DOC as well as microbial biomass between frozen and thawing soil. Only exchangeable NH_4^+ concentrations showed pronounced peaks during thawing irrespective of soil water status and thawing cycle ($P < 0.05$, Fig. 1, Table 1). During the second soil freezing period, there was a significant increase of both microbial biomass C and N (Fig. 1). Both higher WFPS and fertilization increased average soil exchangeable NH_4^+ and NO_3^- contents and microbial biomass throughout the entire experimental incubation, while only fertilization significantly increased DOC and DON concentrations ($P < 0.05$, Table 1).

N_2O and N_2 emissions

Thawing always triggered sharp N_2O emission pulses with peak emissions (up to 12 μg N kg^{-1} dry soil h^{-1}) being observed after first few days, followed by a gradual decline of emission levels to 0–2 μg N kg^{-1} dry soil h^{-1} depending on treatment and number of cycles. N_2O emissions showed similar levels of ca. 0–2 μg N kg^{-1} dry soil h^{-1} at 0 °C during the third cycle (Fig. 2). The maximal magnitude of the N_2O emission pulse as well as total cumulative emissions following a FTC declined with each cycle (Fig. 2, Table 2). Both increased WFPS and fertilization significantly increased N_2O emissions across the three FTC cycles (Table 2). Over the entire incubation period and for all three FTCs, increasing soil moisture from 40 to 80% WFPS and addition of slurry increased the cumulative N_2O emissions by 147.0% and 41.5%, respectively (Table 2).

Dinitrogen emissions showed generally similar response patterns to thawing and the different experimental manipulations, but exceeded N_2O emissions by a factor of 5–30. However, in contrast to N_2O, the cumulative N_2 emissions during the second thawing period were significantly higher than those in first thawing cycle ($P < 0.05$, Table 2). In particular, for the 80% WFPS + F treatment and the first two FTCs, emissions only slowly declined and hardly fell below background emission levels of ca. 0–10 μg N m^{-2} h^{-1} (Fig. 2). Over the entire incubation period including all three FTCs, increasing soil moisture from 40% to 80% WFPS and the addition of slurry increased the cumulative N_2 emissions by 101.9% and 76.8%, respectively (Table 2). The N_2/N_2O ratios

Table 1 Average soil inorganic N, dissolved organic carbon (DOC), and dissolved organic N (DON) contents and microbial biomass C/N in different treatments and *P* values of ANOVA on the effect of freeze-thaw events on these soil parameters

Treatments	NH_4^+-N (mg kg^{-1} SDW)	NO_3^--N (mg kg^{-1} SDW)	DOC (mg C kg^{-1} SDW)	DON (mg N kg^{-1} SDW)	Microbial biomass C (mg C g^{-1} SDW)	Microbial biomass N (mg N g^{-1} SDW)	Microbial biomass C/N ratio
40% WFPS	2.74 ± 0.12a	14.58 ± 0.64a	149.89 ± 5.55a	45.09 ± 2.83a	1.51 ± 0.05a	0.11 ± 0.01a	21.98 ± 8.30a
80% WFPS	3.40 ± 0.27b	17.61 ± 0.92b	159.19 ± 5.98a	49.12 ± 3.35a	2.28 ± 0.07b	0.16 ± 0.01b	16.22 ± 3.77a
80% WFPS + F	3.83 ± 0.19c	20.12 ± 1.23c	195.23 ± 7.31b	76.98 ± 4.22b	2.43 ± 0.06c	0.18 ± 0.01b	20.26 ± 3.67a
P (freeze thaw)							
40% WFPS	*0.041*	0.864	0.256	0.250	0.448	0.770	0.242
80% WFPS	0.066	0.523	0.608	0.821	0.589	*0.035*	*0.033*
80% WFPS + F	*0.048*	0.065	0.555	0.515	0.355	0.201	0.165

Values are means with standard errors. Different letters indicate significant differences among three treatments ($P < 0.05$, $N = 3$). *P* (freeze thaw) is providing results of ANOVA analyses on significance of freeze-thaw events on soil parameters with significant effects being highlighted in italics ($P < 0.05$)

WFPS water filled pore space, *F* fertilization with cow slurry, *SDW* soil dry weight

gradually increased during the first thawing period for all three treatments, while an increase of N_2/N_2O ratios during the second thawing period was only observed from the 80% WFPS + F treatment (Fig. 3a). The $N_2/(N_2 + N_2O)$ ratios were rather stable with values of 0.7–0.92, irrespective of different soil moisture and fertilization treatments (Fig. 3b).

Cumulative N gas emissions from our lab study were used to provide an approximation of N emissions due to slurry incorporation. Comparing the cumulative N gas emissions from the 80% WFPS with the 80% WFPS + F treatments revealed additional N losses of 0.85 and 8.98 mg N kg^{-1} dry soil as N_2O and N_2 due to the fertilization, respectively (Table 2). These are corresponding to 4% and 45% of the applied slurry-N (20 mg N kg^{-1} dry soil or 36 kg N ha^{-1}), i.e., 1.5 and 16.2 kg N ha^{-1} during the incubation period of ca 3.5 months.

Abundance and activity of nitrifiers and denitrifiers

To improve the understanding of the role of nitrifers and denitrifers for N gas fluxes during FTCs, the abundance and expression of functional genes involved in nitrification and denitrification were quantified. In general, the genetic potential (based on the abundance levels of the investigated genes) was not significantly affected by FTCs (Fig. S2). In many cases, the abundance even tended to decrease in thawing compared to frozen soil (Fig. S2). In contrast, the dynamics of the related transcription exhibited remarkable temporal dynamics and treatment responses, i.e., transcripts of *cnorB* and *nosZ* clade I showed pronounced increases in response to both slurry application and increased WFPS, which were similar to the effects observed for gaseous N emissions (Fig. 2). Furthermore, transcripts of these genes showed a pronounced increase due to thawing, despite we failed to sample during the

Table 2 Cumulative soil N_2O and N_2 fluxes during the three freeze-thaw cycles of different treatments

Treatments	Cumulative N_2O flux (mg N kg^{-1} SDW)				Cumulative N_2 flux (mg N kg^{-1} SDW)			
	Cycle 1	Cycle 2	Cycle 3	Three cycles	Cycle 1	Cycle 2	Cycle 3	Three cycles
40% WFPS	0.33 ± 0.02a	0.34 ± 0.03a	0.16 ± 0.01a	0.83 ± 0.05a	1.72 ± 0.17a	2.07 ± 0.34a	2.00 ± 0.34a	5.79 ± 0.84a
80% WFPS	0.90 ± 0.08b	0.70 ± 0.06b	0.45 ± 0.05b	2.05 ± 0.19b	2.98 ± 0.31b	5.02 ± 0.93b	3.69 ± 0.91b	11.69 ± 2.13b
80% WFPS + F	1.64 ± 0.09c	0.71 ± 0.05b	0.55 ± 0.04c	2.90 ± 0.18c	6.72 ± 0.98c	9.56 ± 1.82c	4.39 ± 1.06c	20.67 ± 3.85c

Values are means with standard errors. Different letters indicate significant differences among three treatments ($P < 0.05$, $N = 4$)

WFPS water filled pore space, *F* fertilization with cow slurry, *SDW* soil dry weight

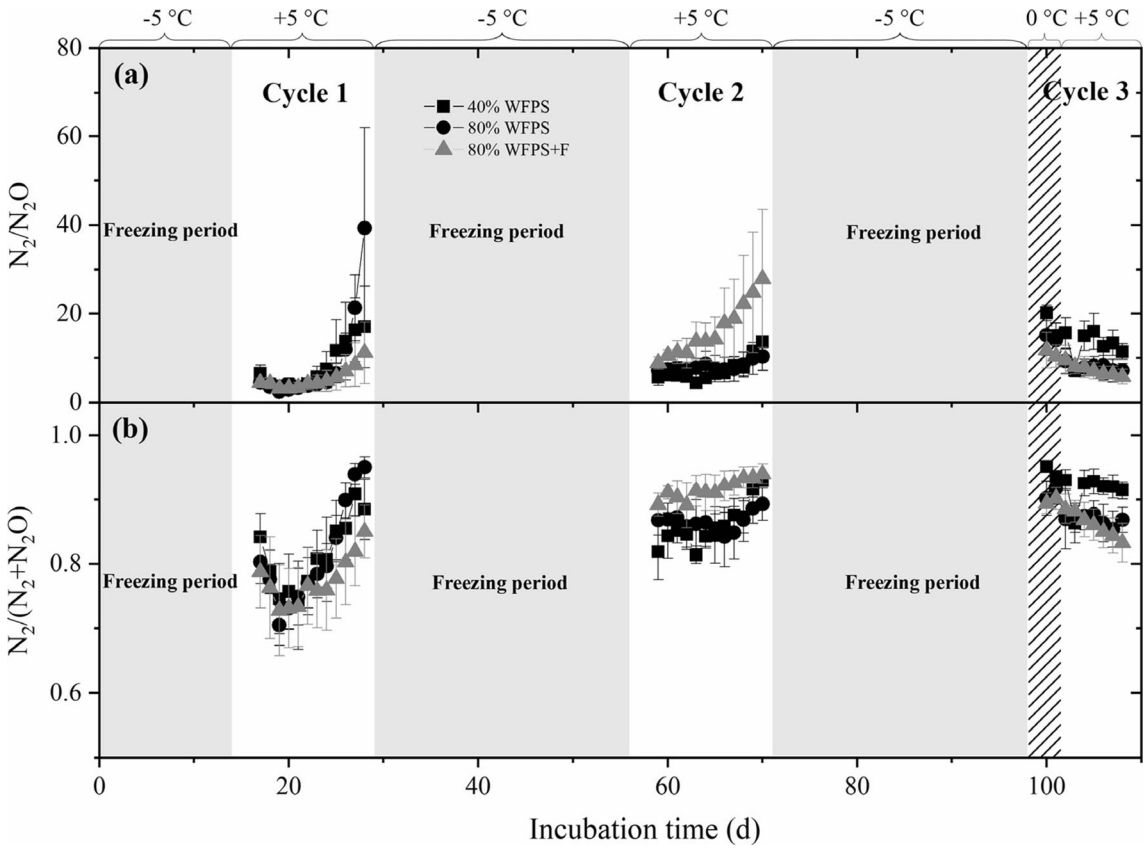

Fig. 3 Dynamics of N_2/N_2O (**a**) and $N_2/(N_2 + N_2O)$ (**b**) ratios during the freeze-thaw cycles of different treatments. WFPS: water filled pore space; F: fertilization with cow slurry. Gray color indicates freezing periods, the shaded area is indicating incubation at 0 °C

peak gaseous N emissions (Fig. 2). The number of transcripts for the archaeal *amoA* gene also strongly increased during the first freeze period (Fig. 2). In contrast, overall activity pattern of soil bacteria as indicated by the abundance of 16S rRNA did not show substantial change during the three FTCs; however, slurry and water addition significantly increased the amount of 16S rRNA in our study ($P < 0.05$, Fig. S3).

The mRNA/DNA ratios of *cnorB* and *nosZ* clade I showed generally similar temporal dynamics as observed for gene transcripts with substantially increased ratios after water and slurry addition as well as during thawing periods (Fig. S4).

Relationships between gene transcripts and the associated N gas fluxes

There were no significant correlations between the measured abundances of key populations driving denitrification and the associated N gas fluxes (data not shown). In contrast, the *nosZ* clade I and *cnorB* gene transcripts showed good explanatory power for N_2O and N_2 emissions (Fig. 4). Specifically, N_2O emissions were significantly correlated to both *cnorB* and *nosZ* clade I transcripts ($P < 0.01$, Fig. 4a, b) as well as the ratio of mRNA/DNA ratio of the respective genes ($P < 0.05$) when data from both FTC cycle 1 and 3 were analyzed. The relationship between the abundance of *nosZ* clade I transcript and N_2 emission was marginally significant when all data were used ($P = 0.07$), while the correlations were significantly improved when data were separated for single FTC ($P < 0.05$, Fig. 4c).

Discussion

Effect of soil moisture and temperature on N_2O and N_2 fluxes during FTCs

It has been well established that FTCs can trigger N_2O pulse emissions, with reported emissions ranging from ca. 10–4200 μg N_2O-N m^{-2} h^{-1} during FTCs (Grogan et al. 2004; Matzner and Borken 2008; Kim et al. 2012; Congreves et al. 2018). N gas pulse emissions during the thawing period after freeze events might include both the physical effects (e.g. substrate release) as well as the effect of temperature increase on enzymatic activities (Risk et al. 2013). However, field measurements of N_2O emissions at the investigated site showed generally low N_2O background fluxes of few μg N m^{-2} h^{-1} with no effects of temperature changes (Unteregelsbacher et al. 2013). This might indicate that also the N gas flux peaks in this study were primarily attributed to freeze-thaw effects sensu stricto, i.e., to physical soil

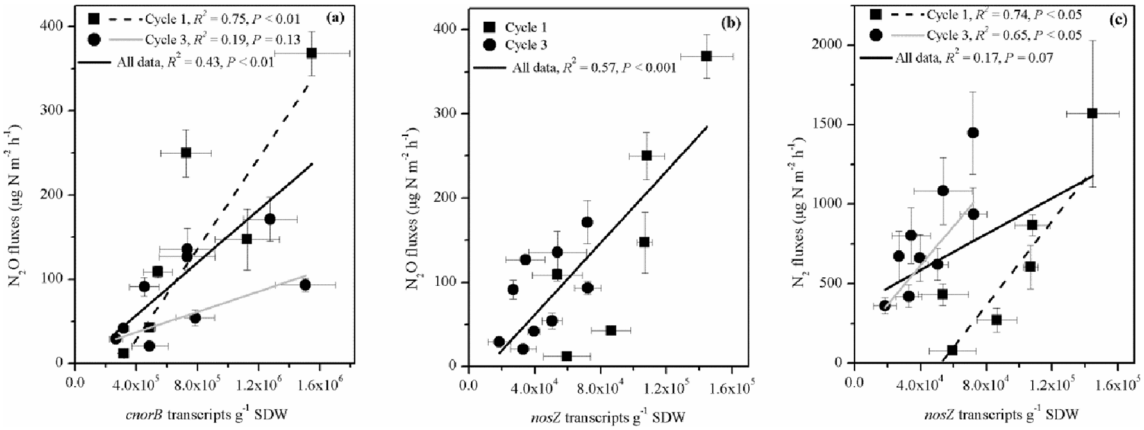

Fig. 4 Relationships between *cnorB* mRNA transcripts and N_2O fluxes (**a**), between *nosZ* clade I mRNA transcripts and N_2O fluxes (**b**) and between *nosZ* clade I mRNA transcripts and N_2 fluxes (**c**). SDW: soil dry weight

disturbance and associated substrate liberation rather than to temperature increases.

Our mechanistic study used sieved soil to improve the comparability of N gas emission measurements and parallel gene analyses in separate subsamples. Despite we re-packed cores to the original bulk density, sieving soil might change soil structure, and thus, distribution of water and oxygen. Furthermore, homogenization of soil might have facilitated substrate release effects but neglects plant effects on soil N turnover. All of this can potentially alter N gas fluxes. Consequently, extrapolation to field fluxes of both N_2O and N_2 is generally uncertain. Therefore, the N_2O and N_2 fluxes in this study were generally expressed on soil dry weight basis. However, when expressed on area units, maximum N_2O peak emissions of 600 μg N_2O-N m^{-2} h^{-1} are observed in this study (only first FTC, treatment 80% WFPS + slurry), while all other FTC peaks reach up to maximal values of 300 μg N m^{-2} h^{-1}. Hence, the magnitude of N_2O emissions reported in our study is generally in line with earlier field studies on FTC at other sites. For the investigated soil, in situ N_2O emissions during FTC were not yet published.

There are only a few earlier studies on freeze-thaw N_2 emissions. Applying ^{15}N mass balance approaches to a chernozem in Alberta, Nyborg et al. (1997) indicated that a considerable fraction of the N lost via denitrification during spring thaw was most likely as N_2. By using the ^{15}N gas flux method to directly measure N_2 during FTC, Wagner-Riddle et al. (2008) found that the patterns of N_2 fluxes were similar to those of N_2O fluxes during soil thawing, and the peak N_2 fluxes were approximately 5–10 times higher than those of N_2O. Hence, our findings on FTC N_2 emissions are generally in line with the very limited earlier work on that topic. Moreover, for the soils of this study, Zistl-Schlingmann et al. (2019) reported N_2 emissions from intact-plant soil mesocosms of up to 2–4.5 mg N_2-N m^{-2} h^{-1} after heavy summer precipitation events. This is in the same order of magnitude as the FTC N_2 emissions from disturbed soil of ca 1–2.5 mg N m^{-2} h^{-1}. Nonetheless, the actual extent to which results of this study are transferable to field conditions still remains uncertain.

Although it is well known that soil moisture is a key regulator of temporal variability of N_2O fluxes (Wu et al. 2010; Friedl et al. 2016; Kuang et al. 2019), only little is known about the effect of soil moisture on N_2O fluxes during FTCs, and reported results are controversial (Wu et al. 2014). On the one hand, increased freeze-thaw-related N_2O emissions with increasing soil WFPS have been reported in previous investigations in semi-arid steppe and temperate spruce forests (Wolf et al. 2010; Wu et al. 2010). On the other hand, Teepe et al. (2004) reported that N_2O emissions during thawing increased with an increase of WFPS from 42 to 64%, but decreased between 64% and 76% WFPS in agricultural soils. In our study, significantly higher N_2O emissions were observed at 80% compared to 40% WFPS. Enhanced microbial metabolism by substrate supply due to ice-induced physical soil disturbance has been discussed as a reason for the higher N_2O emissions at higher WFPS values, and this might also explain our observations. Soil aggregates are disrupted as a result of ice crystals expanding in pores between particles during FTCs, thereby exposing physically protected organic matter and increasing the amount of accessible substrate for N_2O emissions (Grogan et al. 2004; Kim et al. 2012). This assumption is supported by our results of soil properties, which showed significantly higher soil inorganic N and microbial biomass C/N contents for soils incubated at 80% WFPS as compared to soils incubated at 40% WFPS (Table 1). Moreover, higher soil moisture content might result in a higher share of anaerobic microsites in the topsoil during thawing periods, which are known to promote N_2O emissions by denitrification at least to a certain extent (de Bruijn et al. 2009; Wolf et al. 2012).

In contrast to our hypothesis, the N_2/N_2O ratios did not significantly differ between the 40% and 80% WFPS treatments so that higher soil moisture promoted total

denitrification based on both enhanced N_2O and N_2 emissions (Fig. 3). This indicates that 80% WFPS in combination with FTCs was not sufficient to trigger the strict anaerobicity that is required for a strong promotion of the use of N_2O as alternative electron acceptor in the denitrification chain (Butterbach-Bahl et al. 2013). This is in agreement with previous studies reporting highest N_2O emissions between 80% and 100% WFPS (Ciarlo et al. 2007; Zhu et al. 2013).

The gradually increasing N_2/N_2O ratios observed in particular during the first thawing period could be possibly related to the release of low-temperature inhibition of N_2O reduction to N_2 at increasing temperatures; however, the existence of such a low temperature effect on N_2O reductase is controversially discussed (Risk et al. 2013). The continuously high nitrate availability of > 10 mg N kg^{-1} sdw (Fig. 1) indicates that the changes in electron acceptor availability might not have caused increases in N_2/N_2O ratios.

In contrast to N_2O, there is no earlier experimental evidence of how soil moisture affects N_2 emissions during FTCs. In our study, enhanced N_2 emissions with increasing soil WFPS were observed during the three FTCs, which is consistent with previous observations under unfrozen soil conditions and based on acetylene or ^{15}N techniques on general dependence of N_2 emissions on soil moisture (Ciarlo et al. 2007; Zhu et al. 2013). This matches the hypothesis of many models that the magnitude of N_2 emissions during denitrification increases with increasing soil moisture (Li et al. 2000; de Bruijn et al. 2009). Furthermore, the direct N_2 flux measurements in combination with molecular tools in the present work provide experimental evidence for earlier assumptions that N_2 emissions by denitrification are dominating gaseous N losses during FTCs (Ludwig et al. 2004; Wu et al. 2010) and confirm that such N losses can be of significant importance for the ecosystem N mass balance (Nyborg et al. 1997).

Relationships between abundance of activity of denitrifers and nitrifiers and the formation of N_2O and N_2

Throughout our incubation assay under changing moisture and fertilization/substrate availability, we did not detect significant changes in numbers of nitrifiers and denitrifiers as indicated by the measurements of functional genes for the different processes. Such a decoupling between the genetic structure and N gas production estimates has been reported for soils of various ecosystems (Miller et al. 2009; Henderson et al. 2010; Yoshida et al. 2012) and confirms that regulation of processes occurs more on the level of the activity than on the level of the population size. In contrast to DNA, the mRNA levels of *cnorB* and *nosZ* clade I and the ratios of mRNA/DNA dynamically responded to FTCs and changes in moisture and fertilization and co-varied with corresponding N gas fluxes. We also detected the expression of the archaeal *amoA* gene throughout the FTCs, providing evidence that both nitrification and denitrification were occurring in the FTCs. An earlier study at our field site also found that the archaea are probably triggering nitrification during winter (Wang et al. 2016). Based on both molecular data and evidence from ^{15}N isotope pool dilution gross process studies, the latter study also reported large gross ammonification and nitrification activity in frozen soil, which is in agreement with increasing transcript levels of *amoA* in the first soil frost cycle in this study. However, the missing relationship between N_2O and *amoA* expression indicates rather minor contribution of nitrification to N_2O emission during the FTCs. In contrast, the dynamics in the activity of denitrifers as shown by *cnorB* and *nosZ* clade I transcripts with significant correlation to N_2O- and N_2 fluxes indicates that de novo denitrification contributed strongly to the measured gaseous emissions of N_2O and N_2 during FTCs. Hence, our study illustrates that quantifying transcripts of genes coding for key enzymes of denitrification and nitrification are a valuable approach to explain both N_2O and N_2 gas fluxes during FTCs (Butterbach-Bahl et al. 2013; Chen et al. 2015). However, it has to be considered that only the "classical" *nosZ* clade I gene was investigated, targeting mainly alpha-, beta-, and gamma-proteobacteria. Recently, a second cluster of *nosZ* genes was described (Jones et al. 2013), encoding for enzymes which catalyze the same reaction but have less than 50% amino acid sequence similarity with type I *nosZ* enzymes. These *nosZ* clade II genes were found among a diverse range of bacterial and archaeal phyla (Jones et al. 2013), suggesting that not all N_2O reducers were covered in the present study. Thus, consideration of clade II *nosZ* genes could further improve the explanatory power of transcripts of denitrification genes to predict gaseous N losses, especially answering the question about the importance of non-denitrifying N_2O reducing organisms (which often possess *nosZ* clade II genes) for mitigating N_2O emissions.

Both slurry addition and increased WFPS significantly increased the N_2O and N_2 fluxes, accompanied by enhanced *cnorB* and *nosZ* clade I expression, suggesting enhanced total denitrification. This is well in agreement with our general understanding of the response of denitrification rates to soil moisture (Braker and Conrad 2011; Butterbach-Bahl et al. 2013). As for the positive effects of slurry as fertilizer on N gas fluxes, there might be two potential mechanisms: (1) the substrate addition of inorganic N and DOC that is known to promote denitrification (Braker and Conrad 2011) and (2) the increase of microbial biomass in soil as a result of the introduced microbiome of the slurry itself, which was evident from increased levels of 16S rRNA genes immediately after slurry addition (Fig. S2a). Since the ability to denitrify is widespread among bacteria, with approx. half of the bacteria containing the *narG* gene (Zumft 1997), the addition of denitrifiers by slurry might significantly matter and contribute to the N gas fluxes. The above two aspects could also be synergistic.

Studies indicating a correlation between *nosZ* abundance and/or expression and N_2 emissions are rare, despite the fact that *nosZ* is the only enzyme known to date that utilizes N_2O as a primary substrate (Jones et al. 2013). Henderson et al. (2010) and Dandie et al. (2011) did not detect significant relationships between *nosZ* gene expression and N_2O emissions or denitrification rates in agricultural soils. This is not surprising, considering that *nosZ* gene expression as a measure of gross N_2O consumption and gross N_2 formation may not necessarily be related to net N_2O loss (Wen et al. 2016). In a study on FTCs, Németh et al. (2014) indeed found that N_2O emissions were negatively related to the *nosZ* transcript levels. Consequently, *nosZ* gene expression should be closely related to N_2 production, which was demonstrated in this study for the first time during FTCs, while such a relationship had been revealed for growing season fertilization events already in our previous work (Chen et al. 2015). The fact that both *cnorB* and *nosZ* clade I are closely correlated to both N_2O and N_2 might be related to the relatively stable N_2O/N_2 ratios in our study and is indicating relatively stable relationships between gross N_2O soil production and net N_2O emissions. The relatively stable ratios of N_2O/N_2 observed during FTCs might serve to estimate in situ FTC N_2 emissions based on in situ N_2O monitoring during FTC events in the studied ecosystem.

Effect of fertilization on N_2O and N_2 fluxes during FTCs

As expected, cow slurry incorporated into montane grassland soil significantly increased N_2O and N_2 emissions during FTCs (Table 2). Over three FTCs in 3.5 months, the additional gaseous N losses in the 80% WFPS plus fertilizer treatment compared to the 80% WFPS treatment without fertilizer amounted to almost half of the added fertilizer N. This is in line with our general understanding that fertilization stimulates denitrification, especially at high soil moisture levels (Ciarlo et al. 2008; Wang et al. 2013; Pan et al. 2018).

With only 0.3 kg N ha^{-1} $year^{-1}$ background, N_2O field emissions at the site analyzed are very low (Unteregelsbacher et al. 2013). Keeping in mind that a comparison of field fluxes with this incubation study based on sieved soil requires great care, the estimated N_2O fertilizer emissions of ca 1.5 kg N_2O-N ha^{-1} or 4% of fertilizer N during FTCs observed here nonetheless point at an important role of FTC fertilizer N_2O emissions for the non-CO_2 greenhouse gas balance in this ecosystem. This is also confirmed by emission factor considerations, i.e., the 4% N_2O emission factor obtained in this study is—probably due to the clayey texture—higher than the average emission factor for addition of animal slurry to agricultural soils of ca 1.2% (Charles et al. 2017).

Increased N_2 losses due to combined occurrence of manuring and FTCs will not affect atmospheric radiative forcing, but might affect the ecosystem N balance and fertilizer N use efficiency. With a potential loss of up to half of slurry N application, a potential maximal N_2 loss of 16 kg N ha^{-1} appears possible at a moderate autumn slurry addition of 36 kg N ha^{-1} followed by intense FTCs. This share of slurry-N lost by N_2 within 3 FTCs is slightly higher than the N_2 loss of 31–42% that was detected within 2 weeks in a laboratory experiment simulating a surface slurry addition (of 50 kg N ha^{-1}) to the soil of this study in summer under incubation conditions of 70% WFPS and 18 °C (Zistl-Schlingmann et al. 2019). Despite transferability of our findings to field conditions remain limited, our findings imply that the farmers' practice of applying slurry late in autumn could involve the risk of limited N use efficiency when there are several severe FTC in the subsequent winter.

Conclusions

Linking N_2O/N_2 flux measurements from montane grassland soil under controlled laboratory conditions over three intense FTCs with analysis of denitrifier gene expression provided a solid mechanistic basis to conclude that FTCs can trigger pulse emissions not only of N_2O but also of N_2. Since freeze-thaw N_2 pulse emissions exceeded those of N_2O by a factor of 5–30, these N_2 losses could be relevant for N use efficiency and ecosystem N mass balances. Increasing soil moisture and fertilization can strongly increase both FTC N_2O and N_2 emissions. To more broadly understand the relevance of FTC N_2 emissions and to accurately represent them in biogeochemical models, direct field measurements of FTC N_2 emissions in different ecosystems are needed, thereby also considering different freezing temperatures. This, however, still poses severe methodological challenges.

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