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N_2O emissions and product ratios of nitrification and denitrification as affected by freezing and thawing

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Abstract

Agricultural soils contribute significantly to atmospheric nitrous oxide (N_2O). A considerable part of the annual N_2O emission may occur during the cold season, possibly supported by high product ratios in denitrification ($N_2O/(N_2+N_2O)$) and nitrification (N_2O-N/(NO_3^--N + NO_2^--N)) at low temperatures and/or in response to freeze–thaw perturbation. Water-soluble organic materials released from frost-sensitive catch crops and green manure may further increase winter emissions. We conducted short-term laboratory incubations under standardized moisture and oxygen (O_2) conditions, using nitrogen (N) tracers (^{15}N) to determine process rates and sources of emitted N_2O after freeze–thaw treatment of soil or after addition of freeze–thaw extract from clover. Soil respiration and N_2O production was stimulated by freeze–thaw or addition of plant extract. The N_2O emission response was inversely related to O_2 concentration, indicating denitrification as the quantitatively prevailing process. Denitrification product ratios in the two studied soils (pH 4.5 and 7.0) remained largely unaltered by freeze–thaw or freeze–thaw-released plant material, refuting the hypothesis that high winter emissions are due to frost damage of N_2O reductase activity. Nitrification rates estimated by nitrate (NO_3^-) pool enrichment were 1.5–1.8 μg NO_3-N g^{-1} dw soil d^{-1} in freeze–thaw-treated soil when incubated at O_2 concentrations above 2.3 vol% and one order of magnitude lower at 0.8 vol% O_2. Thus, the experiments captured a situation with severely O_2-limited nitrification. As expected, the O_2 stress at 0.8 vol% resulted in a high nitrification product ratio (0.3 g g^{-1}). Despite this high product ratio, only 4.4% of the measured N_2O accumulation originated from nitrification, reaffirming that denitrification was the main N_2O source at the various tested O_2 concentrations in freeze–thaw-affected soil. N_2O emission response to both freeze–thaw and plant extract addition appeared strongly linked to stimulation of carbon (C) respiration, suggesting that freeze–thaw-induced release of decomposable organic C was the major driving force for N_2O emissions in our soils, both by fuelling denitrifiers and by depleting O_2. The soluble C (applied as plant extract) necessary to induce a CO_2 and N_2O production rate comparable with that of freeze–thaw was 20–30 μg C g^{-1} soil dw. This is in the range of estimates for over-winter soluble C loss from catch crops and green manure plots reported in the literature. Thus, freeze–thaw-released organic C from plants may play a significant role in freeze–thaw-related N_2O emissions.

Keywords: Nitrous oxide; N_2O; Freeze–thaw; Denitrification; Nitrification; ^{15}N; Respiration; Catch crop

1. Introduction

The increasing N_2O concentration in the atmosphere causes concern due to its contribution to global warming (Hansen and Sato, 2004) and destruction of stratospheric ozone (Dyominov and Zadorozhny, 2005). Since agriculture contributes about 50% to the anthropogenic N_2O emission (IPCC, 2001), modern agriculture must strive to reduce its contribution to atmospheric N_2O.

Freeze–thaw events during winter and spring are known to induce a pulse of N_2O emitted at or shortly after thawing (Goodroad and Keeney, 1984; Christensen and Tiedje, 1990; Dörsch et al., 2004), partly attributable to release of physically trapped N_2O (Goodroad and Keeney, 1984), but primarily due to biological activity (Röver et al., 1998; Öquist et al., 2004). The phenomenon is quantitatively significant; winter emissions may exceed 50% of the

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annual emission (Flessa et al., 1995; Röver et al., 1998) and the mechanisms must be taken into account when evaluating agricultural practices for their mitigation potential.

Freeze–thaw events result in transient pulses of C and N mineralization, due to frost release of physically protected soil organic material (Christensen and Christensen, 1991) and frost damage to microorganisms (Mackey, 1984; Skogland et al., 1988; Schimel and Clein, 1996). Enhanced O_2 consumption, combined with high water content of thawing soil, will increase the anaerobic volume and thus enhance denitrification. The freeze–thaw-induced emission of N_2O could thus be a straightforward result of enhanced denitrification. In addition, the relative activity of N_2O reductase can be lowered at near-freezing temperatures (Melin and Nommik, 1983; Holtan-Hartwig et al., 2002a), possibly resulting in high $N_2O/(N_2+N_2O)$ ratios in thawing soil. A high $N_2O/(N_2+N_2O)$ ratio in thawing soil could also be a "post-freezing trauma"; N_2O reductase (NOS) appears to be more vulnerable to perturbations than the other denitrification enzymes, and if this holds for frost damages, it would result in a higher $N_2O/(N_2+N_2O)$ product ratio after freezing. Freeze–thaw also enhances N mineralization and hence nitrification (DeLuca et al., 1992). Lowered oxygen availability during freeze–thaw-induced respiration bursts could lead to such high N_2O ratios, since the $N_2O/(NO_3^- + NO_2^-)$ ratio of nitrification increases sharply in response to oxygen limitation (Goreau et al., 1980; Davidson, 1991; Dundee and Hopkins, 2001).

Substantial over-winter losses of aboveground material from various catch and cover crops have been reported (Dejoux et al., 2000; Korsæth et al., 2002), a part of which will be available for microorganisms as melt water infiltrates the soil. Frost-vulnerable plants could thus further stimulate N_2O emission from nitrification/denitrification during thawing.

Here we describe a series of experiments designed to explore how process rates and product ratios of nitrification (N_2O-N/(NO_3^--N + NO_2^--N)) and denitrification ($N_2O/(N_2+N_2O)$) are affected by freezing and thawing of the soil and/or the release of water-soluble material from frost-damaged plants. ^{15}N tracer techniques were used to discriminate between nitrifier- and denitrifier-produced N_2O under controlled temperature and O_2 conditions. The following questions were asked. Is freeze–thaw-induced N_2O due to increased nitrification, denitrification, or both? Does frost-released plant C or N contribute to soil freeze–thaw-induced N_2O emissions? Does freeze–thaw increase the $N_2O/(N_2+N_2O)$ ratio of denitrification and thus contribute to the freeze–thaw N_2O pulse?

2. Materials and methods

2.1. Soils

Soil 1 was a mollic gleysol (FAO/ISRIC/ISSS, 1998) with 13% clay (<2 μm), 68% silt (2–60 μm), 19% sand (>60 μm) in the mineral fraction; the soil contained 11.4% organic C and 0.62% organic N and pH (H_2O) was 4.5. The soil was sampled in late autumn from the plough layer of a field previously grown with potatoes after 1 y with fallow. The soil was air dried with frequent mixing by hand until crumbling was possible. The dried soil was shaken in a sieve rack, and the 2–6 mm aggregate size fraction was recovered and stored in plastic bags at 4–5 °C until use. The moisture content of the air-dried aggregates was 0.54 ml g^{-1} dw. Prior to incubation, the moisture content was adjusted to 0.68 ml g^{-1} dw (corresponding to approximately −10 kPa soil moisture tension) by addition of 0.14 ml water or plant extract g^{-1} dw.

Soil 2 was a mineral soil with 5% clay, 86% silt and 9% sand, containing 6% organic C and 0.4% N. The pH (H_2O) was 7.0. Soil 2 was used in one experiment only as a pH contrast to soil 1 (see Section 2.8). Unless stated otherwise, soil 1 was used.

2.2. Plants

Red clover was grown in pots with Vermiculite (expanded mica) in a greenhouse. The plants were grown both unlabelled and ^{15}N labelled. Dissolved $NH_4^{15}NO_3$ in mixture with mineral fertilizers was added in regular doses throughout the entire growth period in order to achieve homogenous labelling. This resulted in plants with 2.79 ± 0.04 at% ^{15}N in homogenized bulk dry matter. Unlabelled plants were given the same growth conditions and nutrient solutions, but without isotopic labelling. At the end of the growth period, plants were set at 6 °C for 4 weeks and then to 0.5 °C for 2 weeks to simulate autumn adaptation (Junttila et al., 1995). After harvesting, the plants were immediately frozen (−18 °C) and stored in plastic until further use (2–3 months).

2.3. Plant extract

We simulated winter loss of plant N + C by freezing, thawing and leaching the plants: 10 g (1.49 g dw) of whole, frozen (−18 °C), aboveground clover material was put in a 150 ml centrifuge tube with a Whatman GF/A filter on a perforated filter holder above a 35 ml sap collection container. The material was gently compacted, 10 ml of deionized water was added and the containers were immediately centrifuged at low speed (500 × *g*, 5 min) at +1 °C as the plants thawed. This extraction was repeated three times for each portion of plants, and the extracts were pooled and immediately frozen to −18 °C. The extract was analyzed for pH (6.0), total C (2.36 ± 0.062 mg C ml^{-1}), total N (0.197 ± 0.009 mg N ml^{-1}), NO_3^- (13.7 μg NO_3^--N ml^{-1}), and at% ^{15}N of total N (2.72 ± 0.04 at%) and NO_3^- (0.99 ± 0.03 at%).

Denitrification activity in the plant extract was tested by anaerobic incubation at 20 °C with 30 μg NO_3^--N ml^{-1} under 0.1 hPa acetylene (C_2H_2). No N_2O and CO_2

production was observed before 40 h, hence this source of N_2O was ignored in our experiments (10–30 h).

As the isotope experiments (Section 2.7) required the absence of NO_3^- in the plant extract, NO_3^- had to be removed with otherwise minimal changes in the plant extract. A *Pseudomonas chlororaphis* culture lacking NOS was incubated anaerobically with the plant extract at 20 °C for 1 d, followed by heat treatment to 82 °C to kill the bacteria. NO_3^- removal was complete and the heat treatment was effective as tested by reincubation experiments (not shown). The plant extract without NO_3^- was used only in the ^{15}N tracer experiments.

2.4. Analysis

NO_3^- and NH_4^+ contents in soils were measured colorometrically in 2 M KCl extracts (1:3, soil:extractant ratio). N_2O and CO_2 were analyzed on an automated GC–ECD/TCD setup described by Sitaula et al. (1992). This system allows semi-continuous monitoring of N_2O and CO_2 during incubation of up to 26 reaction bottles in a thermo-regulated water bath. Sampling was done automatically with a Gilson XL 222 auto-sampler connected to a loop injection system with 0.5 ml sample injected per measurement, which was replaced by He.

2.4.1. Isotope analysis

Plant material was analyzed on a ThermoQuest NCS 2500 elemental analyzer coupled to a VG Isotech continuous flow isotope ratio mass spectrometer (CF-IRMS). Samples were dried and homogenized by grinding before combustion. Precision on standard analysis was better than 0.3‰ ($\delta^{15}N$). NH_4^+ and NO_3^- from soil extracts were prepared by the diffusion method of Mulvaney and Khan (1999) with some modifications. Recoveries of NH_4^+ and NO_3^- in test solutions were $94.3 \pm 1.5\%$ and $97.9 \pm 1.9\%$, respectively. The standard deviations of $\delta^{15}N$ analysis of diffused standard were 0.58‰ and 0.53‰ for NH_4^+ and NO_3^-, respectively, when the other ion in the solution (NO_3^- or NH_4^+, respectively) was ^{15}N enriched to a $\delta^{15}N$ value of 3000–4000. $\delta^{15}N$ in N_2O was analyzed on a Thermo Finnigan Delta XP plus CF-IRMS coupled to an online gas concentration system (Thermo Finnigan Precon). The standard deviation of 30 air samples was less than 0.3‰.

2.5. Anaerobization and adjustment of O_2 concentrations prior to incubation

The anaerobization procedure consisted of five cycles of evacuation (2 min) and filling with He (0.5 min) and venting of the final He overpressure. To adjust initial O_2 concentrations, aliquots of the headspace were withdrawn with a gas-tight syringe and equal amounts of O_2 were injected. N_2O reductase activity was inhibited by injecting C_2H_2 to a final concentration of 10 vol%, followed by venting.

2.6. Experiment 1: CO_2 and N_2O production in response to freezing and thawing, plant extract and variable O_2 concentrations

Experiment 1 was conducted to compare plant extract and freeze–thaw as to their effect on respiration (CO_2 production) and net N_2O production. 10 g (ww) of soil 1 (6.5 g dw) was weighed into 120 ml acid-washed serum bottles and capped with butyl rubber caps with metal crimps. The soil was compacted by centrifugation ($300 \times g$, 5 min). The compaction was done only in experiment 1. Half of the samples were frozen at −20 °C for 5 d, and the other half was kept at 5 °C.

After thawing, 0.93 ml water or clover extract was added (resulting in a final soil moisture tension of −10 kPa), the bottles were recapped and, except for the aerobic treatment, anaerobized immediately, and headspace O_2 concentrations adjusted to either 21, 7, 2.3, 0.8 or 0 vol% ($n = 3$). The clover extract added represents an input of 338 ± 8.8 μg C and 28 ± 1.3 μg N g^{-1} dw soil. N_2O and CO_2 accumulations (5 °C) were measured every 2 h for 9 h and production rates were calculated from the change in concentration over time (linear regression for each bottle).

The quantitative effects of soil freeze–thaw and plant extract on denitrification, freeze–thaw-induced denitrification and respiration rates were compared with those evoked by a range of plant extract concentrations. The plant extract was diluted 1:5, 1:7.5, 1:10, 1:12.5 and 1:15. Treatments were (1) Control, (2) freeze–thaw-treated soil, (3) undiluted plant extract, (4–8) diluted plant extracts. Equal amounts of liquid (0.93 ml per flask containing 10 g soil fresh weight) were used for all treatments, either as deionized water (controls and freeze–thaw treatments) or as plant extract. All treatments were incubated aerobically and anaerobically at 20 °C, with and without 10 vol% C_2H_2.

The plant extract (13.7 μg NO_3^--N ml^{-1}) increased the nitrate concentration in the soil from 4.0 μg NO_3-N g^{-1} to 6.0 μg NO_3-N g^{-1}. In a separate experiment, we tested whether denitrification would be limited by NO_3^- concentrations by measuring denitrification rates under anaerobic conditions as affected by adding 0, 5 or 15 μg NO_3^--N g^{-1}.

2.7. Experiment 2: tracing organic N and NH_4 as sources of N_2O

Experiment 2 was designed to differentiate between the N sources of the emitted N_2O. Portions of 10 g (ww) soil in serum bottles were frozen for 5 d. Three different amendments were used to determine whether NH_4^+ or organic N contributed significantly to N_2O emission: (1) ^{15}N-labelled plant extract, (2) non-labelled plant extract with ^{15}N-labelled NH_4^+ (5 μg NH_4^+-N ml^{-1}, 5 at% ^{15}N) and (3) ^{15}N-labelled NH_4^+ solution (5 μg NH_4^+-N ml^{-1}, 5 at% ^{15}N). After anaerobization, three replicates of each treatment were adjusted to either 21, 7, 2.3, 0.8 or 0 vol% O_2. The bottles were incubated at 5 °C for 24 h to ensure sufficient

amounts of N_2O for isotope analyses. N_2O concentration was measured every third hour in one of the three parallels per treatment/O_2 concentration. At the end of the incubation, N_2O was sampled for $\delta^{15}N$ analysis by transferring headspace gas to an evacuated 120 ml bottle through a double tip needle (>5 min to avoid fractionation). Gas samples and KCl extracts were analyzed for $\delta^{15}N$ in N_2O, NH_4^+ and NO_3^- as described above.

2.8. Experiment 3: freeze–thaw effects on the denitrification product ratio

Experiment 3 was designed to investigate whether the observed stimulation of N_2O emission by freeze–thaw and plant extract could be attributed to a change in denitrification product ratio rather than to a change in denitrification rates. Since soil 1 was found to have a stable high $N_2O/(N_2+N_2O)$ product ratio, we included a second soil with a higher pH, which we expected to be more susceptible to changes in the $N_2O/(N_2+N_2O)$ ratio.

Soil 1: 10.0 g ww (6.5 g dw) was incubated anaerobically at 5 °C with or without freeze–thaw treatment and not diluted plant extract additions as described for experiment 1. A higher incubation temperature treatment (20 °C) was included to check whether high product ratios could be due to the low incubation temperature. Soil 2: 5 g ww (3.12 g dw) was incubated anaerobically at 5 °C for 2 days with or without freeze–thaw and plant extracts (0.46 ml). All treatments were incubated anaerobically, with or without C_2H_2 ($n=3$).

2.9. Calculations

N_2O and CO_2 production rates were calculated from the linear slope of concentration change over time, corrected for gas sampled and dissolution in soil water. The production of gas was largely constant within the evaluated time spans.

The relative contribution of nitrification to N_2O emission was estimated based on the assumption that NH_4^+ and NO_3^- were the only sources of N_2O. Thus Eq. (1) applies:

$$(a+b)\times {}^*N_2O = (a\times {}^*NH_4^+)+(b\times {}^*NO_3^-), \quad (1)$$

where a is the relative share of N_2O-N originating from NH_4^+-N, b is the relative share of N_2O-N originating from NO_3^-, $^*N_2O$ is at% ^{15}N in N_2O, $^*NH_4$ is at% ^{15}N in NH_4^+ and $^*NO_3$ is at% ^{15}N in NO_3^-. Since $a+b=1$, we get

$$a = ({}^*N_2O - {}^*NO_3^-)/({}^*NH_4^+ - {}^*NO_3^-). \quad (2)$$

Eq. (2) gives the amount of N_2O derived from NH_4^+ relative to the total N_2O production. As the enrichments of NO_3^- and NH_4^+ change during incubation due to nitrification and mineralization, the average of NH_4^+ and NO_3^- isotope values measured at the start and end of the experiment were used. The N_2O produced at anaerobic conditions, at which no nitrification was detected, was 29.5‰ lighter than the initial nitrate, which can be ascribed to isotopic fractionation during denitrification. Barford et al. (1999) found a fractionation factor of 28.6‰ N_2O production by *Paracoccus denitifricans*. As this would influence the calculated contribution of nitrification to N_2O production, a value of 29.5‰ was subtracted from all measured $\delta^{15}N$ values of NO_3^- before applying $^*NO_3$ in Eq. (2). The calculations are analogous to that described by Shearer and Kohl (1993) and based on the assumptions that the N_2O produced under anaerobic conditions is entirely due to denitrification, and that the fractionation is identical for all O_2 concentrations.

To estimate nitrification rates from NO_3^- pool enrichment, the fraction c of the NO_3^- pool at the end of the experiment originating from labelled NH_4^+ was calculated analogously to Eq. (2):

$$c = ({}^*NO_{3End}^- - {}^*NO_{3Start}^-)/({}^*NH_4^+ - {}^*NO_{3Start}^-), \quad (3)$$

where c is the relative share of NO_3^--N at experiment end originating from NH_4^+-N, $^*NO_{3End}$ is at% ^{15}N in NO_3^- at experiment end, $^*NO_{3Start}$ is at% ^{15}N in NO_3^- and $^*NH_4$ is average at% ^{15}N in NH_4^+ during incubation. To estimate nitrification rates, c was multiplied with the NO_3^- concentration at the end of the incubation and divided by incubation time. This estimate of nitrification may be slightly underestimated, as it does not account for removal of NO_3^- (e.g., through denitrification).

Statistical differences between treatments were tested by Student's t tests in JMP 5.0 (SAS Institute).

3. Results

3.1. Experiment 1: CO_2 and N_2O production in response to freezing and thawing, plant extracts and variable O_2 concentrations

Addition of plant extract induced a 21 times increase in aerobic CO_2 production in non-frozen soil (Fig. 1A) and a similar increase in frozen soil (Fig. 1B). This plant extract induced respiration declined with decreasing O_2 concentrations. Freezing and thawing of the soil (full bars, Fig. 1B versus A) resulted in a 5–6 times increase in CO_2 production. In contrast to the plant-extract-induced respiration, the freeze–thaw-induced respiration was remarkably unaffected by the O_2 concentration in the headspace.

Assuming a molar ratio of 1 between emitted CO_2 and consumed O_2, we found that the total O_2 consumption throughout the incubation period was less than 30% of the initial O_2 present in the 0.8 vol% treatments. For the other treatments, the relative reduction was 10% or less.

Rates of N_2O production were enhanced by the addition of plant extract (open versus full bars, Figs. 1C and D), and the effect increased gradually with decreasing O_2 concentration. The freeze–thaw effect on N_2O production (full bars, Fig. 1D versus C) was only significant at low (0–2.3 vol%) initial O_2 concentration.

With decreasing concentrations of plant extract added to soil 1, the aerobic CO_2 and anaerobic CO_2 and N_2O

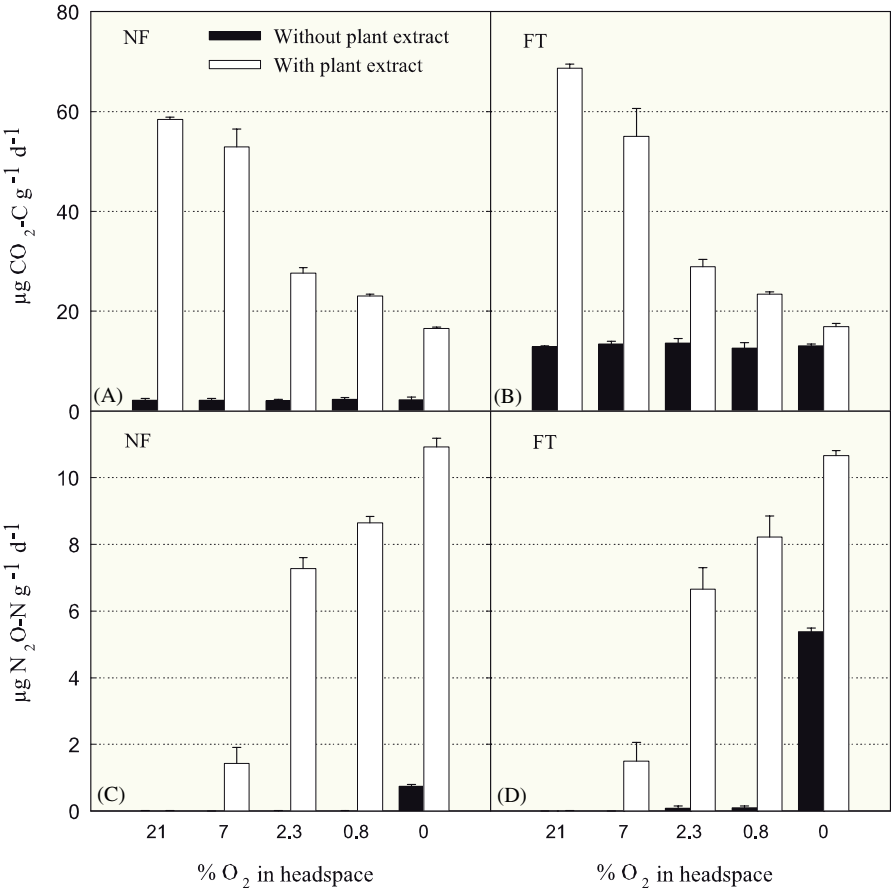

Fig. 1. Gas production rates as a function of soil treatments and initial O_2 headspace concentrations. Non-frozen (NF) and freeze–thawed (FT) soil was either amended with deionized water (full bars) or equal volumes of plant extract (open bars) and incubated for 9 h at 5° C. Production rates of CO_2 (A and B) and N_2O (C and D) were calculated by linear regression for each single bottle. Error bars show standard deviation ($n = 3$).

production rates gradually declined towards similar low values (Fig. 2). Plant extract induced CO_2 and N_2O production rates comparable to those measured after freezing and thawing in a dilution corresponding to about 20–30 μg C g^{-1} dw soil (1:10–1:15 dilution, Fig. 2, Table 1). Paired *t*-tests show that denitrification in 1:12.5 and 1:15 dilutions was not different from that in freeze–thaw-treated soil. For aerobic CO_2, all treatments were different from freeze–thaw, whereas for anaerobic CO_2, 1:10, 1:12.5 and 1:15 were not different from the freeze–thaw treatment ($P < 0.05$). A similar quantitative relationship was observed for soil 2 at anaerobic conditions and 5 °C (data not shown). Table 1 also gives the ratios between CO_2 and N_2O production measured under anaerobic conditions in the presence of C_2H_2, both for soils 1 and 2 (see also experiment 3). Assuming that the C source has an oxidation state of 0 (e.g., as glucose), the general equation for denitrification with N_2O as the sole end product

$$(CH_2O + NO_3^- \rightarrow CO_2 + 0.5\ N_2O + 1.5\ H_2O)$$

predicts a molar ratio of 0.5 between CO_2 production and N_2O production. The observed ratios were very close to 0.5 for soil 1 and slightly lower for soil 2, and quite stable across the treatments in both soils, suggesting that denitrifying activity accounted for a very large share of the measured C mineralization.

There was no significant effect of nitrate concentration on denitrification rates in any of the treatments during 24 h incubation (data not shown).

3.2. Experiment 2: tracing organic N and NH_4 as sources of N_2O

Depending on O_2 availability, plant extract treatments resulted in 2–4 times higher NH_4^+ concentrations by the end of the incubation (6–8 μg N g^{-1}) compared with the water plus NH_4^+ treatment (1.4–2.9 μg N g^{-1} soil). As only 0.7 μg NH_4^+-N g^{-1} was added initially, this demonstrates that N was mineralized in all treatments.

The $\delta^{15}N$ values of the N pools (NH_4^+, $NO_3^- + NO_2^-$ and N_2O) before and after 28 h of incubation are given in Table 2 and the estimated nitrification rates and nitrifier N_2O production rates are given in Table 3. Nitrification was found in the treatment without plant extract ($H_2O + {}^{15}NH_4^+$) as an increase ($P < 0.01$) in ^{15}N enrichment of the NO_3^- pool at 21–2.3 vol% O_2 (compared with the anaerobic treatment), and a visible but not significant increase in ^{15}N enrichment at 0.8 vol% O_2. The estimated

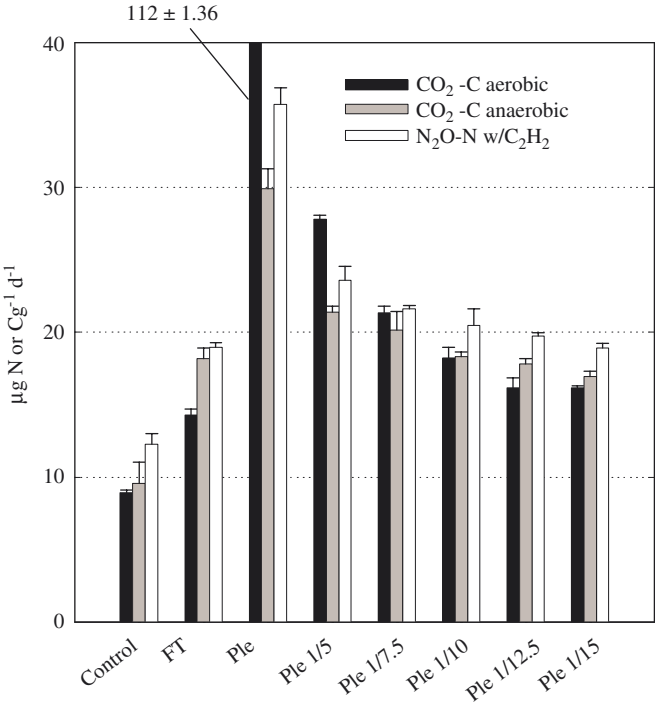

Fig. 2. Effects of plant extract concentration on CO_2 production and denitrification in soil 1 in the presence and absence of oxygen. Rates of CO_2-C production under aerobic (CO_2-C aerobic) and anaerobic (CO_2-C anaerobic) conditions, and the denitrification rates measured as N_2O-N under anaerobic conditions in the presence of 10% acetylene (N_2O w/C_2H_2) over 24 h of incubation at 20 °C are presented. Denitrification under aerobic incubation was negligible and is not shown. Treatments indicated in the graph are FT: freeze–thaw treated, Ple: plant extract amendments (1–1:15 dilutions).

Table 1
Ratio between N_2O and CO_2 production under anaerobic incubation with acetylene

Treatment	Plant C added (mg C g^{-1} soil)	Molar ratio N_2O/CO_2	
		Soil 1 (pH 4.5)	Soil 2 (pH 7)
Control	0	0.46	0.28
Freeze–thaw	0	0.42	0.36
Plant extract	0.338	0.46	0.26
1:5 Ple:H_2O	0.068	0.44	
1:7.5 Ple:H_2O	0.045	0.45	
1:10 Ple:H_2O	0.034	0.44	0.36
1:12.5 Ple:H_2O	0.027	0.46	
1:15 Ple:H_2O	0.023	0.46	

Data for soils 1 and 2 are from experiments 1 and 3, respectively.

nitrification rates based on these data were similar for the three highest O_2 concentrations (1.5–1.8 µg NO_3-N g^{-1} d^{-1}), an order lower at 0.8 vol% O_2, and zero at 0 vol% O_2 (Table 3).

The N_2O pool in the $H_2O + {}^{15}NH_4$ treatment had $\delta^{15}N$ values ranging from −23‰ at 0 vol% O_2 to 52.4‰ at 0.8 vol% O_2. The nitrifier contribution to N_2O production was significant ($P<0.01$) at 0.8, 2.3 and 20 vol% O_2. The estimated share of nitrifier-produced N_2O in the 0.8 vol% O_2 treatment was 4.35 ± 0.30%, with an $N_2O/(NO_3^- + NO_2^-)$ ratio of 0.27, and 0–1% for the higher O_2 concentrations. Due to low, and thus uncertain, N_2O production rates, combined with very low nitrification contribution to N_2O production, no nitrification product ratios could be calculated for the O_2 concentrations above 0.8 vol%. However, a nitrification product ratio of 10^{-3} or lower is probable.

In the treatments with added plant extract, the final $^{15}NO_3$ enrichment values were unaffected by oxygen concentrations. No initial data for $\delta^{15}NO_3$-N were available for this treatment, but since there was no difference in $\delta^{15}NO_3$ enrichment between the aerobic and anaerobic treatments, we may assume that nitrification was insignificant in the ^{15}N-Ple treatment at any of the O_2 concentrations. The slight ^{15}N enrichment of NO_3^- in the ^{15}N-Ple treatment compared with the other treatments ($\delta^{15}N = 37$‰) is probably due to traces of labelled NO_3^- remaining in the plant extract. Accordingly, there was no significant nitrification contribution to the N_2O emission from the ^{15}N-Ple treatments, except for 21 vol% O_2 ($P<0.01$, Table 2). The $\delta^{15}N_2O$ value for this treatment (95.4 ± 77‰) represents an NH_4^+ contribution to N_2O production of 2.5 ± 1.5%. In conclusion, less than 4.4% (Table 3) of the N_2O, and in most cases no N_2O, originated from nitrification.

3.3. Experiment 3: freeze–thaw effects on the denitrification product ratio

Table 4 shows the denitrification product ratios for anaerobic incubation. Soil 1 showed consistently high $N_2O/(N_2O + N_2)$ ratios (0.69–0.99) for all treatments. The ratio was unaffected by freeze–thaw, but increased with a value of ~0.2 by the addition of plant extracts with freeze–thaw ($P = 0.05$). The time course of N_2O accumulation (Fig. 3) showed linear increases with time (high R^2 in Table 4).

Soil 2, which had higher pH (7.0) than soil 1, had a lower $N_2O/(N_2 + N_2O)$ ratio than soil 1 (Table 4). As for soil 1, an almost linear increase in N_2O accumulation was observed within the relatively long incubation time (Fig. 3), and the response in N_2O emission and denitrification to plant extracts and freeze–thaw were similar to that in soil 1 (Table 4). The product ratio in soil 2 was reduced substantially by freeze–thaw ($P = 0.05$), but largely unaffected by plant extract and plant extract in combination with freeze–thaw.

4. Discussion

4.1. Nitrifications contribution to freeze–thaw emissions of N_2O

For nitrification to be of any significance for N_2O emission, the nitrification rates must be either very high or

Table 2
Average ($n = 3$) $\delta^{15}N \pm SD$ (‰) of NH_4^+, NO_3^- and N_2O before and after 28 h of incubation at 5 °C at different initial headspace O_2 concentrations

Treatment	Pool	Initial values	Final values after 28 h				
			21 vol% O_2	7 vol% O_2	2.3 vol% O_2	0.8 vol% O_2	0 vol% O_2
$H_2O + {}^{15}NH_4^+$	NH_4^+	2005±91	1177±29	1175**	1157±18.9	1366±27.0	1645±138
	NO_3^-	8.7±4	58±5	58±4	59±5.9	13±4.9	7±6.5
	N_2O	ND	3.6±7	−9.6±0.5	18.7±12.0	52.9±3.2	−23.3±14.9
Ple + $^{15}NH_4^+$	NH_4^+	1609±22	766±11	772±41	821±15.9	844±11.4	893±32.7
	NO_3^-	5.2±0.3	8±3	7±0.4	6±4.0	8±0.7	7±7.0
	N_2O	ND	−8.1±19	−17.3±11	−23.9±3.5	−26.1±0.9	−25.3±0.6
^{15}N-Ple	NH_4^+	ND	3940±125	3974±75	4099±73.6	3958*	4078±39
	NO_3^-	ND	30±3	68±35	43±7.8	49*	37±14.6
	N_2O	ND	95.4±77	1.7±14	−18.4±3.6	−9.2*	−17.1±4.1

The three treatments were addition of water and $^{15}NH_4^+$, unlabelled plant extract (Ple) and $^{15}NH_4^+$ and ^{15}N-labelled Ple. The $\delta^{15}N$ of labelled Ple was 6437‰.
ND, not determined; there was no initial N_2O, whereas NH_4^+ and NO_3^- samples were lost.
*One or **two replicates measured.

Table 3
Average rates of nitrification (±SD; $n = 3$), N_2O production (R^2 in parentheses), percent of N_2O production (±SD) that stems from nitrification and product ratio of nitrification (N_2O-N/NO_3-N + NO_2^--N)

Treatment (% O_2)	Nitrification (ng N $g^{-1}d^{-1}$)	N_2O production (ng N $g^{-1}d^{-1}$)	Total N_2O from nitrification (%)	Product ratio of nitrification (N_2O-N/NO_3-N)
21	1794±324	0.9 (0.37)	0.1±0.5	*
7	1535±78	1.9 (0.77)	−0.7±0.1	*
2.3	1662±131	0.4 (0.04)	1.1±0.09	*
0.8	172±118	1070 (0.98)	4.35±0.30	0.27±0.19
0	10±129	3050 (1.00)	0.04±0.65	—

Only data for the $H_2O + {}^{15}NH_4^+$ treatment (Table 2) are presented, since there was no detectable nitrification in the other treatments.
*Not significantly different from 0, but probably lower than 10^{-3}.

Table 4
Average N_2O production rates (±SD; $n = 3$) with and without acetylene (C_2H_2), and product ratios of denitrification ($N_2O/(N_2 + N_2O)$) in response to freeze–thaw and plant extract addition

Soil	Treatment	Temperature (°C)	No C_2H_2 (ng N_2O-N g^{-1} d^{-1})	With C_2H_2 (ng N_2O-N g^{-1} d^{-1})	$N_2O/(N_2 + N_2O)$	R^2
Soil 1	Water	5	1502±166	2040±152	0.74±0.10	0.89–0.91
	Water, freeze–thaw	5	4368±226	6379±526	0.69±0.07	1.00
	Plant extract	5	8301±525	9607±172	0.86±0.06	0.99–1.00
	Plant extract, freeze–thaw	5	6770±244	7831±514	0.87±0.07	1.00
	Water	20	7157±136	7212±278	0.99±0.04	0.98–1.00
Soil 2	Water	5	1021±160	1938±212	0.53±0.10	0.99–1.00
	Water, freeze–thaw	5	2802±55	7365±93	0.38±0.01	0.99–1.00
	Plant extract	5	5621±83	10224±90	0.55±0.01	0.99–1.00
	Plant extract, freeze–thaw	5	4131±6	8274±304	0.50±0.02	0.99–1.00

All ratios are based on linear regression of N_2O accumulation versus time under anaerobic conditions. The minimum and maximum R^2 of the linear regression with time for the six N_2O production rate estimates (±C_2H_2) within each treatment are given.

the product ratio of nitrification must be higher than the low values around 10^{-3} observed for most ammonia oxidizing bacteria under optimal conditions (Jiang and Bakken, 1999). In theory, freeze–thaw events could result in high rates and product ratios due to release of substrates for both ammonia-oxidizing bacteria and heterotrophic organisms in relatively wet soils. This could result in O_2 limitation of nitrification, which is known to raise the product ratio of nitrification (Goreau et al., 1980; Davidson, 1991; Dundee and Hopkins, 2001). The results did demonstrate such an increased product ratio for nitrification, but only for one O_2 concentration (0.8 vol%,

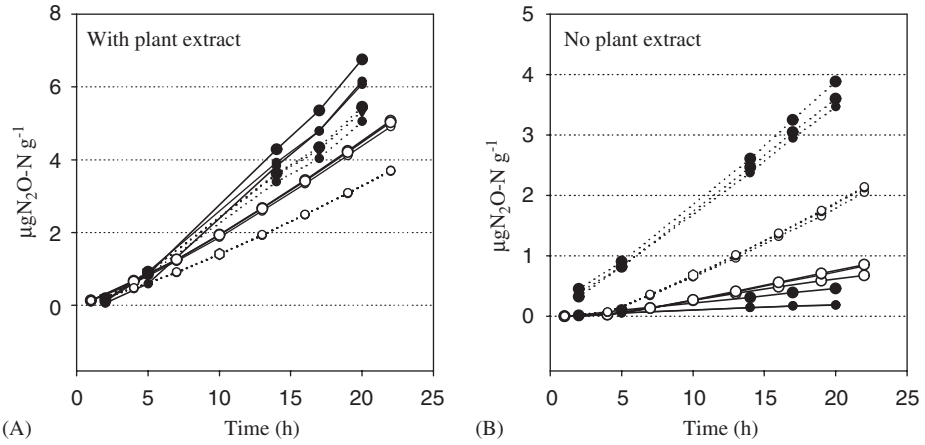

Fig. 3. Typical N_2O production curves from anaerobic incubations (experiment 3) without acetylene (C_2H_2). Left: with plant extract, right: without plant extract; filled symbols: soil 1, open symbols: soil 2; dashed lines: freeze–thaw-treated soil, continuous lines: non-frozen soil. Three replicates are shown for all treatments. Note the different scales on the *y*-axes.

Table 3), resulting in a moderate (4–5%) contribution of nitrification to the measured net N_2O production. At O_2 concentrations higher than 0.8 vol%, nitrification was apparently not O_2 limited and no nitrification N_2O production was detected.

In the treatments with plant extracts (Ple + $^{15}NH_4^+$ and ^{15}N-Ple), no significant nitrification rates could be determined by NO_3^- pool enrichment. This was not due to a shortage of NH_4^+ as net mineralization was shown. An explanation could be that plant extract amendment lowered O_2 concentrations and hence nitrification rates to levels below the detection limit of the ^{15}N pool enrichment method. In contrast, ^{15}N detection in N_2O was much more sensitive, explaining the minor nitrification contribution in the ^{15}N Ple treatment at 21 vol% O_2 (Table 2). In conclusion, the data suggest that N_2O emission from nitrification following freeze–thaw events is insignificant in this low pH soil except under O_2-limiting conditions, where it's contribution was still minor. Our results deviate from those of Bollmann and Conrad (1998), who found that nitrification was the major contributor to N_2O emissions over 0.5 and 1 vol% O_2 concentration in two neutral to alkaline (pH 7.5 and 8.5) soils, but their experiments contained no freeze–thaw or low-temperature treatment. In general, we would expect a higher nitrification $N_2O/(NO_3^- + NO_2^-)$ ratio in acid than in alkaline soils (Jiang and Bakken, 1999). Our findings are more comparable to the freeze–thaw experiments of Ludwig et al. (2004), who found denitrification to contribute 83% of the produced N_2O immediately after thawing and 72% after 70 h incubation in undisturbed soil cores. Priemé and Christensen (2001) found denitrification to be the dominant source of freeze–thaw-released N_2O from three out of four organic soils. The measured nitrification rates in our soil were in the same range as those reported for various soils (60–2500 ng N g^{-1} d^{-1}) under variable but comparable experimental conditions (Maag and Vinther, 1996; Cookson et al., 2002; Ludwig et al., 2004). Thus, the low nitrification contribution to N_2O emission is not due to a particularly low nitrification rate.

4.2. Freeze–thaw effects on denitrification product ratio

Freezing and thawing represents a severe perturbation that may kill a significant fraction of soil microbes (Skogland et al., 1988; DeLuca et al., 1992). We hypothesized that this perturbation could also result in a transient reduction in the relative activity of NOS and hence increased $N_2O/(N_2 + N_2O)$ product ratios, as has been demonstrated for low-temperature and heavy metal stresses (Holtan-Hartwig et al., 2002a, b). Our results give no evidence to support this hypothesis; the two soils tested had distinct $N_2O/(N_2 + N_2O)$ product ratios that were either unaltered or somewhat lowered by the freeze–thaw treatments (Table 4). Near-zero incubation temperatures were deliberately avoided in our freeze–thaw experiments, since several authors (Melin and Nommik, 1983; Holtan-Hartwig et al., 2002a; Dörsch and Bakken, 2004) have found that near-zero temperatures in themselves (without any previous freezing and thawing) may result in high $N_2O/(N_2 + N_2O)$ ratios due to apparent dysfunctioning of N_2O reductase (NOS). By using moderately high (5 °C) incubation temperatures, we were able to test whether frost had any selective damage to NOS. The results show that this is clearly not the case.

4.3. Freezing and thawing compared with organic C amendments

In agreement with numerous studies on freeze–thaw effects (e.g., Christensen and Tiedje, 1990; Schimel and Clein, 1996; Priemé and Christensen, 2001), we observed a substantial increase in CO_2 production compared with the non-frozen soil (Figs. 1, 2 and 3B). Adding undiluted extract from frozen clover plants resulted in substantially higher CO_2 production than in the freeze–thaw treatment

(Fig. 1), and this plant-extract-induced respiration was strongly dependent on O_2 concentration (Fig. 1). This contrasts the soil freeze–thaw-induced respiration, which was virtually unaffected by O_2, and could be taken to suggest qualitative differences between the substrates. Refinement of the experiment (Fig. 2), however, clearly demonstrated that this was not the case. By lowering the input of the frost-released plant material to a concentration where it induced an aerobic CO_2 production rate equal to that induced by freezing and thawing of the soil (Fig. 2, Table 1), the O_2 dependency of the CO_2 production rate disappeared. Thus, the apparent contrast between the two substrates is due to a difference in substrate concentration. The CO_2 and N_2O production in response to varying O_2 concentrations in the headspace reflects the interdependency of O_2 concentration and respiration in creating anaerobic sites in the soil. At low respiration rate (i.e., soil without plant extract), O_2 diffusion was clearly sufficient to sustain aerobic metabolism through the entire soil volume at all O_2 concentrations above zero, since practically no N_2O production occurred. In contrast, the plant extract amendment resulted in O_2 consumption which clearly exceeded the diffusion of O_2, thus resulting in increasing anaerobic fractions with decreasing headspace O_2 concentrations, reflected in the increasing rates of N_2O production (Figs. 1C and D). With increasing anaerobicity, the CO_2 production gradually declined (Figs. 1A and B), suggesting that the denitrification capacity was unable to compensate for the lack of O_2. In contrast, the CO_2 production in the soil with lower respiration rates (unamended, Figs. 1A and B, soil with diluted plant extracts Fig. 2) was virtually unaffected by the absence of O_2, suggesting that the denitrification capacity was able to replace O_2-based metabolism completely. This conclusion is supported by the fact that practically all the CO_2 production in the anaerobic treatment was attributable to denitrification, as judged by the ratio between denitrification rate and CO_2 production rate during anaerobic incubation (Table 1).

The amounts of decomposable soil organic matter released by our freeze–thaw treatment is unknown, but can be tentatively calculated by comparison of respiration rates as induced by freeze–thaw with those induced by the added plant extracts (assuming approximately equal mineralization rates). The comparison (Fig. 2) would suggest that the freeze–thaw treatment released 20–30 μg readily decomposable C g^{-1} soil. The freeze–thaw-released decomposable material is probably in part attributable to lethal effects on a fraction of the microflora (Skogland et al., 1988; DeLuca et al., 1992; Herrmann and Witter, 2002). The microbial biomass C (C_{mic}) was not measured in our soil, but can be assessed by the relationship between biomass C and soil organic C reported for a range of soils by Anderson and Domsch (1989) to be C_{mic} = 2.3–2.9% of soil organic C. This would mean that soil 1 contained 2–3 mg C_{mic} g^{-1} (since its total organic C content was 114 mg C g^{-1}). The estimated frost-released amount of 20–30 μg decomposable organic C g^{-1} soil thus represents only ~1% of the estimated biomass C. This exercise illustrates that minor freeze–thaw-induced lethal effects on the microbial biomass can explain the observed respiratory flush and enhancement of N_2O emission.

Another important aspect of freeze–thaw could be the distribution of freeze–thaw-susceptible C within the soil matrix. Sehy et al. (2004) extracted DOC from soil after freeze–thawing and added an equal amount (3.2 μg C g^{-1}) to non-frozen soil to simulate freeze–thaw release of C. They found no increase in CO_2 production and a much smaller increase in N_2O emission than after freeze–thaw treatment. They suggested that the difference between freeze–thaw treatment and DOC amendments was due to the difference in local distribution of the substrate. It seems plausible that freeze–thaw-induced C release at organic-rich microsites of high microbial activity produces greater CO_2 and N_2O emission responses (possibly involving local anaerobiosis) than when spread equally through the soil matrix, as in Sehy et al. (2004) and our experiments with the plant extracts.

In the following, the amounts of plant extract used in our experiments are compared with what can be expected to be lost from plants under field conditions. Korsæth et al. (2002) found that the total C of white clover (above ground biomass) was reduced from 102 g C m^{-2} in the autumn to 65 g C m^{-2} in the following spring. This loss is equivalent to an addition of 0.3 mg C g^{-1} soil, if distributed evenly in the upper 10 cm of the soil profile, which is exactly the same input as in our experiment with undiluted plant extract. This would suggest at first glance that our treatment with undiluted extract is quite realistic. This is not the case, however, since a substantial fraction of the biomass loss will probably be present as plant debris at the soil surface. Our substrate inputs can also be compared with standing crops in the field. The amount of plant material used to produce our plant extract equals 7.1 mg dw plant material soil g^{-1} in the treatment with undiluted plant extract. For a 10 cm soil layer, this would be equal to a plant biomass of approximately 900 g biomass C m^{-2}, which is 9 times more than the plant biomass in the field experiment (Korsæth et al., 2002). A reasonable conclusion would be that anything between undiluted and 1:10 diluted plant extract in our experiment yields realistic concentrations for a field situation, depending on the distribution of the frost-released plant material within the soil profile. The experiment thus strongly suggests that the presence of frost-sensitive plants in fields may greatly enhance denitrification during freeze–thaw events.

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