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HANNU T. KOPONEN

Production of Nitrous Oxide (N_2O) and Nitric Oxide (NO) in Boreal Agricultural Soils at Low Temperature

Doctoral dissertation

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Department of Physics

Author's address: Department of Environmental Science
University Of Kuopio
FI-70211 KUOPIO
FINLAND
Tel. +358 17 163 589
Fax +358 17 163 750
E-mail: Hannu.Koponen@uku.fi

Supervisors: Professor Pertti J. Martikainen, Ph.D.
Department of Environmental Science
University of Kuopio

Docent Kristina Servomaa, Ph.D.
Department of Environmental Science
University of Kuopio

Reviewers: Peter Dörsch, Ph.D.
Department of Plant and Environmental Sciences
Norwegian University of Life Sciences
Aas, Norway

Mats G. Öquist, Ph.D.
Department of Forest Ecology & Management
Swedish University of Agricultural Sciences
Umeå, Sweden

Opponent: Professor Leif Klemedtsson, Ph.D.
Department of Plant and Environmental Sciences
Göteborg University
Göteborg, Sweden

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ABSTRACT

The gaseous nitrogen oxides nitrous oxide (N_2O) and nitric oxide (NO) are produced in microbial nitrification and denitrification. N_2O is a strong greenhouse gas, while NO has importance in atmospheric chemistry. Up to 68% of the land surface of the northern hemisphere experiences soil freezing for variable times. N_2O is known to be produced in soil also at low temperatures and emissions during winter can contribute up to 90 % of the annual N_2O emission. Increased emissions of N_2O during soil thawing have been observed in numerous field and laboratory studies. However, the underlying processes and physical and chemical factors controlling N_2O emissions at low temperatures are not well understood. Studies on emissions of NO at low temperatures, including the effects of freezing-thawing, are lacking.

This study was conducted by manipulating microcosms with agricultural soil in the laboratory. The key focus was on soil physical changes and their effects on N_2O and NO emissions at low temperatures. The results showed that both mineral and organic soils can have high N_2O emissions during soil freezing and thawing. At soil temperatures near zero, the N_2O emission rates can be high, even exceeding the rates at +10ºC. When frozen, soil microbial processes can remain active and produce N_2O at least down to -8ºC. Produced N_2O is not necessary liberated to the atmosphere immediately, but can be stored in frozen soil leading to high N_2O concentrations in the soil atmosphere. A new finding was that agricultural soils can also have high N_2O production rates at low plus degrees without freezing-thawing history. In organic soils, the magnitude of N_2O emissions during thawing was found to depend on both freezing temperature and moisture status of the soil. In contrast, NO emissions at low temperatures were regulated merely by soil temperature.

Denitrification was evidently the major mechanism for N_2O production at low temperatures. The results suggest that denitrification benefits more from freeze-thaw related changes in soil physical and chemical conditions than general heterotrophic microbial activity. Freeze-thaw induced release of easily degradable substrates from cell lyses appeared to be of minor importance. This was supported by the finding that soil freezing and thawing did not cause discernable change in soil microbial biomass or community structure. This stresses the importance of soil microenvironments for controlling soil microbiological activities at low temperatures and ultimately biogeochemial cycling of nitrogen in boreal agricultural soils.

Universal Decimal Classification: 502.521, 631.416.1, 631.433.5, 546.172.5, 546.172.6
CAB Thesaurus: nitrogen oxides; nitrous oxide; nitric oxide; greenhouse gases; agricultural soils; soil physics; soil temperature; freezing; thawing; microbial activities; nitrification; denitrification; soil water; microenvironments; nitrogen cycle

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Kuopio, November 2007

Hannu Koponen

ABBREVIATIONS

C	Carbon
CO_2	Carbon dioxide
FTC	Freeze-thaw cycles
N	Nitrogen
N_2	Nitrogen gas
N_2O	Nitrous oxide
napA	Nitrate permease A
NH_2OH	Hydroxylamine
NH_4^+	Ammonium
nirS	Nitrite reductase S
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOR	Nitric oxide reductase
ppb	Parts per billion (10^{-9})
ppm	Parts per million (10^{-6})
Q_{10}	Relative change in a biological or chemical process rates as a consequence of 10^oC change in temperature
Tg	Tera grams (10^{12}g)
WFPS	Water filled pore space

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their chapter numbers.

Chapter II Hannu T. Koponen, Laura Flöjt and Pertti J. Martikainen. 2004. Nitrous oxide emissions from agricultural soils at low temperatures: A laboratory microcosm study. Soil Biology & Biochemistry 36, 757-766.

Chapter III Hannu T. Koponen and Pertti J. Martikainen. 2004. Soil water content and freezing temperature affect freeze-thaw related N_2O production in organic soil. Nutrient Cycling in Agroecosystems 69, 213-219.

Chapter IV Hannu T. Koponen, Claudia Escudé Duran, Marja Maljanen, Jyrki Hytönen and Pertti J. Martikainen. 2006. Temperature responses of NO and N_2O emissions from boreal organic soil. Soil Biology & Biochemistry 38: 1779-1787.

Chapter V Hannu T. Koponen, Tuula Jaakkola, Minna M. Keinänen-Toivola, Saara Kaipainen, Jaana Tuomainen, Kristina Servomaa and Pertti J. Martikainen. 2006. Microbial communities, biomass, and activities in soils as affected by freeze thaw cycles. Soil Biology & Biochemistry 38: 1861-1871

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CHAPTER I

GENERAL INTRODUCTION

CHAPTER I
GENERAL INTRODUTION

1.1 Background

Nitrogen is a key element for microbes and plants. The main gaseous compound (78%) present in the atmosphere is nitrogen gas (N_2). Atmospheric nitrogen is relatively inert, but it becomes biologically active via biological and anthropogenic nitrogen fixation to ammonium (NH_4^+) which can be incorporated into amino acids and proteins. Mineralization processes convert this organic nitrogen back to ammonium. Ammonium from biological decomposition or fertilisers is converted to nitrate (NO_3^-) in microbial nitrification. Plants utilize ammonium and nitrate for growth. The nitrogen cycle is closed by microbiological denitrification which converts nitrate via nitrite (NO_2), nitric oxide (NO) and nitrous oxide (N_2O) to N_2. Both nitric oxide and nitrous oxide produced in nitrification and denitrification can be emitted from soil and play an important role in atmospheric chemistry (Bouwman, 1990, Derwent, 1995).

In the troposphere, N_2O is an important greenhouse gas, accounting for almost 6% of the anthropogenic greenhouse effect (IPCC, 2001). The atmospheric concentration of N_2O has increased from the pre-industrial era (270 ppb) to a present concentration of 319 ± 0.12 ppb, and is increasing approximately linearly at a rate of 0.8 ppb yr^{-1}, corresponding to about 0.25% yr^{-1} for the past few decades (IPCC, 2007). Global mean atmospheric lifetime of N_2O is 114 years, and it is 298 times more powerful as a greenhouse gas than carbon dioxide (CO_2) in a time horizon of 100 years (IPCC, 2007). In the stratosphere, N_2O participates also in catalytic cycles involved in the destruction of ozone (Cruzen and Ehhalt, 1977).

NO plays an important role in the lower levels of the troposphere. Here NO acts as a precursor for ozone thereby influencing indirectly the oxidation of greenhouse gases (Williams et al., 1992). In the stratosphere, NO is involved in catalytic reactions resulting in the destruction of stratospheric ozone (Cruzen and Ehhalt, 1977). The average atmospheric lifetime of NO is short, being a day or less in the polluted boundary layer of the troposphere, and 5 to 10 days in the upper troposphere (IPCC, 2001). The major source of NO is fossil fuel combustion, but in rural areas biomass burning and emissions from soil are other important sources (IPCC, 2001). Thus, NO from fertlised soils may play an important role in local tropospheric ozone chemistry (Bouwman et al., 2002).

Soils contribute 70 % and 20 % of the total global fluxes of N_2O and NO, respectively (Conrad, 1995). Agricultural soils account for 35 % of the global N_2O emission, of which 14 % (5 % of total soil flux) are attributed to N fixation by agricultural practices (biological fixation + fertilizer production) (Isermann, 1994, Kroeze et al., 1999). Both N_2O and NO formation are linked to the soil microbial processes nitrification and denitrification, while denitrification can theoretically act as a sink for N_2O (Regina et al., 1999, Bowman et al., 2002). Agricultural soils have a great potential to produce nitrogenous gases since soil nitrogen cycling is enhanced by agricultural practises such as fertilization and tilling. Especially, organic agricultural soils in the boreal region can act as a large N_2O source (Kasimir-Klemedtsson et al., 1997).

Soil physical and chemical conditions affect both nitrification and denitrification, and hence NO and N_2O production. Soil denitrification has been suggested to have a positive, though variable correlation with temperature (Granli and Bøckman, 1994). In agricultural soils with high nitrate availability, denitrification may be assumed

to be limited by aeration and temperature. Nevertheless, high N_2O emissions have been reported from cold agricultural soils during thawing, both in field (e.g. Christensen and Tiedje, 1990, Röver et al., 1998) and in laboratory experiments (e.g. Chen et al., 1995, van Bochove et al., 2000). Although this phenomenon is well established, the underlying processes and regulation factors, e.g. moisture content and freezing temperature, are not fully understood. Also, the behaviour of NO emissions from agricultural soils at temperatures near 0ºC has been neglected so far.

In this study, the focus was on agricultural soils and on the physico-chemical and biological factors controlling and driving the NO and N_2O production at low temperatures near 0ºC.

1.2 Processes involved in formation of nitrogenous trace gases in soil

1.2.1 Microbial denitrification

Denitrification is an anaerobic, heterotrophic process which is controlled by the oxygen partial pressure in soil and the availability of carbon (C), nitrate (NO_3^-) and other N oxides (Tiedje, 1988). Denitrifying microorganisms are facultative anaerobic bacteria (e.g. genera *Bacillus, Hyphomicrobium, Paracoccus, Pseudomonas, Thiobacillus,)* which use N-oxides as electron acceptors when oxygen availability is low (Bowmann, 1990). The ability to denitrifiy is wide-spread among phylogentically unrelated bacteria. In denitrification, NO_3^- is reduced to dinitrogen (N_2) via the intermediates nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) (process schema 1 with valences of N).

(1) $NO_3^-(+5) \rightarrow NO_2^-(+3) \rightarrow NO\ (+2) \rightarrow N_2O\ (+1) \rightarrow N_2(0)$

Under oxygen-limited conditions, with water filled pore spaces (WFPS) higher than 60%, denitrification has been considered as the main source of N_2O in agricultural soils (Linn and Doran, 1984, Davidson, 1992, Williams et al., 1998, Wolf and Russow, 2000). The maximum N_2O emission has been suggested to occur at WFPS of 80-85% (Dobbie et al., 1999). Under these conditions, N_2O emission seems to dominate over NO emission although the actual production of NO may exceed that of N_2O (Remde et al., 1989). In acid soils (pH < 5-6) N_2O emissions from denitrification are higher, apparently due to inhibition of N_2O reductase activity at low pH (Granli and Bøckmann, 1994, Flessa, 1998).

1.2.2 Microbial nitrification

Nitrification is defined as "biological oxidation of ammonium to nitrite and nitrate, or a biologically induced increase in the oxidation state of nitrogen" (Soil Science Society of America, 1987) (process schema 2 with valences of N). Nitrification is an aerobic process controlled by the availability of ammonium (NH_4^+) and oxygen (Firestone and Davidson, 1989). In autotrophic nitrification, oxidation of ammonium (e.g. genera *Nitrosococcus, Nitrosolabus, Nitrosomonas, Nitrosopira, Nitrosovibrio)* or nitrite (e.g. genera, *Nitrobacter, Nitrococcus, Nitrospira)* is used for energy production and CO_2 is used as a carbon source. In heterotrophic nitrification (e.g. *Pseudomonas, Aspergillus*), organic substances are used as a source of both carbon and energy. NO and N_2O have been suggested to be produced by oxidation of hydroxylamnine (NH_2OH), a precursor of nitrite (NO_2^-) (Haynes, 1986). Reduction of NO_2^- to N_2 via NO and N_2O at sub-optimal oxygen concentrations (nitrifier denitrification) is also known (Poth and Focht, 1985).

(2) $NH_4^+(-3) \rightarrow NH_2OH\ (-1) \rightarrow NOH\ (+1) \rightarrow NO_2^-(+2) \rightarrow NO_3^-(+5)$

Nitrification has been suggested to be the major source for N_2O in soils at WFPS below 60% (Linn and Doran, 1984). However, Hutchinson et al. (1993) concluded, that nitrification can be a dominant source of NO even at high water contents when oxygen is available. In nitrification the NO production generally dominates over that of N_2O (Anderson and Levine, 1986, Skiba et al., 1993).

1.2.3 Other microbial processes associated with gaseous N production

Some non-nitrifying or non-denitrifying microorganisms can produce N_2O, but not NO (Bleakley and Tiedje, 1982). Robertson and Tiedje (1987) suggested that fungi can be dominant N_2O producers, especially in forest soils. However, there is no evidence that these processes are significant in agricultural soils (Bremner, 1997). Other potential sources for N_2O and NO are dissimilatory nitrate reduction to ammonium (DNRA) and anaerobic ammonium oxidation (anammox) (Burgin and Hamilton, 2007). Some evidence has been found on N_2O production in DNRA (Smith & Zimmerman 1981), but non in anammox so far. Both processes seem to be of minor importance in soils.

1.2.4 Chemodenitrification

Chemodenitrification refers to different chemical reactions of NO_2^- resulting in the formation of NO, N_2O, and N_2. van Cleemput and Baert (1984) concluded that under acidic conditions both soil organic matter and soil mineral phase (increasing the Fe^{2+} concentration in soil solution) stimulate nitrite decomposition, i.e. chemodenitrification. The amount of N_2O produced by chemodenitrification is suggested to be small compared to the formation of NO and N_2 (van Cleemput and Baert, 1984, Davidson, 1992, Bremner, 1997). On the other hand, chemodenitrification is not considered to be a major source of NO (McKenney and Drury, 1997). However, Mørkved et al. (2007) found, that chemodenitrification can contribute significantly to the apparent nitrification-derived N_2O emissions.

1.2.5 Factors affecting the emissions of NO and N_2O from soil

Once formed in the soil, NO and N_2O can escape to the atmosphere. Firestone and Davidson (1989) proposed a conceptual model, the "hole in the pipe", describing nitrification and denitrification as pipes from which the gaseous products (NO, N_2O, N_2) are leaking (Fig.1). In the model there are three levels of regulation of NO and N_2O emission: (i) the factors affecting rates of nitrification and denitrification, which are analogous to the flow of N through the pipe, (ii) the factors (see chapter 1.5.) that affect the relative proportions of the products, which are described by the size of the holes in the pipes, and (iii) the factors affecting gaseous diffusion through the soil to the atmosphere.

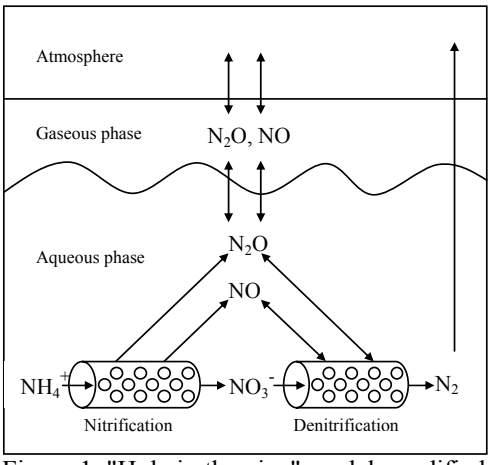

Figure 1. "Hole in the pipe" model, modified from Firestone and Davidson (1989)

The emission rates (i.e. the sum of production from denitrification and nitrification) of NO and N_2O from a soil are dependent on the availability of NH_4^+ and NO_3^- in soil, temperature, pH, soil moisture, soil type, vegetation, land use practices, use

of chemicals (fertilizers), and irrigation practices (Granli and Bøckmann, 1994). With high water content the residence time of NO in soil increases allowing its reduction to N_2O (McKenney and Dury, 1997). The same is true for N_2O. With high water contents, more N_2O will be reduced to N_2 before it escapes to the atmosphere (Davidson et al., 1991).

1.3. Agriculture and nitrogen cycling

Nitrogen cycling is heavily affected by human activities. The most fundamental human-induced change to the global N cycle is the steady increase in reactive nitrogen in the biosphere through production of synthetic nitrogen fertilizers and cultivation of N_2-fixing plants (Vitousek et al., 1997). The majority (55%) of the World's population relies in food which is produced with the help of mineral fertilizers and with the present agricultural input it is possible to satisfy the protein demand of 6.1 billion (10^9) people living on a mixed diet (Isermann, 1994). Global N input into agricultural systems from synthetic fertilizers has increased from less than 2 Tg N yr^{-1} in 1930 to 77 Tg yr^{-1} in 1990 (Kroeze et al., 1999). The industrialization in the late 19th century caused changes in agriculture, mainly due to labour-saving machinery and the change from grain to livestock products, vegetables, and special tropical products (Kroeze et al., 1999). Fast economic growth, a demand for cotton, wool etc. by factory industry, together with rising incomes of the workers in industry created an increasing need for agricultural products (Kroeze et al., 1999). According to the "hole in the pipe" model (chapter 1.2.5), this increase in nitrogen input to soils has in the past and will in the future lead to increased NO and N_2O emission from agriculture. So far, the atmospheric N_2O concentration has increased with the same relative rate as the anthropogenic N fixation (Galloway et al., 1995, Vitousek, 1997). In order to abate a future run-away effect of anthropogenic climate forcing from agriculture, we need to develop mitigation strategies that reduce the gaseous losses per applied unit of fixed nitrogen. A thorough understanding of the soil processes involved in the formation and emission of NO, N_2O, and N_2 is a prerequisite to reach this goal.

Nitrogen fertilizers (NH_4^+ and NO_3^-) have been reported to increase the emissions of N_2O immediately after the addition (e.g. Eichner, 1990, Mummey et al., 1994, Chang et al., 1998). Especially, soils fertilized with ammonium are important sources of NO, and it has been predicted that along with future fertilizer use, agricultural soils will contribute more than 50% of the global NO_x emissions (Skiba et al., 1993, Yienger and Levy, 1995). The low efficiency of use of fertilizer N in agricultural systems is primarily caused by the large losses of N, including N_2O (Minami, 1997).

Studies of N_2O emission rates in agricultural systems have revealed high spatial and temporal variations which seem to be higher than those reported for NO (Johansson and Granat, 1984, Skiba et al., 1992 Velthof et al., 1996). Differences in the variability of NO and N_2O emissions are likely caused by differences in their predominant production processes; N_2O production from denitrification requires low oxygen partial pressures and therefore N_2O emissions are much more variable in time and space than those of NO (Skiba et al., 1992). Both NO and N_2O emissions show diurnal variations, which follow changes in soil temperatures (Skiba et al., 1992, Maljanen et al., 2002). Beauchamp (1997) emphasized the importance of understanding the complex physical, chemical and biological factors that control nitrogenous gas fluxes. This also includes poorly understood mechanisms of N_2O and NO production at temperatures near 0ºC, and their regulation factors.

1.4 Importance of soil freeze-thaw cycles and low temperatures for gaseous N fluxes

In temperate and boreal regions, soils undergo several freeze-thaw cycles (FTC) during winter. Up to 68% of the land surface of the northern hemisphere experiences freeze-thaw cycles and the length of these cycles varies from a few days to several months (Zhang et al., 2005). Under an insulating snow cover, soil temperatures often fluctuate around 0ºC, resulting in small scale FTCs (Dörsch et al., 2004, Regina et al., 2004). When frozen, soil moisture status remains relatively constant (Schürmann et al., 2002) or increases during snow melt due to reduced infiltration in partially frozen soil **(**Ruser et al., 2001**).**

In boreal regions soil temperatures remain at a low level for several months, and small changes in soil temperature during winter season may be extremely important for N_2O and NO emissions (Martikainen, 2002). Estimates of the annual share of N_2O emitted during the cold season in various terrestrial ecosystems vary from 5 to 90% (Table 1). The impact of low temperatures on NO emissions has not been studied. For the time being, N_2O emissions from cold soils are thought to be one of the main uncertainties for annual N_2O budgets (Ruser et al., 2001).

1.5 Factors controlling N_2O and NO production and emissions at low temperatures

1.5.1 Biological factors

1.5.1.1 Microbiological processes

The main source for N_2O emissions during soil freeze-thaw cycles are microbial processes (Röver et al., 1998). Microbial activity, including nitrification and denitrification, are thought to increase with temperature. However, McGarity (1962) reported an increase in denitrification activity measured by gas production after soil freezing-treatment. Dorland and Beauchamp (1997) concluded that denitrification can occur in soil below 0ºC temperatures, and that the rate of denitrification at any temperature is dependent on the supply of organic substrates. The Q_{10} values for soil denitrification reported in literature vary from 2.0 to 12.3 at a temperature range from 0 to +15ºC (Mahli et al., 1990, Dorland and Beauchamp, 1991). A Q_{10} greatly different from 2 may indicate that physical and chemical factors affect the reaction rates (Granli and Bøckman, 1994).

Denitrification includes several reductative steps, regulating the gaseous composition of denitrification products stoichiometrically by the expression and kinetics of the reductases. The N_2O reductase is the last enzyme in the denitrification sequence, reducing N_2O to N_2. It is thought to be more sensitive to changes in environmental conditions than the other reduction enzymes (Knowles, 1982). Decrease in temperature increases the ratio of N_2O to N_2 in denitrification by suppressing the N_2O reductase (Melin and Nômmik, 1983, Maag and Vinther, 1996). However, changes in the N_2O to N_2 ratios with decreasing temperature are not due to specifically higher activation energies for N_2O reductase but due to some unknown anomalies at critically low temperatures (Holtan-Hartwig et al., 2002, Öquist et al., 2004). Holtan-Hartwig et al. (2002) also showed that soil denitrifying communities may vary in their ability to reduce N_2O at 0^oC.

Table 1. Winter-time share of annual N_2O-N emissions from various terrestrial ecosystems, data from the literature

Location	Management	Soil type	Vegetation	Mean annual T (ºC)	Precipitation (mm)	Annual N_2O-N flux (kg ha^{-1})	Duration of winter (months)	N_2O winter (% of annual)
Central Germany[1]	Agriculture	loam with silt	oil seed rape			4.8	4	58
	Fallow		grass			3.2		45
	Forest		oak forest (*Quercus petrea*)			1.4		50
Lower Saxony, Germany[2]	Agriculture	silty loam	winter wheat			3.7-7.0	3	70
Lower Saxony, Germany[3]	Agriculture	loamy silt	sugar beet/winter wheat		ca. 644	1.5-3.6/ 1.1-3.5	5	50
Southern Germany[4]	Agriculture	coarse loam	spring barley/sunflower	+7.4	833	9.3-16.8	2	46
Southern Germany[5]	Agriculture	fine loam	potato/corn/wheat	+7.4	833	2.41/3.64/ 6.93	5	49
Eastern Finland[6]	Agriculture	organic	grassland	+2.0	600	12.2	7	38
	Forested (fen)	organic (peat)				0.9-1.5		28
Eastern Finland[7]	Agriculture	organic	barley/grass	+2.6	643	8.3-11	6,5	15-60
	Forested	organic (peat)	birch (*Betula pendula* Roth)			4.2		36
Western Finland[8]	Agriculture	organic	barley/grass	+2.4	561	8.5/2.8	6	5-99
Southern Finland[9]	Agriculture	organic	grass/spring barley/fallow	+4.3	607	7.3/15/ 25	7	55
Northern Finland[9]	Agriculture	organic	grass/spring barley/fallow	0	537	4.0/13 /4.4	7	52
Southern Finland[10]	Agriculture	clay	grass/spring barley/potato	+4.3	607	3.7-7.8	6	37-68

[1]Teepe et al. (2000) [2]Röver et al. (1998) [3]Kaiser et al. (1998)
[4]Flessa et al. (1995) [5]Ruser et al.(2001) [6]Alm et al. (1999)
[7]Maljanen et al. (2003) [8]Maljanen et al. (2004) [9]Regina et al. (2004)
[10]Syväsalo et al. (2004)

The commonly observed burst of N_2O during thawing of frozen soil is often attributed to increased denitrification activity triggered by a transient increase in substrate availability during thawing (Müller et al., 2002, Müller et al., 2003, Sehy et al., 2004, Mørkved et al. 2007). In the presence of high nitrate levels, the induction of N_2O reduction enzymes can be retarded, thereby increasing the N_2O to N_2 ratio (Blackmer and Bremner, 1978, Dendooven et al., 1994).

1.5.1.2 Supply of carbon and nutrients

Edwards and Killham (1986) discussed the possibility of soil freeze-thaw in mobilizing the available carbon with respect to enhanced activity of denitrifiers. Physical release of easily degradable C has been suggested to be one essential factor for high N_2O emissions following soil freeze-thaw, as suggested by Sehy et al. (2004). This carbon may originate from microorganisms or plant roots killed by soil freezing, or detritus that becomes available by the freeze-thaw process (Christensen and Tiedje, 1990). Mørkved et al. (2007) concluded that the freeze-thaw induced release of decomposable organic C is the major driving force for N_2O emissions. In general, easily available carbon may even increase the production of N_2O when *de novo* synthesis of the reductase enzyme is inhibited (Dendooven and Andersson, 1995). Even though the release of nutrients by FTC may be small, soil bacteria that are normally in a stationary state can be triggered by small amounts of extra nutrients thus explaining the respiratory flush commonly observed upon thawing of frozen soil (e.g. Skogland et al., 1988). Release of nutrients by cell lysis has been suggested to be greatest after the first freeze-thaw cycle and to decline when additional freeze-thaw cycles are applied (Schimel and Clein, 1996, Larsen et al., 2002). Soil microbial biomass C has been reported to decrease in the freeze-thaw treatment (Larsen et al., 2002). However, there exist also results that soil microbial biomass is unaffected by soil freeze-thaw events (Lipson and Monson, 1998, Grogan et al., 2004). Even though the nutrient release from cell lysis is discussed widely in the literature, there is no clear experimental evidence for the significance of microbial lysis to FTC induced N_2O emissions. Hermann and Witter (2002) could not find any measurable changes in the amount of microbial biomass, but based on the ^{14}C labelling they suggested that microbial biomass could contribute to 65% of the carbon flush upon soil freeze-thaw cycles. This represented only 5 % of the microbial biomass carbon in soil.

Soil freezing-thawing, like soil drying-rewetting, can disrupt soil aggregates thereby releasing protected soil organic matter and exposing it to microbial attack. This may explain the increased availability of inorganic and organic substrates occasionally measured in soils after mechanical perturbation (Soulides and Allison, 1961). van Bochove et al. (2000) reported a burst in denitrification activity after freezing-thawing, and they concluded that this burst was sustained by C mineralization from organic matter released by disruptive forces induced by freezing and thawing of micro- and macroaggregates. Aggregate stability has been observed to decrease in medium and fine textured soils with increasing water content at freezing (Lehrsch et al., 1991).

1.5.2. Abiotic factors

1.5.2.1 Water

Biological communities in soil live in a complex, three dimensional physical framework with variable geometry, composition and stability over small spatial scales (Young and Ritz, 1998). The temperature ranges and gradients to which soil microbes are exposed in boreal regions

range from minus degrees to above 20ºC. At sub-zero temperatures, the importance of water availability becomes one of the crucial parameters for microbial activity.

Microbial processes require water, and liquid water can exist in soils at sub-zero temperatures. The freezing point of soil water is lower than the freezing point of pure water, due to the presence of solutes in the soil water and due to matric potential induced by the soil matrix (porosity, surfaces etc.) (Edwards and Cresser, 1992). During freezing, the concentration of the soil solutes increases due to exclusion of solutes from the growing ice grid, leading to a decrease in the freezing point temperature of the remaining liquid water (Sähli and Stadler, 1997).

At temperatures below 0ºC unfrozen water can exist in the soil matrix, although the bulk soil water is frozen. This water is believed to be associated with soil particle surfaces or small pores, and the proportion of unfrozen water in the soil increases with e.g. increasing proportions of humus material (Sparrman et al. 2004). Ice layers between the soil particles may prevent O_2 diffusion, resulting to anoxia because of biotic oxygen consumption in unfrozen soil microenvironments (Clein and Schimel, 1995, Teepe et al., 2001). Several studies (e.g. Teepe et al., 2001, Öquist et al., 2004) stresses the importance of these unfrozen water films, and the ice layer covering this thin water film, which can create conditions that are favourable for denitrification.

Salting out effect; the increase in ionic strength in the remaining unfrozen water, causes a reduction in the solubility of non-polar gases due to the polarizing effect of salts on the solvent. In exceptional situations, e.g. after fertilizer application or during soil freezing, when salts concentrate in liquid water films, increased N_2O emissions may be due to decreased solubility (Heincke and Kaupenjohann, 1999). Conversely, decrease in temperature increases the solubility of N_2O. At 0ºC, the solubility of N_2O is approximately twice to that at 19ºC (Heincke and Kaupenjohann, 1999). Both salting out (a decrease in solubility) and higher solubility due to low temperature can affect N_2O emissions at temperatures near 0°C, but the significance of these processes is difficult to delineate.

1.5.2.2 Gas diffusion

An ice layer can act as a diffusion barrier, enabling produced N_2O to accumulate below the frozen layer in the soil profile and preventing oxygen to penetrate into the soil (Goodroad and Keeney, 1984, Cates and Keeney, 1997). Ice layers divide the soil profile into two sections: 1) frozen layer in the soil surface, which is highly variable in N_2O concentrations due to presence or absence of diffusion barrier and 2) unfrozen subsurface region, where the accumulation of N_2O can occur (Burton and Beauchamp, 1994). This sub-surface production can cause increased emissions through the escape of gases from frost induced cracks (Kaiser et al., 1998). The ice layer may also prevent the soluble N_2O to escape from the liquid water film, resulting in supersaturated soil solutions (Teepe et al., 2001).

1.6 Aims and overview of the experiments

N_2O emissions at low temperatures are reported in many field and laboratory experiments (e.g. Christensen and Tiedje, 1990, Chen et al., 1995, Röver et al., 1998, van Bochove et al., 2000). However, the processes behind freeze-thaw related emissions are not fully understood yet. The main aim of this study was to examine the factors regulating N_2O and NO emissions at temperatures near 0ºC. The key focus was on the soil physical changes, i.e. soil moisture, soil freezing temperature, and their effects on soil microbiological processes, denitrification and nitrification, and the possible changes in microbial

biomass, community structure and general microbial activity. To this end, the composition of the gaseous products, N_2O and NO, as well as the microbiological processes were studied at various temperatures, with the special emphasis on temperatures around 0ºC.

All four experiments were performed under laboratory conditions, in incubator cabins using soil microcosms. This approach allowed performing the experiments under controlled temperature and moisture conditions. Soils used in the experiments were from agricultural sites (Chapter II, III, V) or from sites which have an agricultural land-use history (Chapter IV). In one experiment (Chapter II) the main focus was on the effect of temperature on N_2O emissions from different soil types. The effects of moisture content on FTC related N_2O emissions (Chapter III) and temperature responses of NO and N_2O emissions (Chapter IV) were conducted using organic soils (histosol). In one experiment (Chapter V) the effects of soil freeze-thaw cycles on the soil microbiology was studied in more detail. The experiments and main parameter are summarized in Table 2 and described in more detail in the corresponding chapters.

Table 2. The experiments and their main research topics

Chapter	Soil type	T range	Research topic/questions to be solved
II	Organic[1] Clay[1] Silt[1] Loam[1]	+15 to -8ºC +2.5 to -4ºC -8 to +10ºC -2ºC to +4ºC	Does low temperature affect N_2O production similarly in different soil types? Does low temperature modify the N_2O emission near 0ºC without soil freezing?
III	Organic[1]	-1,5 to +4ºC -15 to +4ºC	What is the effect of soil moisture content and severity of frost on the freezing-thawing related N_2O emissions?
IV	Organic[2 and 3]	+9.5 to -4.9ºC -4.9 to +5.5ºC	Are the effects of temperature and freezing-thawing similar on NO and N_2O emissions?
V	Peat[1] Loamy sand[1]	-17.3 to +4.1ºC	What are the effects of soil freezing-thawing on chemical variables, microbial activity, microbial biomass and microbial community structure?

[1]agricultural soil [2]afforested site [3]abandoned site

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CHAPTER II

NITROUS OXIDE EMISSIONS FROM AGRICULTURAL SOILS AT LOW TEMPERATURES: A LABORATORY MICROCOSM STUDY

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Nitrous oxide emissions from agricultural soils at low temperatures: a laboratory microcosm study

Hannu T. Koponen*, Laura Flöjt, Pertti J. Martikainen

Department of Environmental Sciences, Research and Development Unit of Environmental Health, University of Kuopio, BioTeknia 2, P.O. Box 1627, FIN-70211 Kuopio, Finland

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Abstract

We studied in laboratory microcosms (intact soil cores) N_2O and CO_2 emissions from four different agricultural soil types (organic soil, clay, silt and loam) at low temperatures with or without freezing–thawing events. When the temperature of the frozen soil cores was increased stepwise from −8 °C the N_2O emissions began to increase at −0.5 °C, and peaked at −0.1 °C in the organic, clay and silt soils, and at +1.6 °C in the loam soils. However, a stepwise decrease in soil temperature from +15 °C also induced an increase in the N_2O emissions close to the 0 °C. These emissions peaked between −0.4 and +2.5 °C depending on the soil type and water content. However, the emission maxima were from 2 to 14.3% of those encountered in the experiments where frozen soils were thawed. Our results show that in addition to the well-documented thawing peak, soils also can have a maximum in their N_2O emission near 0 °C when soil temperature decrease. These emissions, however, are less than those emitted from thawing soils. The correlations between the N_2O and CO_2 emissions were weak. Our results suggest that N_2O is produced in soils down to a temperature of −6 °C.

Keywords: Temperature; N_2O emissions; CO_2; Freezing–thawing; Agricultural soils

1. Introduction

Nitrous oxide (N_2O) is an efficient greenhouse gas. Its global warming potential is 340 times that of CO_2 when calculated for a time horizon of 100 yr (Jain et al., 2000). Nitrous oxide also participates in the depletion of stratospheric ozone, which has importance in absorbing hazardous UV-B radiation (Beauchamp, 1997). The atmospheric concentration of N_2O has increased over the past decades and continues to increase annually at the rate of 0.2–0.3% (IPCC, 1994). Agricultural soils contribute approximately 80% of the total N_2O in the atmosphere, and as such are the most important anthropogenic source of N_2O (Isermann, 1994). N_2O is produced in soils mainly by nitrification and denitrification processes. Soil physical and chemical characteristics, e.g. texture, water content and associated O_2 diffusion rate, temperature, availability of NO_3^-, NH_4^+ and organic substrates, plus pH all affect N_2O production (Davidson, 1991).

Microbial activities, including nitrification and denitrification, are generally greatest during seasons with high soil temperatures (Sommerfield et al., 1993). However, N_2O emissions have shown a great temperature anomaly. There are several studies on the high N_2O fluxes at low soil temperatures in northern European and North American soils, showing that from 38 to 70% of the annual emissions can take place during winter (van Bochove et al., 1996; Wagner-Riddle et al., 1997; Röver et al., 1998; Alm et al., 1999; Teepe et al., 2000). The highest N_2O fluxes at low temperatures have been associated with freezing and thawing cycles (Flessa et al., 1995; Kaiser et al., 1998; Premié and Christensen, 2001; Teepe et al., 2001). Several alternative mechanisms have been proposed to explain the high N_2O release during thawing including physical release of the trapped N_2O (Burton and Beauchamp, 1994), an increase in the availability of substrates and associated denitrification activity (Christensen and Tiedje, 1990; Christensen and Christensen, 1991), a combination of physical N_2O release and increased microbial activity (Goodroad and Keeney, 1984; Kaiser et al., 1998) and chemical production of N_2O (Christianson and Cho, 1983).

* Corresponding author. Tel.: +358-17-163589; fax: +358-17-163750.
E-mail address: hannu.koponen@uku.fi (H.T. Koponen).

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We have recorded many observations of on high N_2O emissions from Finnish agricultural soils in situ during winter without freezing–thawing cycles (Maljanen et al., 2003). The mechanism for these emissions is unknown. In well-controlled laboratory experiments we have studied the soil conditions allowing high N_2O production at low temperatures. We studied N_2O production in boreal mineral and organic agricultural soils at low temperature both without and with freezing–thawing events. We found that the freezing–thawing cycles induce high N_2O release but there also can be high N_2O production at temperatures close to 0 °C without freezing–thawing events.

2. Materials and methods

2.1. Experimental soils

Profiles (0–20 cm depth) of clay, silt, loam and organic soils were taken into PVC cylinders (diameter 105 mm, length 300 mm) with a stainless steel corer. There were 10 replicate cores for each soil type. Mineral soils were cored on 7th July 1998 from experimental fields of Agrifood Research Finland in southern Finland (clay 60°45′N, 23°22′E; silt 61°15′N, 24°57′E; loam 61°25′N, 24°11′E). The average annual precipitation (1961–1990) in this region is 581 mm, of which 213 mm is snow. The average annual air temperature is +3.9 °C. Organic soil cores were taken on 8th July 1998 from an experimental field in Siikasalmi, eastern Finland (62°55′N, 29°30′E). The average annual precipitation of this region for the period 1961–1990 was 612 mm, of which 232 mm was snow. The average annual air temperature was +2.2 °C. All the sites were growing barley in 1998. The soil cores were stored at +4 °C for 4–6 weeks before the experiments.

The gravimetric moisture content was determined from the collection sample (0–20 cm depth, five replicates from each field, 50 g FW) by drying the soil samples for 24 h at 105 °C. Bulk density was determined by using a Kopeck-drill (Blake and Hartge, 1986) and particle density with pyknometers (Blake, 1965). Soil pH was measured from soil-water suspensions (1:5 v/v). Ammonium–N and NO_3^-–N were extracted from integrated soil samples (0–20 cm) with 1 M KCl (soil:KCl 1:5 v/v, 175 rev min^{-1}, 1 h). The extracts were filtered (Blauband 589[3] BlueRibbon filter paper) and stored at −20 °C until analyzed. Ammonium–N was analyzed from the extracts spectrophotometrically (Philips PU 87501 UV/VIS at 630 nm wavelength); (Fawcett and Scott, 1960). The NO_3^-–N content in the extracts was determined with a nitrogen analyser (Lachat Instruments Quick Chem 800, 520 nm wavelength) after reduction to NO_2^-–N (Cd reduction method).

The soils were sampled after a dry period and were therefore well aerated; their WFPS ranged from 42 to 66%. Soil pH was 5.3–6.6, and total contents of C ranged from 2.4 to 26% and for N from 0.2 to 1.6% (Table 1).

Table 1
Physical and chemical characteristics of soil types (0–20 cm), $n = 5$

	Organic	Clay	Silt	Loam
Gravimetric moisture content (%)	60.1	24.9	23.2	19.6
WFPS (%)	61	66	57	42
pH	6.3	5.4	6.6	5.3
BD (g cm^{-3})	0.33	1.14	1.11	1.05
PD (g cm^{-3})	1.82	2.64	2.69	2.60
C (%)	26.4	4.02	2.36	2.48
N (%)	1.56	0.32	0.21	0.26

WFPS: water filled pore space, BD, bulk density, PD, particle density.

The content of NO_3^-–N at the beginning of the experiment varied from 6 μg N g^{-1} in the clay soil up to 120 μg N g^{-1} in the organic soil. All soils also contained NH_4^+–N (Fig. 3).

2.2. General experimental set-up and the gas flux measurements

Before experiments began, the aboveground vegetation (barley) was cut from the cores. N_2O and CO_2 fluxes were measured from the cores by a closed chamber technique (Nykänen et al., 1995). At the beginning of the measurements, the cores were sealed in gas tight PVC chambers (Fig. 1). The volume of the headspace varied from 0.6 to 0.9 l. After closing the chamber, gas samples of 20 ml were taken into 60 ml polypropylene syringes (Terumo) equipped with 3-way stopcocks (Connecta) through the rubber septum in the lid after 5, 30 and 60 min. The chamber was equipped with a capillary tube (1 mm diameter, 1.5 m long) to avoid underpressure inside the chamber during gas sampling. Temperatures of the atmosphere of the incubation cabin (LMS Cooled Incubator, model 250) and at a depth of 5 cm in one core of each soil type were monitored continuously by temperature sensors (Fluke) and by data loggers (HOBO®) from the air.

After changing the incubator temperature, the soil cores were allowed to stabilize to the new temperature for 3–4 d before gas flux was measured. This procedure ensured homogenous temperature throughout the soil profiles, and that the measured flux reflected gas production from soil at the set temperature. Without this stabilization period, the flux would show merely the diffusion of the stored N_2O without a close association to the actual gas production.

Gas concentrations in the syringes were determined in the laboratory within 24 h from sampling with a Shimadzu GC-14A gas chromatograph equipped with thermal conductivity detector for CO_2 and electron capture detector for N_2O. Peak areas were integrated with the GLASS-CR 10 program (Shimadzu Corp) as described in Maljanen et al. (2001). The gas flux rates were calculated from the linear increase in the gas concentrations in the chamber with time (Nykänen et al., 1995).

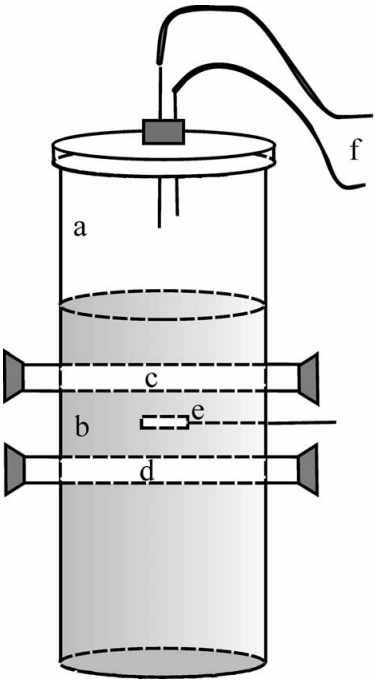

Fig. 1. The structure of the cylinder used in the experiments. (a) Headspace, (b) soil profile, (c and d) perforated plastic tubes for soil gas sampling, (e) temperature sensor, (f) sampling line inserted through rubber septum.

2.3. The experiments

Three experiments were conducted. In addition to the N_2O release CO_2 fluxes were also measured simultaneously to demonstrate the effect of experimental conditions on the general heterotrophic microbial activity in the soils.

2.3.1. Experiment 1: effects of temperature reduction from 15 °C to −8 °C

The hypothesis for this experiment was that the N_2O release will decrease when the temperature was dropped but close to 0 °C some increase would take place based on the in situ observations. The temperature was dropped stepwise from +15 °C down to −8 °C, using 12 different temperatures. There were 10 replicate soil cores for each soil type with the natural water content (42–66% water-filled pore space, WFPS) prevailing at the sampling time (Table 1).

At a temperature of +2.5 °C a gas sampling system was inserted into one silt core and into one loam core to study the concentration of N_2O within the soil at temperatures just above 0 °C and at temperatures below 0 °C, to determine subsurface production of N_2O below freezing point. The system consisted of a perforated plastic tubes (10 mm diameter, 150 mm long) that were inserted horizontally in the soil cores at depths of 40 and 80 mm from the soil surface (Fig. 1). The tubes were stoppened by rubber septa and their atmosphere was replaced by N_2 (99.5%). Gas samples (10 ml) were taken through the septa by injection needles. Simultaneously, 10 ml of N_2 was added to the tubes to prevent errors in sampling due to pressure changes.

2.3.2. Experiment 2: effects of temperature reduction (+2.5 °C to −4 °C) followed by stepwise temperature increases (−8.0 °C to +10 °C)

We determined if there is any difference in the emissions between decreasing and increasing soil temperatures. Because of the rather low N_2O emissions in the first experiment we ran this experiment at higher soil moisture (90% of the WFPS). Three replicate cores of the four soil types (used in Exp. 1) were used. Soil was moistened, and to omit a possible major N_2O pulse (Davidson, 1992; Mummey et al., 1994; Jørgensen and Jørgensen, 1997), the soils were stabilized for 5 d at +2.5 °C before the gas fluxes were measured at the various temperatures (range +2.5 to −4 °C, seven different temperatures). During the temperature decrease, the development of gas concentrations in the silt and loam cores were followed by the gas sampling systems described in Section 2.3.1. After the experiment at −4 °C, the temperature was lowered to −8 °C for 3 months. In the second part of this experiment, the temperature was increased in a stepwise manner in temperature range of −8 °C to +10 °C (11 different temperatures).

2.3.3. Experiment 3: effects of rapid thawing on N_2O and CO_2 emissions

In this experiment we studied the effect of rapid thawing on the N_2O and CO_2 emissions using three replicate cores of each soil. The soils with their natural water content (Table 1) were first kept at −2 °C for 4 d and then their temperatures were allowed to increase freely for 168 h to +4 °C. The fluxes of N_2O and CO_2 were measured at 1–24 h intervals for up to 7 d.

2.4. Statistical analysis

Statistical analysis was conducted using non-parametric Friedman's two-way analysis of variance by ranks. In first two experiments, soil types were tested individually for whole temperature range. In experiment 1 the most crucial temperatures (+5 to −4 °C) were tested again in order to determine the significance ($P < 0.05$) of freezing related emission maximums, in this case Bonferron's correction for multiple subgroup analyses was also performed. In experiment 3, the statistical difference was tested only for N_2O emissions during soil thawing (first 29 h).

3. Results

3.1. Experiment 1: decrease in temperature

The N_2O emissions at +15 °C, the highest temperature in the experiment, were 70 ± 35, 120 ± 25, 15 ± 5 and 75 ± 15 μg m^{-2} d^{-1} from organic, clay, silt and loam soils, respectively. When the soil temperature was decreased, the organic soil showed some decrease in N_2O emissions down to −0.1 °C and then the emission increased rapidly. Clay soil had very low N_2O emissions and a minor temperature response at −0.4 °C (Fig. 2a). Silt and loam had rather constant N_2O emission down to +4.8 °C whereafter the emissions increased (Fig. 2b). In the organic soil, the maximum emission took place at −0.4 °C. Silt and loam had their maximum N_2O emissions at +2.4 °C (Fig. 2a and b, Table 2). In clay where the N_2O emission was low, there was a slight increase in the N_2O emissions between −0.4 and −1.6 °C (Fig. 2a, Table 2). These increases in N_2O were statistically significant ($P < 0.05$) in organic, silt and loam soils. Substantial emissions of N_2O occurred in organic and silt soils down to −6 °C. In these soils the N_2O emissions between 0 and −6 °C were even higher than those measured at 15 °C (Fig. 2a and b). One interesting observation was that the replicate soil cores generally showed low deviation in their N_2O emissions, but at the emission maximums there was an increase in the deviation (Fig. 2a and b). CO_2 emissions decreased with decreasing temperature without any distinct temperature anomaly (Fig. 2c).

In profiles of silt and loam, the gaseous concentrations of N_2O fell with decreasing temperature (Fig. 3a). Below zero there was some increase in the N_2O content in the soil at the depth of 8 cm in the silt with N_2O maximum between

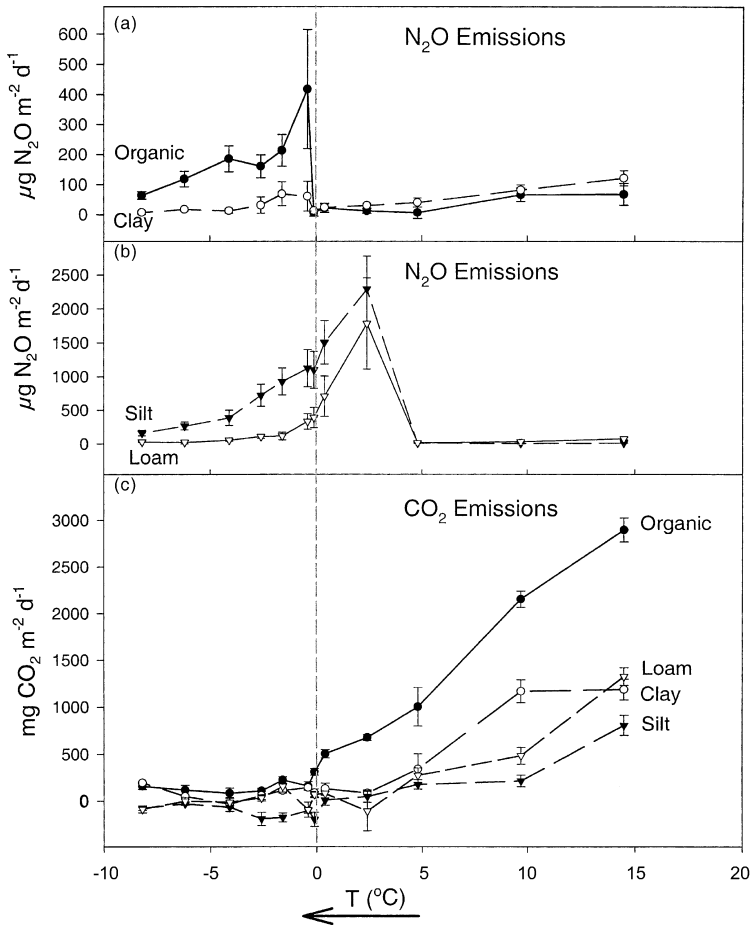

Fig. 2. Nitrous oxide and CO_2 emissions from organic, clay, silt and loam soils at natural soil water content with stepwise lowering of temperature ($n = 10$). Standard deviation is shown with bars. Note different scales.

Table 2
Maximum N_2O-emissions and the corresponding soil temperatures in experiments 1 and 2

Soil type	Experiment 1 (natural soil moisture)[a]			Experiment 2 (90% WFPS)[b]					
	Stepwise decrease in T			Stepwise decrease in T			Stepwise decrease in T		
	T (°C)	μg N_2O m^{-2} d^{-1}	mg CO_2 m^{-2} d^{-1}	T (°C)	μg N_2O m^{-2} d^{-1}	mg CO_2 m^{-2} d^{-1}	T (°C)	μg N_2O m^{-2} d^{-1}	mg CO_2 m^{-2} d^{-1}
Organic	−0.4	420 ± 200	160 ± 50	+0.6	4800 ± 1500	240 ± 90	−0.1	166,500 ± 49,000	650 ± 80
Clay	−1.6	70 ± 40[c]	110 ± 30	+0.3	3900 ± 1700	640 ± 340	−0.1	36,600 ± 5500	960 ± 160
Silt	+2.4	2300 ± 480	50 ± 60	+0.3	600 ± 380	260 ± 60	−0.1	31,900 ± 15,000	470 ± 90
Loam	+2.4	1800 ± 670	90 ± 270	+0.1	7700 ± 1500	280 ± 90	+1.9	83,000 ± 28,900	1300 ± 190

Also the CO_2 emissions at the temperatures with maximum N_2O emissions are shown with standard deviations.
[a] $n = 10$.
[b] $n = 3$.
[c] No sharp maximum emission peak was detected.

0 and −2.5 °C (Fig. 3a). Loam soil showed no similar phenomenon, the concentration of N_2O in the soil profile decreased immediately after reaching 0 °C

Nitrate was available for denitrification during the whole temperature range of the experiment. The content of NO_3^-–N remained constant in loam and even increased slightly in the organic, clay and silt soils. The content of NO_3^-–N was highest in the organic soil, which also showed the highest ammonification rate (Fig. 4). Similarly, there was NO_4^+–N available for nitrification in all soils throughout the experiments (Fig. 4).

3.2. Experiment 2: decrease–increase in temperature

All soils, except the loam, showed an increase in their N_2O emissions when the water content was elevated to 90% WFPS (Figs. 2a,b and 5a,b, Table 2). The N_2O emissions from the organic and clay soils increased immediately after lowering the temperature from +2.5 °C with maxima at +0.7 °C and +0.3 °C, respectively. Loam had an increase in the N_2O emission below +0.5 °C with a maximum at 0.0 °C (Fig. 5a and b). The N_2O emissions from silt were negligible at all the studied temperatures (Fig. 5b). The maxima for the N_2O emissions from organic, clay and loam soils at 90% WFPS were from 4- to 11-fold higher than the maximal emissions without water addition (Table 2). However, the increase in N_2O emissions during temperature decrease was statistically significant ($P < 0.05$) only in organic and clay soils. Water addition had only minor effects on the CO_2 emissions (Figs. 2c and 5c) and as in experiment 1, the CO_2 emissions decreased with temperature without a temperature anomaly (Fig. 5c).

The concentration of N_2O in the soil profiles increased rapidly after reaching 0 °C (Fig. 3b). The concentration maximum in silt soil was at −0.3 °C (−4 cm) and in the loam soil at −0.7 °C (−8 cm).

The soils kept for 3 months at −8 °C all showed negligible N_2O emissions (Fig. 6a and b). When the temperature was increased in a stepwise manner, clay and loam soils began to emit N_2O at high rates between −1.5 and −0.1 °C (Fig. 6a and b). Also the N_2O emissions from organic and silt soils increased in this temperature range although the emission maxima were lower than those from clay or loam (Fig. 6a and b). The highest emissions (Table 2) from organic, clay, silt and loam soils occurred at −0.1, −0.1, −0,1 and +1.6 °C, respectively. In each soil type studied, the N_2O increase was statistically significant ($P < 0.05$). The maxima

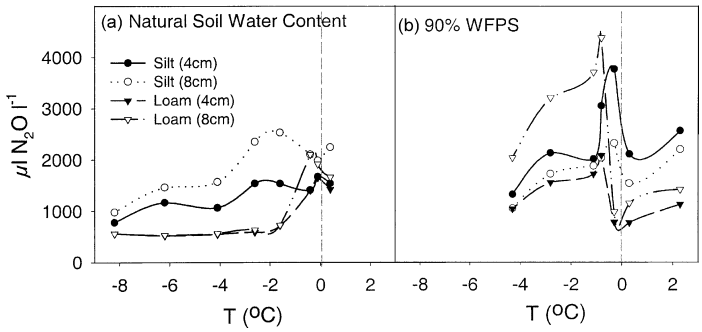

Fig. 3. Concentration of N_2O (μl l^{-1}) in silt and loam soil profiles at depths of 4 and 8 cm, with stepwise lowering of temperature at natural soil water content (a) and at 90% WFPS (b).

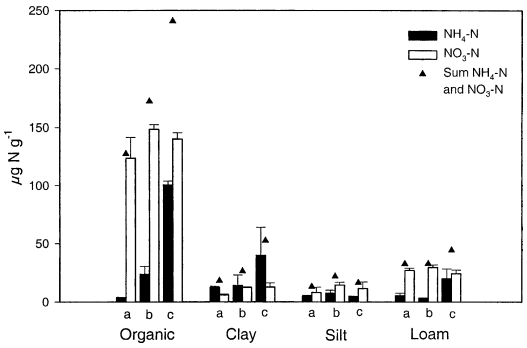

Fig. 4. Ammonium (NH_4^+–N, black bar) and nitrate (NO_3^-–N, white bar) concentrations in soil at +15 °C (Exp.1,) (a), at −8 °C (Exp. 1) (b), and after thawing experiments (Exp. 2) (c) pooled samples from cores, $n = 6$.

emissions when the temperature was increased were from 7- to 53-fold higher than the maxima during lowering of temperature (Table 2).

The CO_2 emissions and their standard deviations were low between −8 and −2.5 °C. Thereafter, the emissions and standard deviations increased with emission peaks between −1.9 and +1.7 °C (Fig. 6c).

3.3. Experiment 3: thawing of soils by increasing temperature continuously

During the thawing within 168 h from −2 to +4 °C the emissions began to increase at temperatures close to 0 °C. Organic soil showed a continuous increase in the N_2O emission up to +3 °C (during the first 30 h from the beginning of thawing, Fig. 7a). The emissions from the organic soil during thawing were the highest measured in

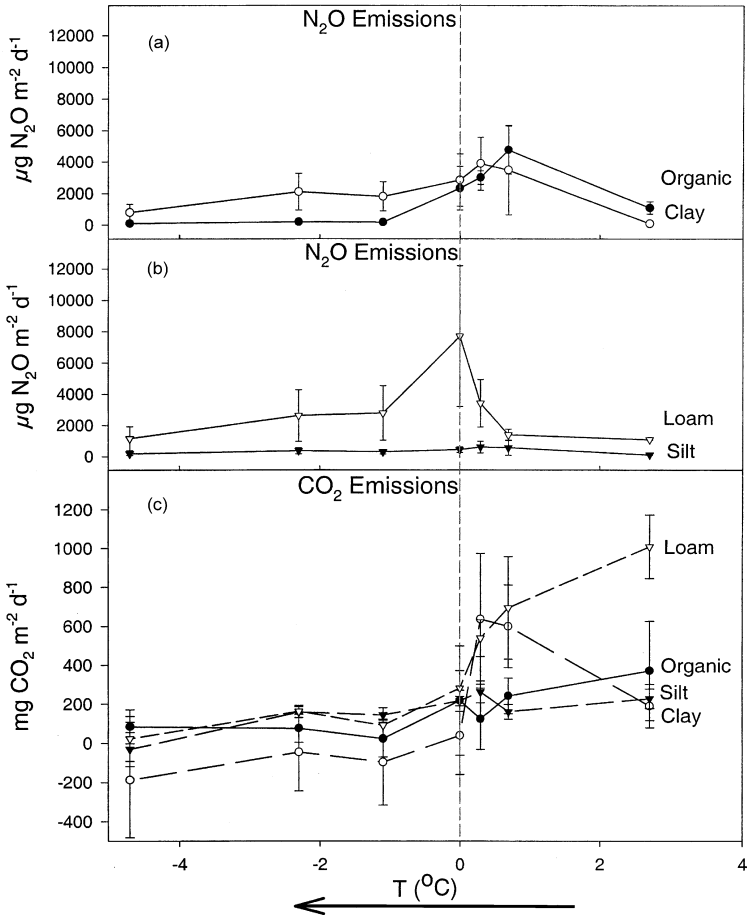

Fig. 5. Nitrous oxide and CO_2 emissions from organic, clay, silt and loam soils with lowering of temperature after adjusting the WFPS to 90% ($n = 3$). Standard deviation is shown with bars.

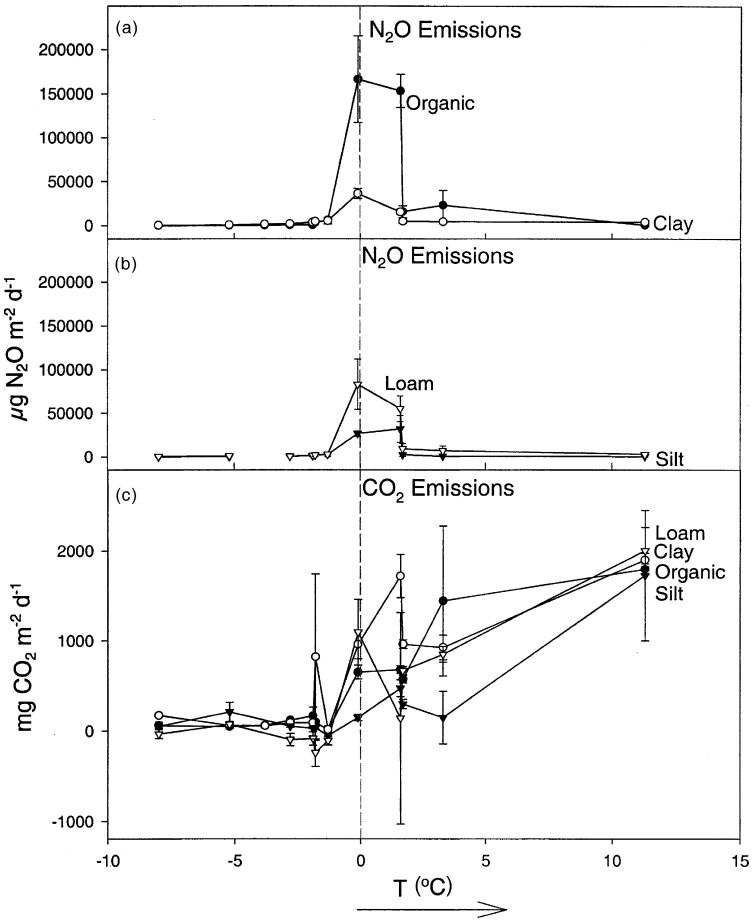

Fig. 6. Emissions of N_2O and CO_2 from soils at 90% WFPS ($n = 3$) with increasing temperature in a stepwise manner. Standard deviation is shown with bars.

this study; the N_2O emissions from other soils were lower (Fig. 7b–d). Clay and silt (Fig. 7c and d) showed peaks in their N_2O emissions between 0 and +2 °C (during the first 50 and 8 h from the beginning of the thawing, respectively). In loam (Fig. 7d) there was only a small N_2O peak during the first 7 h. However, there was a statistical difference ($P < 0.05$) in N_2O emissions (0–29 h, during soil thawing) from organic, silt and loam soils. Emissions of CO_2 increased with the increasing temperature without any distinct peaks (Fig. 7f–i). The emission rates of CO_2 were similar to those recorded in the previous experiments.

4. Discussion

4.1. N_2O emissions above 0 °C without soil freezing history

When soil temperature was lowered in a stepwise manner each of the four soils showed an increase in N_2O release at temperatures near 0 °C. However, the release rates were dependent on the soil type and soil water content. The increase in the N_2O release from the unfrozen soils took place close to just above or below 0 °C. The liberation of N_2O stored in soil does not explain these increases, because the solubility of N_2O in water increases with decreasing temperature. Therefore, we consider that microbial processes were responsible for the increased N_2O production.

Oxygen availability is the key factor regulating denitrification. However, O_2 availability was not the key factor for the observed high N_2O production at low temperatures. When soil temperature is lowered to 0 °C with constant soil water content, the O_2 content normally increases with decreasing temperature as a result of the decrease in microbial O_2 consumption (Smith et al., 1998). This means that the developing O_2 conditions do not favour an increase in the total denitrification rate (sum of N_2O and N_2) when the temperature is lowered close to 0 °C.

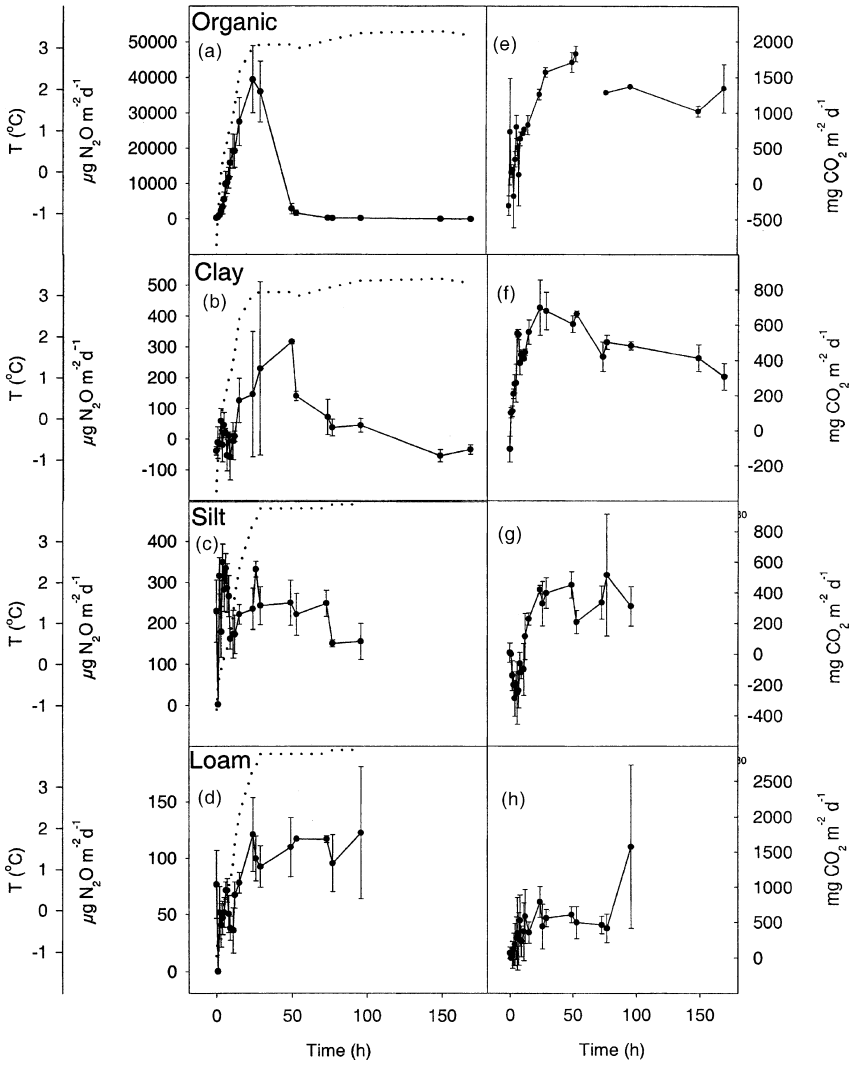

Fig. 7. Emissions of N_2O and CO_2 from organic, clay, silt and loam soils during thawing from −2.5 to +4 °C ($n = 3$). Standard deviation is shown with bars. Note different scales.

Oxygen deficiency, and an associated increase in N_2O production, could occur at lower temperature in soils with high microbial activity. With good soil aeration (WFPS of 42–66%), organic and clay soils revealed increase in N_2O production at a lower temperature than silt and loam, which could be associated with the higher respiration rate (O_2 consumption) in organic and clay soils. High soil water content favours denitrification associated with the limitation of O_2 diffusion. Here, N_2O production was favoured by high soil water content in three out of the four soils. However, there was no systematic shift in the temperature showing the highest emission when the soils were moistened. The increased N_2O release took place at a lower temperature with higher soil water content (lower O_2 diffusion rate) only in loam.

It is known that the ratio of N_2O-to-N_2 in denitrification increases with a decrease in temperature, and thus enhances N_2O production (Keeney et al., 1979; Maag and Vinther, 1996). Presently, we do not have results to show the possible changes in the ratio of N_2O-to-N_2 in our soils.

One possible explanation for the increase in the N_2O emissions close to 0 °C with decreasing temperature may lie in the temperature history of the soil during the experiment. The incubation of soil for 14 d at rather high temperatures (+15, +10 and +5 °C), allows good conditions for growth of the microbial community. When the decreasing

temperature reaches a certain critical point, the populations probably started to decline and the decomposing cells released nutrients for the surviving microbes. This sudden increase in the substrate availability could then account for increase in N_2O production. An interesting observation was that denitrifiers might benefit more from the extra substrates than the other heterotrophic microbes (CO_2 production) in general (see also below).

4.2. N_2O emissions during freezing and thawing of the soil

The high N_2O emissions during freezing–thawing cycles of soils are well documented (Sommerfield et al., 1993; Röver et al., 1998; Teepe et al., 2000; Premié and Christensen, 2001). However, the experiments on freezing–thawing have not differentiated between the actual production and diffusion of N_2O at a particular temperature. We changed the soil temperature in a stepwise manner in order to determine the actual N_2O production, not just only the release of accumulated N_2O. When the temperature of the frozen soils was increased stepwise (Experiment 2. part 2), the N_2O release started to increase at -0.5 °C. These emissions were much higher than the peaks in the N_2O release when the soil temperature was decreased. The key questions are, whether there was an extremely high production of N_2O just below 0 °C, or was there a liberation of the N_2O entrapped in the frozen soils when the soil thawed (Burton and Beauchamp, 1994)?

As a result of the presence of ions in soil solution, thawing begins at temperatures below 0 °C. There obviously was N_2O production below 0 °C, as shown by the N_2O release when the soil temperature was decreased from + to −0 °C. Furthermore, the results from the gas sampling system in the soils show that there was an accumulation of N_2O in the soils below 0 °C. Some of the large amount of N_2O released just below and above 0 °C when the soil temperature was increased stepwise might have originated from the liberation of the N_2O stored in the frozen soils. In our experiments, the CO_2 release also showed a sudden increase just below 0 °C, which might indicate the release of the accumulated CO_2. When soils that had been kept for 4 d at −2 °C were thawed rapidly, without any steps, there were similar peaks in the N_2O release immediately after thawing began.

The possibility cannot be excluded that part of the N_2O released around 0 °C originated from denitrification when soil was thawing. It has been suggested that soil freezing–thawing destroys soil microbes and soil aggregates, increasing the availability of substrates for heterotrophic microbes including denitrifying bacteria (Christensen and Tiedje, 1990; Christensen and Christensen, 1991; Schimel and Clein, 1996; Denef et al., 2001). In fact, CO_2 production close to 0 °C was higher with thawing of the frozen soil than that encountered with decreasing soil temperatures. However, there was no close association between N_2O and CO_2 production, indicating that the denitrifying bacteria had the capability to utilize any extra substrates released during soil temperature stress. The denitrifying bacteria might have increased their activity although the overall microbial communities did not increase their activity as assessed here by CO_2 production.

Our results suggested that there was N_2O production in soils at least down to −6 °C. However, frozen soils may still contain considerable amounts of unfrozen water. The amount of unfrozen water is dependent on pore size distribution, void ratio, particle size and surface area (i.e. soil type) (Burt and Williams, 1976). In clay soils especially, significant amounts of unfrozen water can exist at temperatures below −10 °C (Konrad and Duquennoi, 1993). It is important to note that during freezing the soil solutes (inorganic nutrients, organic substrates) concentrate in the free water around the soil particles (Stähli and Sadler, 1997). Therefore, the unfrozen microsites in soils have the highest content of organic substrates and NO_3^-–N for denitrifying bacteria. The unfrozen microsites are surrounded by ice and therefore have limited gas exchange, which leads to the development of O_2 deficiency and good conditions for denitrification in microsites. The high NO_3^-–N concentration in unfrozen microsites might increase the ratio of N_2O-to-N_2 in denitrification (Blackmer and Bremner, 1978) thus favouring N_2O production. Furthermore, the N_2O and CO_2 produced are entrapped and cannot be released to the atmosphere and they only can be released during thawing. Soil can crack, which was probably associated here with the high deviation in both the N_2O and CO_2 emissions recorded during thawing.

Agricultural soils at temperatures near soil freezing can emit significant pulses of N_2O. In our experiments, intact soil cores had clear N_2O emission maxima both during soil freezing and soil thawing. However, different soil types had different responses. Our results show the importance of low temperatures on annual N_2O fluxes. Neglecting the emissions during soil freezing and thawing can lead to significant underestimation of the annual N_2O fluxes.

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CHAPTER III

SOIL WATER CONTENT AND FREEZING TEMPERATURE AFFECT FREEZE-THAW RELATED N_2O PRODUCTION IN ORGANIC SOIL

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Soil water content and freezing temperature affect freeze–thaw related N_2O production in organic soil

Hannu T. Koponen* and Pertti J. Martikainen
*Department of Environmental Sciences, Research and Development Unit of Environmental Health, University of Kuopio, Bioteknia 2, P.O. Box 1627, FIN-70211 Kuopio, Finland; *Author for correspondence (fax +358-17-163-750; e-mail: Hannu.Koponen@uku.fi)*

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Abstract

An organic agricultural soil was exposed to freeze–thaw cycles (FTC) using either intact soil cores or cores packed with homogenized soil. The cores were first exposed to two mild FTCs (–1.5 °C/+4 °C) with soil water content being 56–85% of the water-filled pore space (WFPS). Both intact and packed soil cores showed high N_2O emissions when the soil was thawing and had high WFPS. The second freeze–thaw cycle induced lower N_2O emission than the first. After the mild FTCs, a deep frost (–15 °C) was applied. This greatly increased the N_2O emissions when the soil was thawing. Freezing–thawing had a smaller effect on CO_2 than on N_2O release. The results show that both soil moisture and the severity of frost modify the N_2O burst after thawing, and N_2O release (denitrification) was favoured more by FTC than heterotrophic microbial activity (CO_2 production) in general. The possible reason for this difference is discussed.

Introduction

In boreal and temperate regions, soils are exposed to several freeze–thaw cycles (FTC) during autumn and spring. Increases in N_2O and CO_2 emissions have been reported during soil thawing, both in field (e.g., Christensen and Tiedje 1990; Röver at al. 1998) and laboratory experiments (e.g., Chen et al. 1995; van Bochove et al. 2000). The FTC-related high N_2O emission events can contribute up to 70% of the annual N_2O emissions (Röver et al. 1998). This phenomenon is well known, but the underlying processes and the conditions that control the production of N_2O are still poorly known.

Soil freezing has been suggested to destroy microbes and/or soil aggregates, leading to increases in nutrient concentrations. The extra nutrient availability then increases the activity of any microbes that have survived after the soil has thawed (Skogland et al. 1988). Schimel and Clein (1996) found with tundra and taiga soils that the respiratory peak decreases with repeated freeze–thaw cycles. A similar phenomenon has been observed with agricultural soils, both for CO_2 and N_2O emissions (Premié and Christensen 2001). Contradictory results, where FTC-related N_2O emission peaks increase with repeated freeze–thaw cycles, have also been reported (Chen et al. 1995). Most studies on FTC-induced N_2O emissions have been done in extreme temperature conditions, with freezing temperatures of -15 °C to -20 °C and thawing at $+10$ °C to $+15$ °C (e.g., van Bochove et al. 2000; Müller et al. 2002), and the effect of soil water content on the FTC-induced gas emissions is still poorly known. However, knowledge of these crucial factors is necessary when evaluating annual N_2O emissions. Moisture is one of the key factors regulating denitrification in soils.

In Finland, organic agricultural soils are mostly drained peatlands, rich in degradable carbon and nitrogen. Due to the oxidation of organic matter, these

soils have a great potential for both N_2O and CO_2 emissions (Kasimir-Klemedtsson et al. 1997). Kasimir-Klemedtsson et al. (1997) estimated that about 25% of the total anthropogenic N_2O emissions in Finland originate from organic soils, although the areal coverage of such soils is only 10% of the agricultural soils in the country.

In this laboratory study, we evaluated the effects of freezing temperature and moisture on FTC-induced N_2O and CO_2 emissions in a Finnish organic agricultural soil. We show how the soil water content and the severity of freezing affect N_2O production after soil thawing, and that thawing has a different impact on N_2O and CO_2 production.

Materials and methods

Sampling

Soil samples for the experiments were taken from an organic soil in Siikasalmi in eastern Finland (62°55′ N, 29°30′ E). The average annual temperature (1971–2001) in this region is +2.6 °C. The coldest month is January, with an average temperature of − 10 °C, and the warmest one is July, when the average temperature is +16.7 °C. Topsoil generally starts to freeze in early November and thaws in early April. The average annual precipitation (1971–2000) is 643 mm, of which 262 mm falls as snow (Finnish Meteorological Institute 2002). The soil pH is 7.0, and total C and N contents are 26% and 1.6%, respectively.

Sampling was done on 20th September 2001. To study undisturbed soil columns, intact soil cores (15 cm in height, 10 cm inner diameter) were hammered into the PVC tubes using a stainless steel corer. For packed cores, soil samples taken from depths of 0 to 20 cm were pooled and sieved (5.6 mm mesh) immediately after sampling. Prior to starting the experiments, the soil was stored for 1 month at +4 °C.

Soil physical and chemical characterization

Gravimetric water content was determined by drying the soil samples (25 g fresh weight) at 105 °C for 24 h. Soil particle density (1.82 g cm^{-3}) was determined using pyknometers (Blake 1965), and field bulk density (0.33 g cm^{-3}) using a volumetric precise cylinder according to Blake and Hartge (1986). Soil water-filled pore space (WFPS) was calculated from soil particle density and bulk density according to Ambus and Christensen (1995). Soil nitrate (NO_3^-) was measured from the soil water suspension (1:5 v/v, 175 rpm, 1 h), and ammonium (NH_4^+) from the soil KCl suspension (1:5 v/v 2M KCl, 175 rpm, 1 h). The extracts were filtered (Blauband 589^3 BlueRibbon filter paper) and stored at +4 °C until analysis. Ammonium was analyzed spectrophotometrically (Ultrospec 3000 pro [Biochrom Ltd., Cambridge, UK] UV/Visible spectrophotometer) (Fawcett and Scott 1960), and nitrate by ion chromatography (Dionex [Sunnyvalley, CA] DX-120 with an AS 9-HC 4-mm anion column and an ASRS-ULTRA 4 mm suppressor).

Freeze–thaw experiments

The experiments were done with intact soil cores and cores packed with homogenized soil at two water contents: at 58% (from the dry weight of soil: this was the sampling moisture) and at 65%. Water was sprayed onto the homogenized soil before packing (packed cores), and water was sprayed onto the soil surface of the intact cores. WFPS ranged from 56 to 75% in the intact soil cores, and from 64 to 85% in the packed soil cores. All soil cores were first incubated at − 1.5 °C for 5 days before thawing at +4 °C. Measurements for gas emission rates (N_2O and CO_2) were carried out at +4 °C during and after the thawing (73–193 h from the beginning of thawing, until the N_2O emissions were at a constant level). Two FTCs were introduced to study the effects of sequential FTCs on N_2O and CO_2 emissions. After the second thawing from − 1.5 °C, one core (65% water content) was frozen to − 15 °C for 2 weeks, then thawed at +4 °C and measured for N_2O and CO_2 emissions.

The temperature of the incubator cabin (LMS Cooled Incubator, model 250) was measured continuously by data loggers (HOBO®). The soil temperature during thawing was monitored by a weather station with temperature sensors (Maws Automatic Weather Station [Vaisala, Finland]), which were inserted to a depth of 5 cm in one replicate core of each treatment.

Gas sampling and analysis for N_2O and CO_2

The soil cores were sealed with gas-tight PVC chambers. The headspaces of the soil cores were flushed continuously with ambient air to avoid the accumulation of gases. Air flush was cut off before the gas flux measurements (closed chamber technique, described

in Crill 1991). Gas samples of 20 ml were taken into 60 ml polypropylene syringes (Terumo) equipped with 3-way stopcocks (Connecta) through a rubber septum at 3, 15 and 35 min after the incubation started. During the first 48 h of thawing, gas measurements were made every 3 to 6 h, then every 8 to 12 h until the emissions of N_2O had settled down to the background level at +4 °C. The concentrations of N_2O and CO_2 were determined with a Hewlett–Packard 5890 Series II gas chromatographic system equipped with ^{63}Ni electron capture (ECD) and thermal conductivity (TCD) detectors (see Nykänen et al. 1995).

Results

Thawing with sampling moisture

At sampling moisture (58% water content), both intact and packed samples showed an increase in N_2O emissions associated with the thawing (Figure 1a, b). The duration of the FTC-related N_2O peaks were less than 48 h in both intact and packed cores. The N_2O maxima for intact and packed cores were 19 μg N_2O m^{-2} h^{-1} and 75 μg N_2O m^{-2} h^{-1}, respectively. In the second FTC, the emissions from the intact cores were lower without any distinct thawing peaks, and only minor emissions (maximum of 8 μg N_2O m^{-2} h^{-1}) were detected. In packed cores, the effect of the FTC was still observed (maximum of 60 μg N_2O m^{-2} h^{-1}), even though the maximum was lower than in the first FTC. Nitrate was available during the whole experiment. At the beginning of the experiment the soil contained 12 ± 0.9 mg NO_3-N g DW^{-1} (average ± SD), and at the end 13 ± 1.8 mg NO_3-N g DW^{-1} in the intact cores, and 27 ± 1.8 mg NO_3-N g DW^{-1} in the packed cores.

Total cumulative production of N_2O after thawing is shown in Table 1. In the first FTC, the cumulative N_2O production in the intact cores was 50% of that in the packed cores. In the second cycle, the peak was observed only in the packed cores, while the intact cores showed no distinct maximum in the emission. In the packed cores, the cumulative N_2O production after the second freeze–thaw was lower, being 70–85% of that in the first FTC.

There were no distinct FTC-induced CO_2 maxima from the intact cores (data not shown). Average CO_2 emissions are shown in Table 1. One of the packed cores showed an increase in CO_2 emissions immediately after the first thawing started (data not shown), but the CO_2 emissions from another core remained constant.

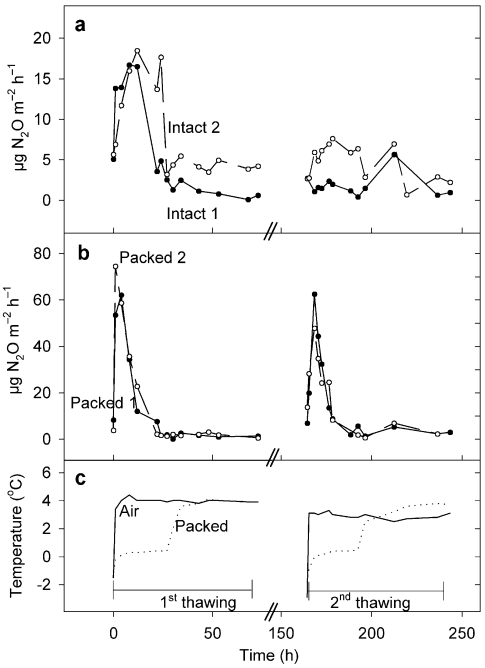

Figure 1. N_2O production of field moist samples (water content 58%) during soil thawing (–1.5 °C/+4 °C). (a) Intact soil cores, (b) packed soil cores, (c) air and soil temperature during soil thawing.

Thawing with moistened samples

Moistened samples (65% water content) showed generally higher N_2O emissions during thawing than the field moist samples. From the intact cores (Figure 2a), the N_2O maxima during the first and the second FTC were 200 μg N_2O m^{-2} h^{-1} and 150 μg N_2O m^{-2} h^{-1}, respectively. The emission peaks were sharp, and thawing-related N_2O emissions were recorded within the first 48 h from the beginning of the thawing. From the packed cores (Figure 2b), the emission maxima were higher during the first FTC (maximum 880 μg N_2O m^{-2} h^{-1}) and the durations of the FTC-related N_2O emissions were longer: increased N_2O emissions were measured up to 168 h from the beginning of soil thawing. In the second FTC, N_2O peaks from both the intact and the packed cores were lower. Nitrate was

Table 1. Thawing related cumulative N_2O production and the average production of CO_2 (average ± sd) in the soil cores during the incubations.

	Cumulative N_2O production during thawing-related maximum[a] (μg N_2O-N m^{-2})			Average CO_2 emission[b] (mg CO_2 m^{-2} h^{-1})		
	Cycle 1 (–1.5 °C)	Cycle 2 (–1.5 °C)	Cycle 3 (–15 °C)	Cycle 1 (–1.5 °C)	Cycle 2 (–1.5 °C)	Cycle 3 (–15 °C)
Sampling moisture						
Individual cores						
Intact 1	100	20 [c]	ND [d]	15 ± 5	16 ± 8	ND
Intact 2	125	50 [c]	ND	47 ± 13	33 ± 8 *[1]	ND
Packed 1	190	160	ND	25 ± 14	32 ± 10	ND
Packed 2	210	150	ND	24 ± 12	21 ± 9	ND
Moistened samples						
Individual cores						
Intact 1	1 200	870	ND	29 ± 7	25 ± 8	ND
Intact 2	1 900	1 900	23 000	33 ± 16	33 ± 16	74 ± 19 *[2]
Packed 1	26 000	420	ND	24 ± 7	17 ± 9	ND
Packed 2	17 000	1 400	9 400	31 ± 12	23 ± 12	25 ± 8

[a] Cumulative production from individual cores, calculated for the duration of the emission maximum (at water content 58% 0–32 h; water content 65% 0–168 h and − 15 °C (water content 65% 3rd cycle 0–151 h); [b] Average emissions for the whole incubation period (at water content 58% 0–79 h and water content 65% 0–193 h, in cycle 3 0–168 h); [c] No sharp emission peak was detected, calculated for production 0–28 h; [d] Not determined; *[1] Significant differences between the CO_2 emissions between the 1st and the 2nd FTC ($p < 0.05$); *[2] Significant differences between the CO_2 emissions between the 2nd and the 3rd FTC ($p < 0.05$).

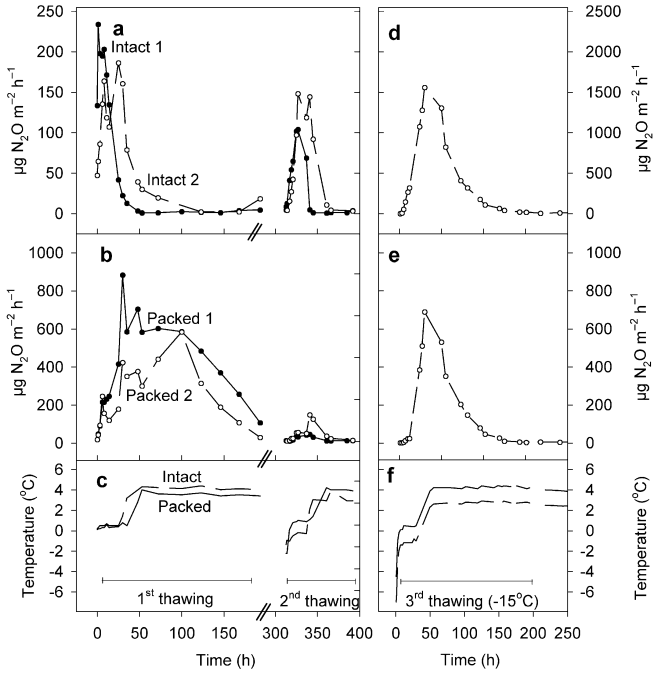

Figure 2. N_2O production of moistened samples (water content 65%) during soil thawing. (a) Intact soil cores (–1.5 °C/+4 °C), (b) packed soil cores (–1.5 °C/+4 °C), (c) soil temperature during soil thawing (–1.5 °C/+4 °C), (d) intact soil core (–15 °C/+4 °C) (notice different scale), (e) packed soil core (–15°C/+4°C), (f) soil temperature during soil thawing (–15 °C/+4 °C).

available during the whole experiment; at the beginning of the experiment the NO_3^- concentration in the intact and packed cores was 20 ± 0.8 mg NO_3-N g DW^{-1}. After the experiment, NO_3^- concentration in the intact core was rather low, 4 ± 0.5 mg NO_3-N g DW^{-1}, whereas in the packed cores it was somewhat higher, 27 ± 1.0 mg NO_3-N g DW^{-1}.

Cumulative N_2O production (Table 1) during emission maxima was greater at higher water content than at lower water content. N_2O production in the moistened intact cores during the first FTC was 14 times higher than that in intact field moist cores. In the packed cores, N_2O production was 106-fold that at the lower water content. Cumulative production from the moistened intact cores was only 7% of that from the packed cores during the first FTC. In the second cycle, N_2O production was lower in the packed cores: cumulative production during the emission maxima was only 2–8% of that during the first cycle. There was no such great difference in the N_2O production from the intact cores during the second cycle, where the production was 70–95% of that during the first FTC.

The CO_2 emission from the moistened cores was similar to that in field moist conditions. Average CO_2 emissions are shown in Table 1. There was no statistical difference in CO_2 emissions between the two freeze–thaw cycles with moistened samples.

Deep frost

Soil frozen at − 15 °C enhanced N_2O fluxes during soil thawing (Figure 2d, e). During soil thawing, the emission maxima from intact and packed cores were 1600 μg N_2O m^{-2} h^{-1} and 690 μg N_2O m^{-2} h^{-1}, respectively. Cumulative production (Table 1) from the intact core was 1.8 times higher than from the packed core. Cumulative N_2O production after the freeze–thaw cycle at − 15 °C/+4 °C in the intact and packed cores was from 6.7 to 12 times higher than that during the previous cycle at − 1.5 °C/+4 °C.

Deep frost modified CO_2 emission in the intact core (Table 1). No clear thawing-related emission maxima were observed, but average CO_2 emission was higher, being 74 ± 19 mg CO_2 m^{-2} h^{-1} during and after the soil thawing (Table 1). The packed core did not show a similar trend, with an average CO_2 emission of 25 ± 8 mg CO_2 m^{-2} h^{-1} (Table 1).

Soil NO_3^- concentration increased during the experiment. Nitrate concentration after the deep frost experiment was 13 ± 0.4 μg NO_3-N g DW^{-1} in the intact core (4 ± 0.5 μg NO_3-N g DW^{-1} in the beginning) and 43 ± 0.3 μg NO_3-N g DW^{-1} in the packed core (27 ± 1.0 μg NO_3-N g DW^{-1} in the beginning). Ammonium was present at low concentrations (< 2 mg NH_4-N g DW^{-1}, data not shown).

Discussion

Soil water content significantly affected N_2O production during soil thawing. High water content reduces oxygen diffusion, favouring denitrification. Van Bochove et al. (2000) concluded that the effect of soil freezing is stronger in small than in large macroaggregates, possibly due to higher water content in small aggregates. Freezing and thawing can also decrease aggregate stability (Oztas and Fayetorbay 2002), and such a disturbance can increase the release of soil inorganic and organic substrates (Soulides and Allison 1961; Denef et al. 2001). It has been suggested that soil freezing can destroy microbial cells, leading to the release of substrates from lysed cells (Christensen and Tiedje 1990; Hantschel et al. 1995). This increase in energy supply is one of the key factors controlling denitrification at low temperatures. When soil is thawed, extra energy becomes available for heterotrophic bacteria (including denitrifiers). Herrmann and Witter (2002) suggest that easily decomposable material becomes available during FTC. They report that only 5% of microbial biomass is destroyed during FTC, but this contributes 65% to the total C flush. Our results show that especially denitrification benefits from this FTC-derived carbon in organic soil.

Though the water content was the same in both intact and packed cores, the WFPS differed. In the field moist intact cores the WFPS was 56%, compared with 64% in the field moist packed cores. In the moistened samples, the WFPS in the intact and packed cores were 75% and 85%, respectively. The difference in WFPS was due to the packing of the cores. Bulk density in packed cores was 0.37 g cm^{-3}, compared with 0.33 g cm^{-3} in intact cores. The difference in N_2O emissions between packed and intact samples was most likely due to the difference in their WFPS. Teepe et al. (2000) found a positive correlation between the WFPS in topsoil (0–5 cm) and total wintertime N_2O emissions. Oxygen diffusion rate played a major role in the FTC-induced wintertime N_2O emissions (Teepe et al. 2000).

The amount of CO_2 emitted during soil thawing reflects the activity of all heterotrophic microbes, including CO_2 from denitrification. Contrary to several other studies (e.g., Skogland et al. 1988; Schimel and Clein 1996) we did not find any significant increase in CO_2 production during soil thawing from − 1.5 °C. However, thawing from − 15 °C doubled the CO_2 emissions from the intact soil core. No such increase in CO_2 production was associated with the deep frost in the sieved/homogenized packed soil core lacking root material. This reflects the increase in the availability of microbial substrates from the root material existing in the intact soil core, which indicates that thawing-related CO_2 emissions can also be dependent on the freezing temperature. Increase in denitrification activity alone did not explain the elevated CO_2 production: the theoretical CO_2 production calculated from the denitrification stoichiometry explained only 0.1–5.2% of the total CO_2 production. In organic soils the availability of C and microbial activity is so high that the relative increase in CO_2 production induced by FTCs is low (see Schimel and Clein 1996). However, as discussed above, this increase in available C is crucial for denitrifiers, which compete for C with aerobic respires.

There are significant amounts of unfrozen soil water (i.e., water films in the soil matrix) down to –20 °C (Rivkina et al. 2000), mostly as a result of the salting out effect (Edwards and Cresser 1992). In this unfrozen water film the nutritional conditions for microbes are good, because both inorganic and organic solutes occur at high concentrations in the unfrozen soil solution. Conditions in the water film especially favor denitrification, since microbial oxygen consumption can create anaerobic conditions in films surrounded by ice.

Soil freezing temperature also had a clear effect on FTC-induced N_2O emission. Though the soil cores had already undergone two additional FTCs at − 1.5 °C, lowering the freezing temperature further induced an N_2O burst. This indicates that freezing temperature plays an important role in FTC-induced N_2O emissions, possibly via the breaking up of macro- and microaggregates and microbial cells. Also, deep frost may destroy fine roots, leading to an increase in soil nutrient supply (Tierney et al. 2001).

At both soil water contents, the thawing-related N_2O emission maximum was higher during the first freeze–thaw cycle. When soil cores were thawed again, N_2O emissions were lower. Priemé and Christensen (2001) found a similar difference in N_2O and CO_2 emissions in their study with agricultural soils. Schimel and Clein (1996) report a similar phenomenon with CO_2 for tundra and taiga soils. They conclude that freeze–thaw cycles cause a flush of microbial C and N during the first cycle, but after repeated cycles the ability of microbial communities to decompose SOM falls. An additional explanation could be that most of the organic and inorganic substrates are released during the first FTC; the amount of substrates falls cycle by cycle, leading to lower thawing-related N_2O (or CO_2) emissions. In packed soil, the peaks were wider at higher moisture content. This may be due to the more anaerobic conditions that favor the activity of denitrifiers.

High FTC-related N_2O emissions have been associated with high concentrations of NO_3^- in soil (Kammann et al. 1998). Such high concentrations ensure that nitrate does not limit denitrification. Furthermore, high nitrate concentrations have been found to increase the N_2O/N_2 ratio in denitrification (Blackmer and Bremner 1978). However, in our study, there was no distinct correlation between NO_3^- content and FTC-induced N_2O emissions, indicating that other factors than the availability of nitrate mostly regulated N_2O production here. Evidently, the soil water content and availability of organic substrates for denitrifying bacteria were among the key factors. However, it is important to note that the availability of carbon for denitrification cannot be predicted well from the total CO_2 production in soil.

Conclusions

Our results show the importance of soil moisture and freezing temperature on freeze–thaw induced N_2O production. However, the intact soil cores showed on average lower N_2O production after the mild frost (–1.5 °C) than the packed cores, resulting probably from the better aeration of the intact cores. Emissions of N_2O with mild frost decreased with repeated FTCs. The thawing-related CO_2 production with mild frost was low, indicating that denitrification benefits more from the FTC-induced substrate increase than soil respiration. However, when root material is available in soil, deep frost (–15 °C) can enhance the substrate availability of heterotrophic microbes in general, increasing both CO_2 and N_2O production.

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CHAPTER IV

TEMPERATURE RESPONSES OF NO AND N_2O EMISSIONS FROM BOREAL ORGANIC SOIL

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Temperature responses of NO and N_2O emissions from boreal organic soil

Hannu T. Koponen[a,*], Claudia Escudé Duran[a], Marja Maljanen[a], Jyrki Hytönen[b], Pertti J. Martikainen[a]

[a]*Department of Environmental Sciences, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland*
[b]*Finnish Forest Research Institute, Kannus Research Unit, P.O. Box 44, FI-69101 Kannus, Finland*

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Abstract

Both NO and N_2O are produced in soil microbial processes and have importance in atmospheric physics and chemistry. In recent years several studies have shown that N_2O emissions from organic soils can be high at low temperatures. However, the effects of low temperature on NO emissions from soil are unknown. We studied in laboratory conditions, using undisturbed soil cores, the emissions of NO and N_2O from organic soils at various temperatures, with an emphasis on processes and emissions during soil freezing and thawing periods. We found no soil freezing- or thawing-related emission maxima for NO, while the N_2O emissions were higher both during soil freezing and thawing periods. The results suggest that different factors are involved in the regulation of NO and N_2O emissions at low temperatures.

Keywords: Organic soil; Nitrous oxide; Nitric oxide; Temperature; Freezing–thawing

1. Introduction

Nitrous oxide (N_2O) is a greenhouse gas which contributes to global climatic warming and to the depletion of ozone in the stratosphere (Bouwman, 1990; Crutzen and Enhhalt, 1997). The current atmospheric concentration of N_2O is 312 ppb but it is increasing at the rate of 0.2–0.3% yr^{-1} (IPCC, 2001). The global warming potential of N_2O is 296 times that of CO_2 for a time horizon of 100 years, and its average atmospheric lifetime is 120 years (Volk et al., 1997, IPCC, 2001). The influence of nitric oxide (NO) on the greenhouse effect is negligible, but it has importance in chemical reactions in the troposphere (Derwent, 1995), where NO reacts with ozone and free radicals in reaction chains producing nitrate and nitric acid (Meixner, 1994). The average atmospheric lifetime of NO is short, around 1.5 days (Yamulki et al., 1995).

Soil contributes 70% and 20% to the global fluxes of N_2O and NO, respectively (Conrad, 1995). There are differences between soil types in their capacity to produce these trace gases. Organic (peat) soils with high organic nitrogen content (1–2%) have high capacity to produce N_2O. Organic agricultural soils have the greatest potential to act as N_2O sources in boreal regions (Martikainen et al., 1993, Kasimir-Klemedtsson et al., 1997), but little is known about their NO fluxes. The area of cultivated organic agricultural soils in Finland is about 300 000 ha, representing 14% of the total area of agricultural soils (Myllys and Sinkkonen, 2004). These soils are responsible for 25% of the total anthropogenic N_2O emissions in Finland (Kasimir-Klemedtsson et al., 1997). Since 1969, more than 240 000 ha (Finnish Statistical Yearbook of Forestry, 2004) of agricultural fields have been afforested in Finland in order to reduce the cultivated area in the country.

N_2O and NO are produced in soil mainly in bacterial nitrification (an aerobic process) and denitrification (an anaerobic process). Several factors affect the emissions of these nitrogenous gases. Soil physical and chemical

*Corresponding author. Tel.: +358 17 163589; fax: +358 17 163750.
E-mail address: hannu.koponen@uku.fi (H.T. Koponen).

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characteristics such as temperature, moisture, pH, availability of ammonium (NH_4^+) and nitrate (NO_3^-) are the most important factors determining the emission rates of N_2O and NO. Several field studies have shown an increase in NO and N_2O emissions immediately after fertilization (McKenney and Drury, 1997, Regina et al., 1998, Williams et al., 1998). In organic agricultural soils, the high nitrogen mineralization rate provides a good supply of ammonium for nitrification, and denitrification then benefits from the nitrate produced in nitrification.

Seasonal variation in N_2O and NO emission rates from soils has been attributed to temperature fluctuations in soils (Yamulki et al., 1995). In boreal and temperate regions, soils are exposed to freeze–thaw cycles during autumn and spring. Laboratory experiments have shown surprisingly high N_2O production rates during freezing–thawing cycles (FTC) (e.g. Teepe et al., 2000, Koponen and Martikainen, 2004) as well as at temperatures close to 0 °C even without FTCs (Koponen et al., 2004). The temperature anomaly in N_2O emissions is also known from field experiments (e.g. Flessa et al., 1995; Maljanen et al., 2003). Sullivan et al. (1996) reported an increase in NO emissions with an increase in soil temperature, but little is known about NO emissions from soil at low temperatures.

In the present study, we explored the temperature responses of NO and N_2O emissions from organic soils with an agricultural land-use history, using laboratory microcosm experiments. The temperature response of NO emissions, including temperatures below 0 °C, was compared with that of N_2O to discover whether there are differences in the effects of temperature on the processes and emissions of these two gases. We show here that temperature regulates differently the emissions of NO and N_2O. This observation has importance when considering the annual NO and N_2O fluxes and the processes responsible for the production of these gases in boreal soils with temperatures fluctuating between plus and minus degrees.

2. Materials and methods

2.1. Sampling

Soil samples for the experiments were taken from Kannus, western Finland. The long-term (1971–2000) mean annual precipitation for the study region is 561 mm, of which approximately 230 mm is snow, and the mean temperature is +2.4 °C. The coldest month is February (−9.2 °C) and the warmest one is July (+15.8 °C). Topsoil is generally frozen from early December to mid-April (Finnish Meteorological Institute, 2002).

We studied two organic soils which had been used for cereal and grass cultivation for decades up to late 1970s. The first site (63°56′N, 23°53′E) was abandoned for 3 years and then afforested with silver birch (Betula pendula L.) 17 years before our experiments. The average height of trees was 6.9 m and the average peat depth of this site is 80 cm. Dominant undergrowth species in this area were Viola palustris, Rubus arcticus, Urtica dioica, Galeopsis bifida and Poa pratensis. The second site (63°54′ N, 23°57′E) with an average peat depth of 20–30 cm was abandoned and left uncultivated 25–30 years ago. This site was almost treeless. Dominant undergrowth species there were Juncus filiformis, Cirsium palustre, Deschampsia cespitosa, Poa pratensis and Epilobium angustifolium.

Four undisturbed soil cores were taken on 20 October 2003 from both sites using PVC cores (12.5 cm in height, inner diameter 19 cm) by hammering the cores into the soil. The soil cores were stored at +4 °C for 2 weeks before the experiments started. Four more undisturbed soil cores were sampled in mid-winter, on 11 February 2004, from the afforested site. At the sampling, both sites were covered with snow (snow depth in abandoned site was 34 cm and in afforested site 38 cm). Soil cores were transported to the laboratory where they were kept at −3.3 °C for 1 week before the experiments.

2.2. Soil physical and chemical characterization

Gravimetric moisture content was determined by drying the soil samples (25 g fresh weight) for 24 h at 65 °C. Soil particle density was determined using pycnometer (Blake, 1965), and soil bulk density using volumetric precise cylinders (Blake and Hartge, 1986). Soil water filled pore space (WFPS) was calculated from soil particle and bulk densities according to Ambus and Christensen (1995). Soil nitrate (NO_3^-) was measured in a soil:water suspension (1:4 v/v, 175 rev min^{-1}, 1 h), and ammonium (NH_4^+) in a soil:KCL suspension (1:4 v/v 2 M KCl, 175 rpm, 1 h). The soil extracts were filtered for the NO_3^- and NH_4^+ analyses using Blauband 589^3 BlueRibbon filter paper. KCl extracts were stored at +4 °C, and water extracts were frozen (−20 °C). Ammonium-N was analyzed spectrophotometrically (Ultrospec 3000 pro [Biochrom Ltd., Cambridge, UK] spectrophotometer) (Fawcett and Scott, 1960), and nitrate-N by ion chromatography (DX-120 with an AS 9-HC 4-mm anion column and an ASRS-ULTRA 4 mm suppressor [Dionex, Sunnyvalley, CA, USA]). Separate soil samples composed of 5-6 sub-samples taken from the study sites were dried and homogenized by passing them through a sieve with 2 mm mesh size. Soil pH was measured in a soil:water suspension (1:2.5 v/v) and total N concentration was determined by the Kjeldahl method, and the total C concentration was measured using a LECO® CHN 1000 analyser [Leco Corp., St. Joseph, MI, USA].

2.3. Experimental design

The experiments at various temperatures were conducted in an incubation cabin (LMS Cooled Incubator, model 250). The soil cores were sealed with gas-tight PVC hats to create a chamber (Fig. 1). The headspaces (1.2–1.3 l) were flushed continuously with NO-free air throughout the whole experiment to avoid the accumulation of gases in the

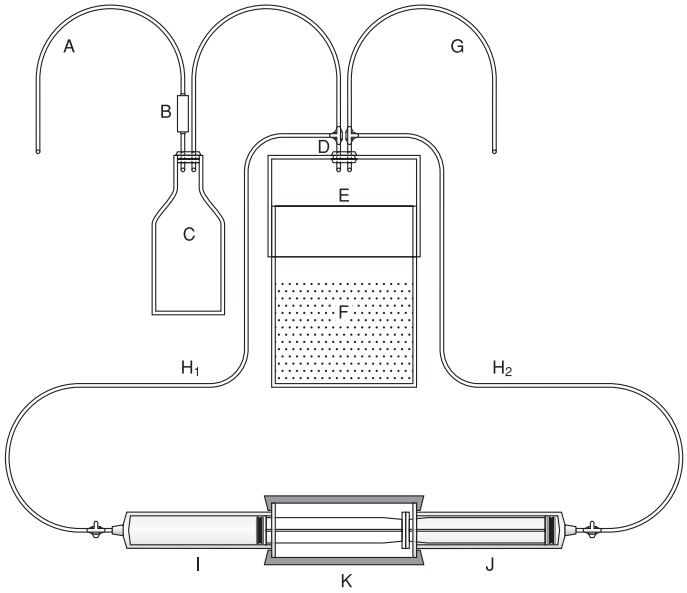

Fig. 1. Experimental design. Replacement air (A) is led though Purafil® filter (B) to remove the NO. Bottle (C) allows installation of more than one soil core to the replacement air system. During the incubation replacement air is flowing from the bottle through the headspace (E) to outside (G), avoiding the accumulation of the gases produced in undisturbed soil profile (F). When sampling for N_2O (and CO_2) starts, three-way stopcocks (Connecta) (D) are turned to position where replacement air is cut off. Two sampling lines (H_1 and H_2) both lead to 60 ml polypropylene syringes (Terumo) (I, J) equipped with 3-way stopcocks (Connecta). Two syringes plungers are connected to each other (K) on the bottom. In the beginning, when replacement syringe (I) is filled with ambient gas, syringe J is empty. When sampling to syringe J, plunger in syringe I slide into syringe, releasing the replacement air to the headspace (E). This system allows sampling from the headspace without any changes in pressure.

headspace. The temperature of the incubator cabins and soil temperature at a depth of 6 cm in two replicates of each soil type were monitored continuously by data loggers (HOBO®).

The temperature was decreased in a stepwise manner, using temperatures +9.5 (±0.4), +4.4 (±0.6), +0.4 (±0.3), −2.2 (±0.3) and −4.9 °C (±0.5 °C). This temperature pattern was selected to mimic the natural temperature conditions in the area. After a temperature change, the soil cores were allowed to stabilize in the new temperature for 6 days. Gas emission rates (N_2O, CO_2, and NO) at temperatures above 0 °C were measured once at each temperature. The soil cores were incubated at −2.2 °C for 3 weeks and at −4.9 °C for 6 weeks (mimicking temperature conditions during winter). At −2.2 °C, gas emission rates were measured twice a week, and at −4.9 °C after 6, 30 and 40 days.

After 6 weeks at −4.9 °C, the soil cores were allowed to thaw at +5.5 °C (±1.6 °C) and gas emissions rates (N_2O, CO_2 and NO) were monitored for 7 days for the afforested soil and 14 days for the abandoned soil. The thawing experiment with the afforested soil, sampled in the winter was done similarly to that for the afforested soil sampled in autumn. The soil cores were insulated with Styrofoam (polyester, 5 cm thick) to limit the heat flux through the walls and the bottom of the cores. This procedure ensured thawing mainly from the soil surface, resembling the natural thawing process in situ. Gas emission rates were measured every 4–12 h until the soil was completely thawed and the emissions were stabilized at a constant level.

2.4. Gas sampling and analysis for N_2O and CO_2

Measurements for N_2O and CO_2 were done with a closed chamber technique using the PVC chambers installed for the air flushing (see above). The air flush was cut off before the gas flux measurements were taken. Gas samples of 20 ml were taken into 60 ml polypropylene syringes (Terumo) equipped with 3-way stopcocks (Connecta) through a rubber septum at 3, 20 and 45 min after the initiation of incubation. For gas sampling a special sampler was designed. There was no pressure change in the chamber because the sampler allowed replacing the sample volume by ambient air at sampling (See Fig. 1). The concentrations of N_2O and CO_2 were determined from 20 ml gas samples with a Hewlett Packard 5890 Series II gas chromatograph equipped with ^{63}Ni electron capture (EC) and thermal conductivity (TC) detectors for N_2O and CO_2, respectively. Two loops (0.5 ml) were flushed with sample air prior to loading into the GC using a ten port Valco valve (see Nykänen et al., 1995). The flux rates were

calculated from the linear increase in the gas concentrations during the measurement period.

2.5. Gas sampling and analysis for NO

Measurements for NO were done with a dynamic chamber technique, with chamber volume of 7.2–7.5 l and flow rate of 0.6 l min^{-1}. The ambient air flush was cut off before the gas flux measurements were taken; NO-free air was used as replacement air during the measurements. Above 0 °C, NO concentration was analyzed by LMA-3D NO_2 analyzer [Unisearch Associates Inc., Ontario, Canada] equipped with an LNC-3D NO_x converter (CrO_3) at wavelength 425 nm. The analyzer was calibrated weekly for concentrations of 0–100 nl l^{-1}. Below 0 °C, NO was analyzed using an AC30 M chemiluminescent nitrogen oxides analyzer [Environment SA, Poissy, France]. The NO flux rate was calculated from the difference in the inlet (ambient concentration) and outlet NO concentrations in a dynamic equilibrium with constant gas flow rate.

2.6. Statistical analysis

In order to examine the effect of temperature, Friedman's test was used to compare the repeated measures from the same subjects in different temperatures. Soil types were tested individually for the whole temperature range during the decrease in temperature. In the soil freezing phase the most crucial temperatures (+0.4 to −4.9 °C) were tested again in order to determine the significance of freezing-related N_2O and NO emissions at these temperatures: in this case, Bonferron's correction for multiple subgroup analysis was used. The significance of differences in NO and N_2O emissions over time, starting from the beginning of the soil thawing, was tested using Friedman's test.

3. Results

3.1. Physical and chemical properties of the soil

Physical and chemical properties of the soils are shown in Table 1. Though the gravimetric moisture content in both afforested and abandoned soils was similar, their WFPS% differed, being higher in abandoned soil. WFPS of the soils was highest in the samples taken in winter. There was minor difference in pH of the soils. Total carbon (C-tot) content in the afforested soil was almost two times higher than in the abandoned soil. N-tot in the afforested soil was almost 3 times higher than in the abandoned soil.

Ammonium content (Table 1) in both soils was approximately 4 times higher at the end than at the beginning of the experiment. In mid-winter samples, NH_4-N content in the afforested soil was 5 times higher at the end than at the beginning of the experiment. The nitrate content (Table 1) at the beginning was 22 times higher in the afforested soil than in the abandoned soil. At the end of the experiment, NO_3-N was higher than in the beginning in afforested and abandoned soils. The NO_3 content in the afforested soil sampled in the mid-winter decreased during the incubation experiment, being approximately 5 times lower at the end of the incubation period.

3.2. N_2O emissions

At the highest temperature (+9.5 °C), the N_2O emissions were 12.3±8.6 μg N_2O-N m^{-2} h^{-1} for the afforested soil and 16.8±4.5 μg N_2O-N m^{-2} h^{-1} for the abandoned soil (Fig. 2a, Table 2). Decreasing the temperature down to 0 °C diminished the N_2O emissions. The Q_{10} (from 9.4 to 0.4 °C) values calculated for N_2O emissions were 1.6±0.5 and 6.4±2.0 for the abandoned and the afforested soils, respectively (Table 3).

Table 1
Physical and chemical characteristics of the soils (0–10 cm, Average±SE)

Soil	*n*	Afforested	Abandoned	Afforested (winter)
Soil type (FAO)		Histosol	Histosol	Histosol
Gravimetric moisture content (%)	4	60±3	59±2	73±0.3
Water-filled pore space (%)	4	44±6	71±7	78±1
Bulk density (g cm^{-3})		0.24	0.37	0.24
pH		4.7	5.0	4.7
C-tot (%)		33	19	33
N-tot (%)		2.56	0.98	2.56
μg NH_4-N g DW^{-1}				
Beginning	4	4.6±0.4	2.1±0.1	4.1±0.4
End	4	21.3±2.0	8.5±2.2	22.3±1.3
μg NO_3-N g DW^{-1}				
Beginning	4	2.7±0.1	61.6±6.0	59.3±13.6
End	4	6.6±2.1	77.5±11.3	12.6±3.8

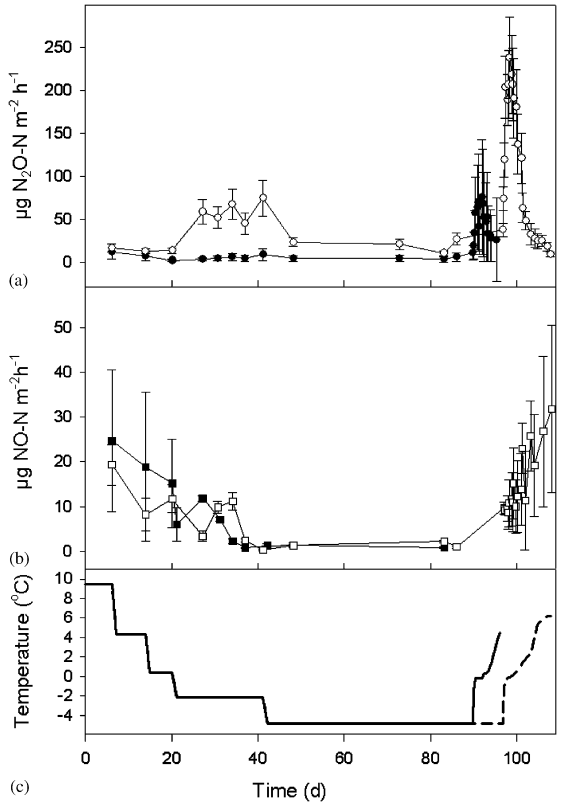

Fig. 2. N_2O-N (μg N_2O-N $m^{-2} h^{-1}$) with standard errors ($n = 4$) from afforested (●) and abandoned (○) soils (a). NO-N (μg NO-N $m^{-2} h^{-1}$) with standard errors ($n = 4$ from afforested (■) and abandoned (□) soils (b) with corresponding temperatures (c) from afforested (solid line) and abandoned (dashed line) soils.

During the freezing period, the temperature response was more marked in the abandoned soil than in the afforested soil. At −2.2 °C, abandoned soil showed an increase in N_2O emissions (58.7 ± 14.5 μg N_2O-N $m^{-2} h^{-1}$, Fig. 2a, Table 2) already 6 days after the decrease in temperature, and the N_2O emissions remained high for 3 weeks. At this temperature, there was a slight increase in the N_2O emissions (average 5.5 ± 1.8 μg N_2O-N $m^{-2} h^{-1}$) from the afforested soil. When the temperature was decreased to −4.9 °C, N_2O emissions decreased in both soils. During the 6-week period at −4.9 °C, N_2O emissions were constant in both soil types (1.3 ± 0.2 μg N_2O-N $m^{-2} h^{-1}$ from the afforested soil and 20.7 ± 2.9 μg N_2O-N $m^{-2} h^{-1}$ from the abandoned soil). There were no statistical differences in the N_2O emissions from the afforested soil at various temperatures, whereas emissions from the abandoned soil decreased ($P < 0.05$) with decreasing temperature. There was also a statistical difference ($P = 0.08$) in the freezing-related emissions between

Table 2
Average (±SE, min–max) NO and N_2O emission and NO/N_2O-ratio (average ±SE (variance)) at different temperatures

	Afforested					Abandoned					Afforested (winter)				
	# of samplings	n	μg NO-N $m^{-2} h^{-1}$	μg N_2O-N $m^{-2} h^{-1}$	NO/N_2O	n	μg NO-N $m^{-2} h^{-1}$	μg N_2O-N $m^{-2} h^{-1}$	NO/N_2O	n	μg NO-N $m^{-2} h^{-1}$	μg N_2O-N $m^{-2} h^{-1}$	NO/N_2O
9.5 °C	1	4	24.7 ± 15.8 (7.7–72.4)	12.3 ± 8.6 (1.8–37.8)	2.0 (2.0)	4	19.3 ± 4.5 (12.9–32.6)	16.8 ± 4.5 (10.0–29.6)	1.1 (0.4)		ND	ND	ND
4.4 °C	1	4	18.9 ± 16.6 (0.7–68.7)	6.9 ± 5.1 (1.2–22.3)	2.7 (2.3)	4	8.2 ± 3.7 (1.6–18.6)	12.9 ± 3.0 (5.4–17.6)	0.6 (0.9)		ND	ND	ND
0.4 °C	1	4	15.2 ± 9.9 (4.8–44.9)	2.1 ± 1.4 (0.7–6.3)	7.1 (0.3)	4	11.8 ± 3.0 (5.6–19.7)	13.6 ± 3.3 (3.9–18.4)	0.9 (1.3)		ND	ND	ND
−2.2 °C	4	4	5.6 ± 1.1 (0.5–17.5)	5.5 ± 1.8 (0.1–19.2)	1.2 (0.7)	4	5.7 ± 1.1 (0.2–15.2)	59.6 ± 6.7 (19.1–112.0)	0.1 (0.0)		ND	ND	ND
−4.9 °C	6	4	1.3 ± 0.2 (0.5–2.3)	5.2 ± 1.8 (0.2–21.3)	0.2 (0.1)	4	1.5 ± 0.2 (0.3–2.7)	20.7 ± 2.9 (7.1–45.8)	0.1 (0.0)		ND	ND	ND
P			0.013	0.136	ND		0.004	0.034	ND		ND	ND	ND
Thawing 0–3 days	10	4	ND	51.9 ± 13.2 (0.2–257.2)	ND	4	10.7 ± 0.8 (3.9–24.9)	168.4 ± 14.2 (23.7–311.8)	0.1 (0.0)	4	1.6 ± 0.2 (0.1–3.6)	100.0 ± 11.0 (9.8–255.3)	0.02 (0.00)
Thawing 4–8 days	7	4	10.3 ± 0.9[a]	36.3 ± 13.4[a] (5.0–22.8)	0.3 (0.2)	4	26.4 ± 1.9 (5.6–31.0)	87.3 ± 13.6 (9.9–237.3)	0.3 (0.3)	4	3.0 ± 0.2 (0.7–5.7)	120.0 ± 7.9 (51.4–207.8)	0.03 (0.00)
Thawing 9–11 days	3		ND	ND	ND	4	29.3 ± 5.9 (12.9–55.0)	20.9 ± 3.7 (5.8–48.6)	1.6 (0.6)	4	2.5 ± 0.2 (1.4–3.5)	85.3 ± 16.4 (27.9–183.6)	0.03 (0.00)

ND—not determined.
[a]6 samplings.

Table 3
Average (±SE) Q_{10} values for NO, N_2O and CO_2 with values from the literature

	Temperature range	NO	N_2O	CO_2	Reference
Afforested soil	+0.4 to +9.4 °C	1.9 (±0.2)	6.4 (±2.0)	5.2 (±0.3)	This study
Abandoned soil	+0.4 to +9.4 °C	2.1 (±0.4)	1.6 (±0.5)	4.1 (±0.3)	This study
Agricultural and forest soils	+1 to +20 °C	2.2–3.6	—	—	1,2,3
Denitrification (soil)	+10 to +30°C	—	1.5–3.0	—	4
Terrestrial and wetland ecosystems, forest soil	+4 to +28 °C	—	—	1.7–2.4	5,6
Tundra and taiga soils	+5 to −2 °C	—	—	5.2–10.0	7

1 = Regina (1998), 2 = Johansson and Granat (1984), 3 = Gasche and Papen (1999), 4 = Knowles (1982), 5 = Raich and Schlesinger (1992), 6 = Winkler et al. (1996), 7 = Clein and Schimel (1995).

various temperatures (temperature range from +0.4 to −4.9 °C) in abandoned soil.

N_2O emissions increased immediately after the soil thawing started. The emissions from the abandoned soil were highest (238.0±47.2 μg N_2O-N $m^{-2} h^{-1}$) 2 days after the thawing started (Fig. 2a). At this time the topsoil had already thawed but the deeper soil, especially the interior of the soil cores was still partly frozen. In this thawing phase a slight increase in the N_2O emissions also occurred from the afforested soil (75.5±66.9 μg N_2O-N $m^{-2} h^{-1}$) (Fig. 2a). There was no statistically significant difference in the thawing-related N_2O emissions from the afforested soil, whereas such emissions from the abandoned soil did differ statistically ($P<0.05$). The afforested soil sampled in mid-winter showed a similar sudden increase in the N_2O emissions ($P<0.05$) immediately after the thawing process started (Fig. 3a). The maximum N_2O emission from the afforested soil sampled in mid-winter soil was 128.7± 17.9 μg N_2O-N $m^{-2} h^{-1}$ after 5 days' thawing.

3.3. NO emissions

At the highest temperature (+9.5 °C), NO emissions were 24.7±15.8 μg NO-N $m^{-2} h^{-1}$ from the afforested soil and 19.3±4.5 μg NO-N $m^{-2} h^{-1}$ from the abandoned soil (Fig. 2b, Table 2). The emissions decreased with decreasing temperature down to +0.4 °C. When the temperature was lowered below 0 °C, there was a slight increase in the NO emissions, but this increase diminished when incubation was continued. When the temperature was decreased to −4.9 °C, NO emissions during the 6-week period were constantly low from both in the abandoned and afforested soils. In both soils, NO emissions at various temperatures differed statistically ($P<0.05$) when tested for the whole temperature range. When tested at critical temperatures near 0 °C (+0.4 to −4.9 °C), there was a nearly significant difference ($P=0.08$) in the NO emissions from the afforested soil, whereas the NO emissions from the abandoned soil differed statistically ($P<0.05$) at these temperatures. The Q_{10} (from +9.4 to +0.4 °C) values for NO emissions were 1.9±0.2 and 2.1±0.4 for the afforested and abandoned soils, respectively (Table 3).

There was no clear thawing-related increase in the NO emissions from either the abandoned or the afforested soils

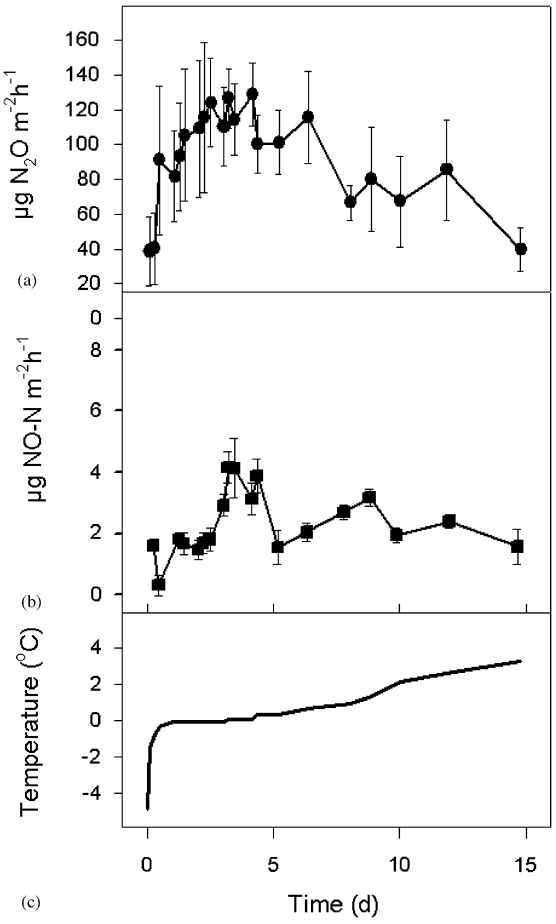

Fig. 3. N_2O-N (μg N_2O-N $m^{-2} h^{-1}$) with standard errors ($n=4$) from afforested soil sampled in mid-winter (a). NO-N (μg NO-N $m^{-2} h^{-1}$) with standard errors ($n=4$ from afforested soil sampled in midwinter (b) with corresponding temperatures (c).

(Fig. 2b). After thawing from −4.9 °C had started, the NO emissions from the abandoned soil increased ($P<0.05$), reaching a constant level of 22.3±2.9 μg NO-N $m^{-2} h^{-1}$ after 10 days with a topsoil temperature of 4.5 °C. Due to

analytical problems, there are no NO emission data from the afforested soil 2 days after thawing started. The afforested soil sampled in mid-winter (Fig. 3b) showed a slight increase ($P<0.05$) in the NO emissions ($4.2\pm1.0\,\mu g\ NO\,m^{-2}\,h^{-1}$) 3 days after thawing started. The NO emissions decreased to $3.4\pm0.2\,\mu g\ NO\,m^{-2}\,h^{-1}$ when the cores were completely thawed.

3.4. NO/N_2O ratio

The NO:N_2O ratio was close to one in both the afforested and abandoned soils at temperatures above 0 °C (Table 2). In the abandoned soil, there was a decrease in the NO:N_2O ratio when soil temperature was decreased to minus degrees, but no such pattern was found in the afforested soil. The NO/N_2O ratio was calculated for different phases of the thawing process. The first phase (0–3 days) covers the beginning of the thawing process. In this phase, the NO:N_2O ratio was low in both soils, 0.1 and 0.02 for the abandoned and afforested (sampled in mid-winter) soils, respectively (we have no data for NO emission from the afforested soil in the 1st phase of the soil thawing). As soil thawing continued (the second phase, 4–8 days), the NO:N_2O ratio increased in the abandoned soil (0.3) but remained the same in the afforested soil sampled in mid-winter (0.03). At 9–12 days after the soil thawing started, the NO:N_2O ratio in the afforested soil sampled in mid-winter was at same level as before.

4. Discussion

NO emissions are typically abundant from soils with good aeration, a condition favoring nitrification, whereas N_2O emissions dominate when denitrification is supported by high water content in soil, creating a lack of oxygen (Davidson, 1992). Andersson and Levine (1986) suggested, based on their studies with pure cultures, that the molar ratio of NO:N_2O in nitrification is usually greater than one, while for denitrifiers this ratio is usually much less than one. Skiba et al. (1993) suggested that the NO:N_2O emission rates obtained from soil experiments do provide a valuable indicator of the importance of nitrification and denitrification as sources of NO and N_2O. During the decrease in temperature down to near freezing (+0.4 °C), NO-N emissions were higher than N_2O-N emissions, i.e. the NO:N_2O ratio was >1, suggesting the importance of nitrifiers in the trace gas production in aerobic conditions when soils are unfrozen. N_2O production increased when temperatures just below 0 °C were reached. There also was some increase in NO emissions in these conditions, although it was much smaller than the increase in N_2O emissions. Below 0 °C, in contrast to the higher temperatures, the NO:N_2O ratio in the abandoned soil was <1, indicating that the N_2O production mechanism was most likely denitrification. No such clear change was observed in the afforested soil, possibly due to lower production of NO and N_2O.

At high water content, where the diffusion rates of O_2 and NO in soil are slow, NO produced is reduced to N_2O before being emitted from soil (Davidson, 1992, Conrad, 1995). Davidson (1992) also suggested that biological consumption of N_2O probably requires more severe reducing conditions than the consumption of NO does. This would explain the low NO emission rates at temperatures below 0 °C. The ice in soil pores can act as a diffusion barrier, enabling especially the reduction of NO in denitrification. The high water content (i.e. high WFPS) often prevailing in afforested organic soils (Wall and Heiskanen, 1998) increases the anaerobic conditions in soil and favors denitrification. At temperatures below zero, limited oxygen diffusion resulting from the ice barrier can enhance N_2O production (Teepe et al., 2000). The production and consumption rates of N_2O and NO could thus change differently at the critical soil freezing point, causing differences in the ratio of NO:N_2O in the emissions.

As mentioned above, the reduced gas diffusion in frozen soil favors trace gas production. There is also another potential mechanism which might enhance the production of gases at low temperatures. The freezing of soil destroys microbial cells and liberates the organic compounds of cells which are good substrates for heterotrophic microbes (Christensen and Tiedje, 1990, Skogland et al., 1988, Papen and Butterbach-Bahl, 1999). It is important to note that recent studies suggest that denitrifiers benefit more than heterotrophs in general from the extra substrates liberated in soil at low temperature (Koponen and Martikainen, 2004). Also in this study there was no substantial increase in CO_2 emissions (indicating the activity of heterotrophic microbes in general) at low temperatures (data not shown), in contrast to the N_2O emission probably originating from denitrification.

The increase in the N_2O emissions was ten times higher in the abandoned soil than in the afforested soil when the decreasing in temperature reached critical values below 0 °C. This could be associated to the differences in soil moisture. Teepe et al. (2004) suggested, that the retardation of denitrification is more pronounced than the acceleration of the nitrification with increasing oxygen concentration (i.e. lower WFPS) in thawing soil. The average WFPS% was $43.6\pm11.7\%$ in the afforested soil and $70.6\pm13.6\%$ in the abandoned soil. High N_2O emissions occur generally at WFPS 60–80%, while optimum conditions for NO production are at 30–60% WFPS (Davidson, 1991). Hence, the higher soil moisture in the abandoned soil might have enhanced N_2O emission. High water content (80–100% WFPS) may lead to the consumption of N_2O and especially of NO by denitrifiers (Davidson, 1991, Skiba et al., 1997). Abandoned soil had higher WFPS% than afforested soil, and abandoned soil produced more N_2O throughout the experiment. At higher WFPS oxygen diffusion into soil is slower. Abandoned soil had also higher soil respiration (data not shown), and slightly lower response of respiration to temperature (see Table 3). Soil respiration consumes soil oxygen. High water content and higher soil respiration

together create more anaerobic conditions, favouring denitrification. At higher temperatures, soil respiration consumed oxygen creating more anaerobic conditions.

The availability of nutrients in the soils might also differ, affecting N_2O and NO emissions. From only 2 data points in the NH_4^+-N and NO_3^--N concentrations (beginning of the incubation and the end of the experiment), the proper distinguishing between the processes during soil freezing/thawing in not possible. However, the NH_4^+ and NO_3^- content increased in all soils during the experiments, indicating nitrogen mineralization and nitrification. There was a slight difference in ammonium content between the abandoned and afforested soil, being higher in the former. In contrast, NO_3^- content was higher in the afforested than in the abandoned soil, indicating higher nitrification in the afforested soil, or higher denitrification in the abandoned soil. In fact, N_2O emissions were higher from the abandoned soil, suggesting the importance of denitrification in this soil.

A high increase in the N_2O production was observed when the thawing period started, supporting the results of several earlier studies (Flessa et al., 1995, Priemé and Christensen, 2001, Teepe et al., 2001, Koponen and Martikainen 2004, Koponen et al., 2004). In contrast to the N_2O emissions, we did not find any remarkable increase in the NO emissions during soil thawing. Only in the afforested soil sampled in midwinter there was a slight increase in the NO emissions observed when soil was thawing. This can be related to the increase in denitrifying activity, releasing NO and N_2O, since the afforested soil sampled in mid-winter had quite high %WFPS ($\geqslant$75%).

The NO and N_2O emissions at a particular temperature were higher during soil thawing than in the first part of the experiments where the temperature was gradually decreased from plus to minus degrees. The higher microbial activities during thawing were probably associated with the higher availability of ammonium and nitrate (see above) as well as with microbial organic substrates. The good substrate supply in thawing soil favors both nitrification (Müller et al., 2002) and denitrification activities. The NO:N_2O ratio during soil thawing was low, but especially in the abandoned soil the ratio increased after 4 days form the beginning of soil thawing. At the beginning of soil thawing the main process in the soil producing N_2O might have been denitrification, but nitrification dominates once thawing is complete. However, the afforested soil sampled in midwinter with higher moisture content did not show a similar pattern: in this case the high water content favored denitrification also after the thawing was complete. This may be the case in nature as well; the melting of the snowpack increases soil water content, favoring denitrification after soil thawing.

5. Conclusions

Our results show that there are differences in the effects of temperature on NO and N_2O emissions from boreal organic soils. NO did not show thawing-related emission peaks, which are typical for N_2O emissions. The reason for this might be that the NO originated mainly from nitrification, in contrast to N_2O, which is produced in denitrification. Denitrification as a heterotrophic microbial process can rapidly utilize the extra carbon associated with soil freezing–thawing. Our results suggest that soil freeze–thaw cycles enhance the N_2O emission in contrast to the NO emission.

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CHAPTER V

MICROBIAL COMMUNITIES, BIOMASS, AND ACTIVITIES IN SOILS AS AFFECTED BY FREEZE THAW CYCLES

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Microbial communities, biomass, and activities in soils as affected by freeze thaw cycles

Hannu T. Koponen[a,*], Tuula Jaakkola[a,1], Minna M. Keinänen-Toivola[b,2], Saara Kaipainen[a,b,3], Jaana Tuomainen[a,c], Kristina Servomaa[c], Pertti J. Martikainen[a]

[a]*Department of Environmental Sciences, University of Kuopio, P.O. Box 1627, FI-70211 Kuopio, Finland*
[b]*Department of Environmental Health, National Public Health Institute, P.O. Box 95, FI-70701 Kuopio, Finland*
[c]*North Savo Regional Environment Centre, P.O. Box 1199, FI-70211 Kuopio, Finland*

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Abstract

Two Finnish agricultural soils (peat soil and loamy sand) were exposed to four freeze-thaw cycles (FTC), with a temperature change from $-17.3\pm0.4\,^{\circ}C$ to $+4.1\pm0.4\,^{\circ}C$. Control cores from both soils were kept at constant temperature ($+6.6\pm2.0\,^{\circ}C$) without FTCs. Soil N_2O and CO_2 emissions were monitored during soil thawing, and the effects of FTCs on soil microbes were studied. N_2O emissions were extremely low in peat soil, possibly due to low soil water content. Loamy sand had high N_2O emission, with the highest emission after the second FTC. Soil freeze-thaw increased anaerobic respiration in both soil types during the first 3–4 FTCs, and this increase was higher in the peat soil. The microbial community structure and biomass analysed with lipid biomarkers (phospholipid fatty acids, 3- and 2- hydroxy fatty acids) were not affected by freezing-thawing cycles, nor was soil microbial biomass carbon (MIB-C). Molecular analysis of the microbial community structure with temperature gradient gel electrophoresis (TGGE) also showed no changes due the FTCs. These results show that freezing and thawing of boreal soils does not have a strong effect on microbial biomass or community structure.

Keywords: N_2O; CO_2; Freeze-thaw cycle; Biomass; Microbial community structure; Lipid biomarkers; TGGE

1. Introduction

Microbial processes at low temperatures have been suggested to be responsible for up to 70% of annual nitrous oxide (N_2O) emissions from agricultural soils (Röver et al., 1998, Syväsalo et al., 2004). Even though the bulk soil is frozen, the water films on the surfaces of soil particles can remain unfrozen down to $-20\,^{\circ}C$, allowing microbial metabolism, and probably also N_2O production, to take place below 0° (Rivkina et al., 2000). In boreal and temperate regions, soils are exposed to freeze-thaw cycles (FTC) mainly during autumn and spring and also during mild winters. Soil thawing-related N_2O emissions have been reported in several studies (e.g. Christensen and Tiedje, 1990; Röver et al., 1998). N_2O and carbon dioxide (CO_2) emissions have been reported to increase in northern soils during FTC (Schimel and Clein, 1996). These FTC-induced emissions have decreased with repeated FTC (Schimel and Clein, 1996, Priemé and Christensen, 2001, Koponen and Martikainen, 2004). The decrease in gas production with repeated FTC suggests either depletion in microbial nutrient availability or damage to soil microbes.

Soil freezing-thawing events have been suggested to destroy microbial cells, releasing nutrients from the destroyed cells for the surviving microbes, which then are highly active during soil thawing (Christensen and Tiedje, 1990). The extra substrates might also originate from the physical disruption of soil aggregates due to frost action (Christensen and Christensen, 1991, Edwards and Cresser, 1992). Herrmann and Witter (2002) reported that easily

*Corresponding author. Tel.: 358 17 163589; fax: 358 17 163750.
E-mail address: hannu.koponen@uku.fi (H.T. Koponen).
[1]Current address: Finnish Forest Research Institute, P.O. Box 18, FI-01301 Vantaa, Finland.
[2]Current address: PrizzTech Oy/DWI Finland, FI-26100 Rauma, Finland.
[3]Current address: MTT Agrifood Research Finland, Soils and Environment, FI-31600 Jokioinen, Finland.

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decomposable material becomes available during FTCs, and microbial biomass C contributes, ca. 65% of the C flush during FTCs. In agricultural soils, the denitrifying population may benefit more from this extra nutrient load than the overall heterotrophic microbial community (Koponen and Martikainen, 2004). Schimel and Clein (1996) suggested that FTC may have an effect on the composition and function of microbial communities.

We studied the effects of multiple soil FTC on soil chemical and microbiological variables in two Finnish agricultural soils. The key nitrogen transforming processes, nitrification and denitrification, as well as the soil microbial biomass and the community structure were studied to obtain a comprehensive picture of the effects of freezing-thawing on soil microbes.

2. Materials and methods

2.1. Study sites

Two different typical Finnish agricultural soil types were studied (Table 1). The soils originated from the experimental fields of the Agrifood Research Finland in Jokioinen (southern Finland, 60°49′N 23°30′E). The mean annual precipitation (measured in the period 1971–2000) of the area is 607 mm, of which approximately 224 mm is snow. The mean annual air temperature is +4.3 °C, with February being the coldest month (mean −6.5 °C) and July the warmest (average +16.1 °C). The topsoil generally freezes in November and thaws in April (Finnish Meteorological Institute, 2002).

Soil samples were taken from depths of 0–25 cm of the uncultivated sectors of the fields. These sectors were kept free from vegetation by regular ploughing. The samplings were carried out on 29 October 2001 (loamy sand) and on 5 November 2001 (peat). The soils were kept at +4 °C for 4 months before the experiments began.

2.2. Experimental set up

Soil material was homogenised by sieving (mesh size 5.6 mm). The soil moisture content expressed as WFPS was 61±4% in peat and 86±5% in loamy sand. The soils were packed into PVC tubes (inner diameter 105 mm, height 300 mm), and soils were compressed manually to equal to the field value of the bulk density (0.34 g cm^{-3} for peat and 1.33 g cm^{-3} for loamy sand). The FTC consisted of freezing the soil cores (six replicates) to −17.3±0.4 °C (5 d) and thawing them at +4.1±0.4 °C (7 d). This FTC was repeated four times. Temperatures applied were selected to mimic the extreme freezing conditions and natural thawing-temperatures in autumn and spring at our study sites. After the fourth cycle the cores were kept at +4.1±0.4 °C for 23 d, in order to study the longer-term effects of multiple FTCs on soil microbiology. Five replicate control cores from both soils were incubated simultaneously without FTC at +6.6±2.0 °C. After each cycle (72 h after the beginning of thawing), 13 d after the fourth FTC (FTC4+13 d) and 23 d after the fourth FTC (FTC4+23 d), one replicate control and treatment core was destroyed and analysed for soil physical, chemical and microbiological variables.

The temperature of the incubation chambers (LMS Cooled Incubator, model 250) was measured continuously by data loggers (HOBO®).

Table 1
Soil physical-chemical properties

	Peat	Loamy sand
Soil type (FAO)[a]	Terric Histosol	Eutric Cambisol
Total C %[a]	24	2.4
Total N %[a]	1.1	0.16
C/N-ratio[a]	21	15
pH	6.0±0.1	5.4±0.1
WFPS %	61±4	86±5
Bulk density (g cm^{-3})	0.35±0.02	1.33±0.09
Particle density (g cm^{-3})	1.80±0.03	2.56±0.14

[a]From Pihlatie et al. (2004).

2.3. Gas sampling and analysis of CO_2 and N_2O

Measurements of N_2O and CO_2 were done with a closed chamber technique as described by Koponen and Martikainen (2004). Since soil thawing started the measurements were done every 2–4 h during the first 12 h and then 2–3 times a day during the following 2.5 d. The individual cores were covered by chambers, giving each core a headspace of 1.3–1.9 l. The headspaces were flushed continuously with air when incubating the cores in the temperature-controlled chambers to avoid the accumulation of gases in the headspace. The air flush was cut off just before making the gas flux measurements. The concentrations of N_2O and CO_2 were determined with a Hewlett Packard 5890 Series II gas chromatograph equipped with ^{63}Ni electron capture (EC) and thermal conductivity (TC) detectors for N_2O and CO_2, respectively (Nykänen et al., 1995). The flux rates were calculated from the linear increase in the gas concentrations during the measurement period of 35 min, and the cumulative flux was calculated by integrating the fluxes over the entire incubation period of 72 h from the beginning of the soil thawing.

2.4. Soil physical and chemical characterization

Soil particle density was determined using pycnometers (Blake, 1965), and gravimetric moisture content was determined by drying the soil at +105 °C for 24 h. Soil nitrate (NO_3–N) was analysed from the soil:water suspension (1:5 v/v, 175 rpm, 1 h) and ammonium NH_4–N from the soil:KCl suspension (1:5 v/v 2 M KCl, 175 rpm, 1 h). The extractions were filtered (Blauband 589^3 Blue Ribbon filter paper (Schleicher & Schuell MicroScience Gmbh,

Dassel, Germany) and extracts were stored at +4 °C maximum 1 week until analysed. NO_3–N was analysed using an ion chromatograph (Dionex [Sunvalley, CA, USA] DX-120 with an AS 9-HC 4 mm anion column and an ASRS-ULTRA suppressor), and NH_4–N spectrophotometrically (Ultrospec 3000 pro [Biochrom Ltd., Cambridge, UK]) at wavelength 630 nm, according to the method of Fawcett and Scott (1960).

2.5. Biological variables

Soil potential for the oxidation of ammonium was measured using a quick slurry technique as described by Pell et al. (1998) using 25 g (fresh weight, FW) of soil. Soil was weighed as five replicates into a 600 ml infusion flasks, and 100 ml of 1 mM phosphate buffer (pH 7.2) containing 0.4 mM $(NH_4)_2SO_4$ and 15 mM $NaClO_3$, was added. Soil slurries were incubated in a shaker (175 rpm, +25 °C); samples of 4 ml were taken at incubation times of 1.5, 3, 5.5 and 7 h and transferred to test tubes containing 4 ml of 4 M KCl to stop the ammonium oxidation. Liquid phase was separated by centrifugation (1 min, 7000 rpm, Hermle Z233 MK-2, [Hermle Labortechnik, Wehingen, Germany]) and stored at +4 °C. The samples were analysed for nitrite (NO_2–N) spectrophotometrically (Ultrospec 3000 pro [Biochrom Ltd., Cambridge, UK]) within 24 h, using a Greis–Ilosvay method at wavelength 540 nm. The ammonium oxidation rates were calculated from the linear accumulation of nitrite over time.

Soil N_2O production capacity in vitro, denitrification activity in vitro and anaerobic respiration was measured using 25 g (FW) of soil in a 600 ml infusion flask stopped with butyl rubber septa. Four replicate flasks were flushed with 99.5% N_2 to create anaerobic conditions. For N_2O production capacity and anaerobic respiration, N_2O and CO_2 concentrations were measured from two replicate bottles at 1, 2, 3.5 and 4.5 h from the beginning of the anaerobic incubation. N_2O and CO_2 were analysed with a Hewlett Packard 5890 Series II gas chromatograph (see above for details). For denitrification activity in vitro, the amount of N_2 from denitrification was determined using the acetylene inhibition technique after the first and the third FTC. Acetylene (C_2H_2) was injected into two replicate bottles (2.5% v/v) 1 h after the beginning of the anaerobic incubation and gas samples of 10 ml for N_2O were taken from the headspace of the bottles at 1, 2.5 and 3.5 h after the C_2H_2 injection. N_2 emission rates were calculated by subtracting the N_2O production rates without C_2H_2 from the rate with C_2H_2.

Microbial biomass carbon was determined using substrate-induced respiration (SIR) according to the method of Anderson and Domsch (1978) using 25 g (FW) of soil. Soil was weighed as three replicates into 600 ml bottles and glucose concentration was adjusted to optimum level at water-holding capacity (WHC) 60%. Thirty minutes after the glucose addition, the bottles were flushed with ambient air and sealed gas tightly. Gas samples of 20 ml were taken through a rubber septum at 1.5, 3 and 4 h after the beginning of the incubation into 60 ml polypropylene syringes (Terumo) equipped with 3-way stopcocks (Connecta). CO_2 concentration was analysed using a gas chromatograph (see above).

The microbial community structure and biomass of the soil samples were analysed with lipid biomarkers (phospholipid fatty acids, 2-hydroxy fatty acids and 3-hydroxy fatty acids) as presented in Keinänen et al. (2002, 2003). The soil samples were analysed from the FTC-exposed and control soils at the beginning of the experiment (FTC 0), after the first FTC (loamy sand only), the second FTC, and the fourth FTC, and 23 d after the fourth FTC (loamy sand only). The lipids were extracted from 3–5 g (dry weight, DW) of soil (2 replicates/sample) using a modified Bligh and Dyer extraction procedure (28.2 ml of chloroform: methanol: phosphate buffer pH 7.4, 1: 2: 0.8 v/v/v). Lipids were separated from the solvent phase after the addition of chloroform and buffer (final ratios of solvents 1:1:0.9 v/v/v), and fractionated in a silica column to neutral, glyco- and phospholipids with 10, 20 and 10 ml of chloroform, acetone and methanol, respectively. PLFAs were further saponified, methylated, extracted and analysed as methyl esters with a gas chromatograph equipped with mass selective detector (GC-MS, Hewlett-Packard model G1800A, Palo Alto, CA, USA) using total ion monitoring. Hydroxy fatty acids were methylated from the lipid extraction residue by mild acid hydrolysis and analysed with GC-MS using selective ion monitoring.

For TGGE analysis of the microbial community structure, DNA was extracted from 0.25 g of soil using an UltraClean™ Soil DNA Kit (MoBio Laboratories, Inc., CA, USA), with additional purification using a Wizard DNA Clean-Up system (Promega Corp., Madison, WI, USA). Total DNA (50 ng) was amplified with primers GM5F (see Muyzer et al. (1993) for the primer, and Santegoeds et al. (1998) for the GC-clamp sequence) and 907R (Muyzer et al., 1995), using Cy5™-label in the reverse primer (Sigma-Genosys, Sigma-Aldrich, Suffolk, UK). Amplification was performed in a 50 µl reaction mix containing 1 × DyNAzyme buffer (Finnzymes, Espoo, Finland), 20 pmol of each primer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 100 ng µl^{-1} BSA (bovine serum albumin), and 1.2 U DyNAzyme II polymerase (Finnzymes) with a touchdown program (+95 °C denaturation, with 0.5 °C reduction per cycle of the annealing temperature from +72 until +55 °C was reached, which was used as the annealing temperature for the last 10 cycles) in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA, USA).

PCR products were separated in a TGGE gel (8% acrylamide, 8 M urea, 20% formamide and 0.1% glycerol) using a TGGE MAXI System apparatus (Biometra, Goettingen, Germany) with 1 × TAE running buffer and +35 to +46 °C thermal gradient. TGGE analyses were done from two replicate sample series. TGGE gels were scanned with a STORM® imager (Molecular Dynamics, Sunnyvale, CA, USA). The community fingerprint patterns

from the TGGE analyses were analysed with the BioNumerics program (Version 4.0, Applied Maths BVBA, St-Martens-Latem, Belgium). The clustering of the samples was calculated from the curve data based on the normalized genetic population fingerprints, applying different algorithms. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with Pearson coefficient was selected as clustering method according to the results of the cophenetic correlations calculations, performed with the tools available in the BioNumerics program package.

2.6. Statistical analysis

Differences in soil nitrogen (NH_4–N, NO_3–N), N_2O–N and CO_2 emissions, nitrification and denitrification and soil microbial biomass carbon in multiple FTC and control were tested with one-way ANOVA with Tukey's post hoc test, with the soil freeze-thaw cycle as the main factor. Variables were normally distributed according to Kolmogrov–Smirnov goodness of fit test. Differences between control and test groups (FTC treatment) were tested with the paired sample *t*-test. Principal component analyses (PCA) of the results of lipid biomarkers (PLFA, 3-OH-FA, 2-OH-FA) were performed to elucidate major variation in data. Two-way variance analysis, followed by Tukey's test, was used to detect changes in biomass estimated on the basis of PLFAs, 2-OH- or 3- OH-FAs. All the statistical analyses were done using SPSS for Windows version 10.1 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Soil NH_4–N and NO_3–N

At the beginning of the experiment, the NO_3–N content in peat and loamy sand soil (Table 2) were 75.6 ± 2.7 μg NO_3–N g DW^{-1} and 16.1 ± 1.2 μg NO_3–N g DW^{-1}, respectively. In peat, the NO_3–N content increased through the experiment in both FTC-treated and control soils. The NO_3–N content was highest 13 d after the fourth FTC, and decreased afterwards, being 115.1 ± 3.3 7 μg NO_3–N g DW^{-1} and 112.9 ± 4.2 7 μg NO_3–N g DW^{-1} in FTC-treated and control soils, respectively. Loamy sand soil showed a slight but statistically insignificant increase in the NO_3–N content during the experiment. In the control cores, NO_3–N content decreased slightly, falling to 13.2 ± 0.8 μg NO_3–N gDW^{-1} by the end of the incubation period.

At the beginning of the experiment, the soil ammonium content (Table 2) was low in both soils: 2.1 ± 0.9 μg NH_4–N g DW^{-1} and 0.3 ± 0.1 μg NH_4–N g DW^{-1} in peat and loamy sand soil, respectively. In the control peat soils (without FTC treatment), the ammonium content remained at this low level. However, in the control loamy sand, there was a small increase in the ammonium content. In peat, the NH_4–N content after the first FTC differed statistically significantly ($P = 0.031$) from that in the control soil: 13.3 ± 2.4 μg NH_4–N g DW^{-1} and 0.6 ± 0.1 μg NH_4–N g DW^{-1} in FTC-treated and control soils, respectively. After this increase, the NH_4–N content in the FTC-treated soil declined to 0.8 ± 0.3 μg NH_4–N g DW^{-1} by the end of the incubation period. Also, in loamy sand, the NH_4–N content was higher ($P = 0.015$) in the FTC-treated than in the control soil after the first FTC (1.6 ± 0.1 μg NH_4–N g DW^{-1} and 1.4 ± 0.1 μg NH_4–N g DW^{-1} in the FTC treatment and the control, respectively). However, NH_4–N content remained at this higher level until 13 d after the fourth FTC in both the control and the FTC-treated soils. After this, ammonium content declined back to initial level.

3.2. Gas emissions during FTCs

Peat had extremely low N_2O emissions during the 72 h thawing period (cumulative emissions are shown in Fig. 1a). The average N_2O emissions from peat were 4.3 ± 2.2 μg N_2O–N $m^{-2} h^{-1}$, 2.8 ± 0.6 N_2O–N $m^{-2} h^{-1}$, 2.1 ± 0.6 N_2O–N $m^{-2} h^{-1}$ and 2.9 ± 1.4 N_2O–N $m^{-2} h^{-1}$ in the first, second, third and fourth FTC, respectively. N_2O production from loamy sand (cumulative production is shown in Fig. 1b) during the first FTC was 22-fold that from peat, average emission 94.8 ± 26.0 μg N_2O–N $m^{-2} h^{-1}$. The second FTC resulted in the highest N_2O production, with average emission of 462 ± 57 μg N_2O–N $m^{-2} h^{-1}$. After the second cycle, N_2O emission started to decline, falling to 156 ± 84 μg N_2O–N $m^{-2} h^{-1}$ in the third FTC, and 8.3 ± 4.2 μg N_2O–N $m^{-2} h^{-1}$ in the fourth FTC.

CO_2 production during soil thawing was higher in peat than in loamy sand (cumulative emissions are shown in Figs. 1c, 1d). At the beginning of the experiment, average CO_2 emission were 34.8 ± 4.1 mg CO_2 $m^{-2} h^{-1}$ for peat soil, and 17.6 ± 1.7 mg CO_2 $m^{-2} h^{-1}$ for loamy sand. With each freeze-thaw cycle, the CO_2 production decreased in peat, reaching 20.3 ± 3.5 mg CO_2 $m^{-2} h^{-1}$ during the fourth FTC. Such a trend was not observed in loamy sand, as the average CO_2 production remained at a constant level, (average emission after the fourth FTC 17.2 ± 0.4 mg CO_2 $m^{-2} h^{-1}$).

3.3. N_2O production capacity and denitrification activity in vitro, anaerobic respiration and ammonium oxidation

Soil N_2O–N production capacity in vitro (Table 2) was higher in peat than in loamy sand. At the beginning of the experiment, soil capacities to produce N_2O–N were 0.73 ± 0.20 and 0.22 ± 0.10 μg N_2O-N g $DW^{-1} h^{-1}$ for peat and loamy sand, respectively. In peat, FTC treatment had no effect on the N_2O production capacity. In loamy sand, the N_2O production capacity decreased slightly after the first FTC. Denitrification in vitro decreased in both soils from the first to the third FTC. The N_2O:N_2 ratios (calculated from denitrification activity in vitro) after the first FTC were 4 and 6 for peat and loamy sand,

Table 2
Soil nitrate content, ammonium content, ammonium oxidation capacity, N_2O production capacity, denitrification activity in vitro and anaerobic respiration from control (without FTC treatments) and test (FTC-treated) soils (average±SE) in the beginning of the experiment, 72 h after each FTC, 13 d, and 23 d after the 4th FTC

	μg NO_3–N g DW^{-1}				μg NH_4–N g DW^{-1}				Ammonium oxidation (ng N g DW^{-1} h^{-1})				N_2O production capacity in vitro (μg N_2O–N g DW^{-1} h^{-1})		Denitrification activity in vitro (μg N_2O-N g DW^{-1} h^{-1})	Anaerobic respiration (mg CO_2 g DW^{-1} h^{-1})	
FTC	Control	Test	*n*	Sig.	Control	Test	*n*	Sig.	Control	Test	*n*	Sig.	Control	Test	Test	Control	Test
Peat																	
0	75.6±2.7^{a}	75.6±2.7^{a}	3		2.1±0.9^{a}	2.1±0.9^{a,b}	3		91.7±3.2^{b}	91.7±3.2^{b,c}	3		0.73±0.02^{a}	0.73±0.02^{a}		7.9±0.1^{a,b}	7.9±0.1^{a}
1	83.5±0.7^{a}	86.0±0.7^{a,b}	3	0.003	0.6±0.1^{a}	13.3±2.4^{c}	3	0.031	98.4±3.6^{b}	110.5±14.4^{c}	2	ND	0.47	0.55±0.05^{a}	0.68±0.04	8.7±0.2^{b}	13.5±0.8^{b}
2	97.8±0.5^{b}	99.0±2.5^{b,c}	3	0.714	ND	6.6±0.2^{b}	3	ND	ND	93.4±3.8^{b,c}	3	ND	0.65±0.06^{a}	0.66±0.05^{a}	ND	8.4±0.4^{b}	10.0±1.4^{a,b}
3	112.8±1.2^{c}	108.7±6.4^{c}	3	0.641	0.7±0.4^{a}	4.6±0.9^{a,b}	3	0.120	92.6±11.6^{b}	98.7±1.5^{b,c}	3	0.403	ND	0.72±0.02^{a}	0.84±0.02	ND	10.2±0.6^{a,b}
4	130.4±2.5^{d}	113.0±1.8^{c}	3	0.033	2.4±0.0^{a}	4.4±0.5^{a,b}	3	0.047	71.3±8.2^{a,b}	67.4±3.4^{a,b}	2	ND	0.48±0.01^{a}	ND	ND	6.7±0.1^{a}	10.9±0.8^{a,b}
4+13 d	ND	139.6±4.2^{d}	3	ND	ND	0.4±0.0^{a}	3	ND	ND	49.6±6.2^{a}	3	ND	ND	0.69±0.01^{a}	ND	ND	7.9±0.7^{a}
4+23 d	112.9±4.2^{c}	115.1±3.3^{c}	3	0.710	1.9±0.0^{a}	0.8±0.3^{a}	3	0.213	49.6±6.2^{a}	63.2±6.3^{a,b}	3	0.596	0.83±0.26^{a}	0.51±0.11^{a}	ND	7.2±0.1^{a}	8.2±0.6^{a}
Loamy sand																	
0	16.1±1.0	16.1±1.0^{a,b}	3		0.3±0.1^{a}	0.3±0.1^{a}	3		81.2±2.7^{b}	81.3±2.7^{d}	3		0.22±0.01^{a}	0.22±0.01^{d}	ND	1.8±0.0^{a}	1.8±0.0^{a,b}
1	13.2±1.2^{b}	18.4±1.7^{a,b}	3	0.144	1.4±0.1^{a,b}	1.6±0.1^{c}	3	0.015	98.0±1.4^{c}	57.2±5.9^{b,c}	2	ND	0.13±0.01^{a}	0.13±0.00^{b,c}	0.16±0.01	2.5±0.2^{a}	3.0±0.5^{a,b,c}
2	12.3±0.9^{b}	19.3±0.7^{a,b}	3	0.030	1.7±0.4^{b}	0.9±0.4^{a,b,c}	3	0.371	73.2±1.7^{b}	65.2±0.3^{c,d}	3	0.030	0.15±0.01^{a}	0.18±0.01^{c}	ND	2.6±0.4^{a}	3.5±0.2^{c}
3	16.0±1.5^{b}	17.7±0.7^{a,b}	3	0.854	1.0±0.5^{a,b}	1.7±0.0^{c}	3	0.311	75.7±5.0^{b}	35.7±3.5^{a}	3	0.001	ND	0.21±0.01^{d}	0.23±0.01	ND	3.2±0.3^{b,c}
4	13.4±0.4^{b}	20.9±1.0^{b}	3	0.028	0.7±0.0^{a,b}	1.3±0.2^{b,c}	3	0.065	52.3±1.6^{a}	43.8±4.8^{a,b}	3	0.254	0.18±0.13^{a}	0.17±0.01^{c,d}	ND	1.9±0.2^{a}	1.6±0.6^{a}
4+13 d	ND	18.9±0.6^{a,b}	3	ND	ND	0.5±0.2^{a,b}	3	ND	ND	39.6±9.1^{a,b}	3	ND	ND	0.10±0.00^{a,b}	ND	ND	1.7±0.1^{a,b}
4+23 d	13.2±0.8^{a}	15.0±0.8^{a}	3	0.041	0.5±0.1^{a,b}	0.0±0.2^{a}	3	0.257	50.3±1.6^{a}	51.4±2.0^{a,b,c}	3	0.601	0.17±0.02^{a}	0.07±0.01^{a}	ND	2.2±0.0^{a,b}	1.9±0.0^{a}

Statistical difference ($P<0.05$) inside the control and test groups in both soils are marked with a, b and c and d (Tukey post hoc test). Statistical significant difference (*P*-value) between control and test groups is shown (paired *t*-test).
ND- not determined.

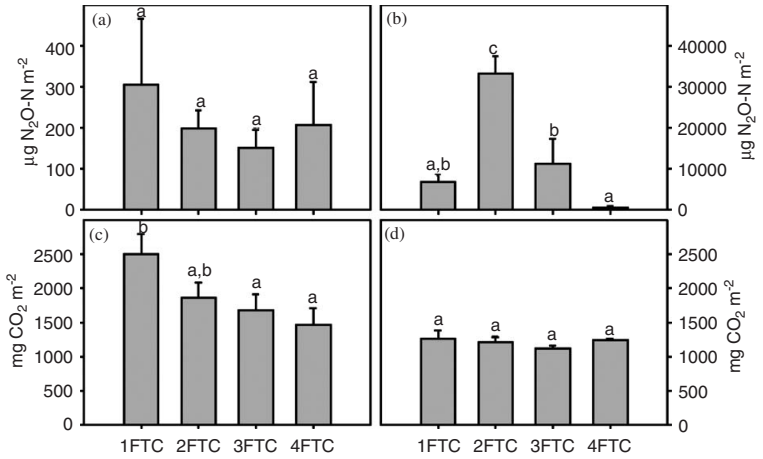

Fig. 1. Cumulative (μg N_2O–N m^{-2}) emission of N_2O in peat (a) and loamy sand (b); cumulative emission of CO_2 (mg CO_2 m^{-2}) in peat (c) and loamy sand (d) during the first 72 h from the beginning of each soil thawing, with standard errors. Statistical difference ($P<0.05$) between the FTC's in both soils in both soils are marked with a, b, and c (Tukey post hock test).

respectively. After the third FTC, the N_2O:N_2 ratios were higher, 6 for peat and 12 for loamy sand.

At the beginning of the experiment, soil anaerobic respiration levels (Table 2) were 7.9 ± 0.1 and 1.8 ± 0.0 mg CO_2 g DW^{-1} h^{-1} in peat and loamy sand, respectively. In the control soils, anaerobic respiration was at a constant level in loamy sand throughout the experiment, while in peat the control was at a higher level after the second and third FTC. In the FTC treatments, anaerobic respiration increased immediately after the first FTC, reaching 13.5 ± 0.8 mg CO_2 g DW^{-1} h^{-1} in peat and 2.7 ± 0.04 mg CO_2 g DW^{-1} h^{-1} in loamy sand, but this increase was statistically significant ($P<0.05$) only in peat. After the first FTC, anaerobic respiration started to decrease in peat, reaching the initial value at sampling 13 d after the fourth FTC. However, in loamy sand soil, anaerobic respiration increased until the third FTC (3.2 ± 0.3 mg CO_2 g DW^{-1} h^{-1}), and then decreased back to the initial level.

Soil potentials for ammonium oxidation (Table 2) at the beginning of the experiment were 91.7 ± 3.2 and 81.2 ± 2.7 ng NO_2–N g DW^{-1} h^{-1} for peat and loamy sand, respectively. The ammonium oxidation potential decreased slowly in both soil types when FTCs were introduced. However, a similar decrease was also observed in the control soils without the FTCs.

3.4. Microbial biomass carbon and amount of lipid biomarkers

At the beginning of the experiment, the amount of microbial biomass carbon (mg MIB-C cm^{-3}, Table 3) was higher in peat (0.28 ± 0.01 mg MIB-C cm^{-3}) than in loamy sand (0.22 ± 0.02 mg MIB-C cm^{-3}). In peat, MIB-C after the third FTC resulted in slightly lower MIB-C (0.22 ± 0.01 mg DW^{-1}), but this did not differ statistically from the amount in the previous or later FTCs. A similar phenomenon was observed in the control soil. At the end of the experiment, the MIB-C in peat was 0.25 ± 0.01 mg MIB-C cm^{-3}. In contrast to the case in peat, MIB-C in loamy sand increased slightly, both in the FTC-treated and control soils. MIB-C increased slightly after the second FTC to 0.27 ± 0.00 mg MIB-C g DW^{-1} and remained at this level in the last two FTCs. Thirteen days after the fourth FTC, the MIB-C was back at the initial level (0.22 ± 0.01 mg MIB-C cm^{-3}).

The amount of viable microbial biomass PLFA (μg PLFA cm^{-3}, Table 3) was higher in loamy sand than in peat soil ($P<0.069$). At the beginning, the amount of PLFA was 34.7 ± 4.3 and 54.0 ± 3.0 μg PLFA cm^{-3} in peat and loamy sand, respectively. No statistical significant changes due to FTCs were observed in either soil type. A similar phenomenon was observed in biomasses estimated on the basis of 3-OH-FAs or 2-OH-FAs. The quantitative amounts of 3-OH-FAs or 2-OH-FAs were statistically significantly higher in peat than in loamy sand soil ($P<0.000$ for both) (Table 4).

3.5. Microbial community structure

No clear differences related to freezing-thawing cycles were found in the profiles of lipid biomarkers using PCA (Fig. 2). However, on the basis of the PLFA profile, the viable microbial communities of peat and loamy sand soils differed (principal component (PC) analyses, PC1, explaining 39.4% of the variation in data, Fig. 2). Peat contained

Table 3
Microbial biomass carbon (mg MIB-C cm^{-3}) and PLFA (μg PLFA cm^{-3}) from control (without FTC-treatments) and test (FTC-treated) soils (average ± SE) in the beginning of the experiment, 72 h after each FTC, 13 d and 23 d after the 4th FTC

	Microbial biomass carbon (mg MIB-C cm^{-3})				Microbial PLFA (μg PLFA cm^{-3})		
FTC	Control	Test	*n*	Sig.	Control	Test	*n*
Peat							
0	0.28 ± 0.01^{b}	0.28 ± 0.01^{b}	3		34.7 ± 4.3^{a}	34.7 ± 4.3^{a}	2
1	$0.24 \pm 0.00^{a,b}$	$0.25 \pm 0.01^{a,b}$	3	0.492	47.1 ± 15.8^{a}	35.2 ± 6.6^{a}	2
2	$0.24 \pm 0.01^{a,b}$	$0.25 \pm 0.00^{a,b}$	3	0.551	ND	ND	
3	0.22 ± 0.01^{b}	0.22 ± 0.01^{a}	3	0.798	ND	ND	
4	0.21 ± 0.01^{b}	0.23 ± 0.00^{a}	3	0.188	29.7 ± 0.9^{a}	40.1 ± 5.9^{a}	2
4 + 13 d	ND	0.25 ± 0.01^{a}	3	ND	ND	ND	
4 + 23 d	$0.24 \pm 0.01^{a,b}$	$0.25 \pm 0.01^{a,b}$	3	0.231	ND	ND	
Loamy sand							
0	0.22 ± 0.01^{a}	0.22 ± 0.01^{a}	3		54.0 ± 3.0^{a}	54.0 ± 3.0^{a}	2
1	0.22 ± 0.00^{a}	0.22 ± 0.01^{a}	3	0.800	52.6 ± 12.0^{a}	44.3 ± 4.2^{a}	2
2	0.21 ± 0.01^{a}	0.27 ± 0.00^{b}	3	0.102	37.3 ± 6.2^{a}	40.6 ± 1.0^{a}	2
3	0.29 ± 0.01^{b}	0.27 ± 0.00^{c}	3	0.224	ND	ND	
4	0.29 ± 0.01^{b}	0.27 ± 0.01^{b}	3	0.230	57.5 ± 2.1^{a}	45.2 ± 7.2^{a}	2
4 + 13 d	ND	0.22 ± 0.01^{a}	3	ND	ND	ND	
4 + 23 d	0.32 ± 0.02^{b}	$0.23 \pm 0.01^{a,b}$	3	0.006	31.4 ± 4.2^{a}	47.7 ± 11.6^{a}	2

ND—not determined.
Statistical difference ($P < 0.05$) inside the control and test groups in both soils are marked with a, b and c (Tukey post hoc test). Statistical significant difference (*P*-value) between control and test groups in microbial biomass carbon is shown (paired *t*-test).

Table 4
Quantitative amounts of lipid biomarkers PLFA (viable microbial biomass), 3-OH-FAs (biomass of gram-negative bacteria) and 2-OH-FAs (biomass of fungi, plants, mycobacteria) in peat and loamy sand soils per volume of the soil (average ± SE)

	Peat	*n*	Loamy sand	*n*
PLFA (μg cm^{-3})	37.4 ± 3.4	10	45.7 ± 2.5	18
3-OH-FA (μg cm^{-3})*	48.8 ± 0.9	10	14.2 ± 0.8	17
2-OH-FA (μg cm^{-3})*	64.2 ± 4.7	10	15.7 ± 0.6	18

*$p < 0.000$.

more cyclopropane PL fatty acids than loamy sand, whereas loamy sand contained more terminally branched saturated PLFAs (Fig. 3). In addition, especially in peat soil, there was a smaller change in the PLFA profile over time. Otherwise, the PLFA profiles were very similar in both soils. The viable microbial community structure contained mainly bacteria (gram-negative bacteria with unsaturated and cyclopropane PLFA, and gram-positive bacteria with terminally and middle branched PLFA), whereas the amount of polyenoic PLFA (eurcaryotes, such as fungi) was very low (Fig. 4). Similarly, the community structure of gram-negative bacteria on the basis of 3-OH-FA differed between the studied soils (PC1, 50.4% of variation, Fig. 3). In peat, the gram-negative bacterial community (3-OH-FAs) in the FTC-treated soil differed from that in the control soil at the fourth FTC on the basis of PCA (Fig. 3). However, 3-OH-FA profiles were rather similar in the soils (data not shown). In the 3-OH-FA profile, 3-OH-12:0, 3-OH-14:0, 3-OH-16:0 and 3-OH-18:0 had highest abundances. In the 2-OH-FA profiles consisting mostly of fatty acids with carbon chain length of 20 or greater and presenting fungi, mycobacteria and dead plant material, there was also a difference between the studied soils but this difference was not as clear as in the PLFA or 3-OH-FA profiles (Fig. 3).

3.6. TGGE analysis of the microbial community structure

The clustering analysis of the microbial community fingerprints was performed with different algorithms, and the most reliable one was selected according to the calculations of the cophenetic correlations. In the phylogram produced with the UPGMA-method, the community fingerprint profiles of the two soil types spread randomly (Fig. 4). Similarly, there was no distinguishable clustering behaviour of the community profiles due to FTC-treatment or control status. Thus there were no clear FTC-driven shifts in the microbial community compositions.

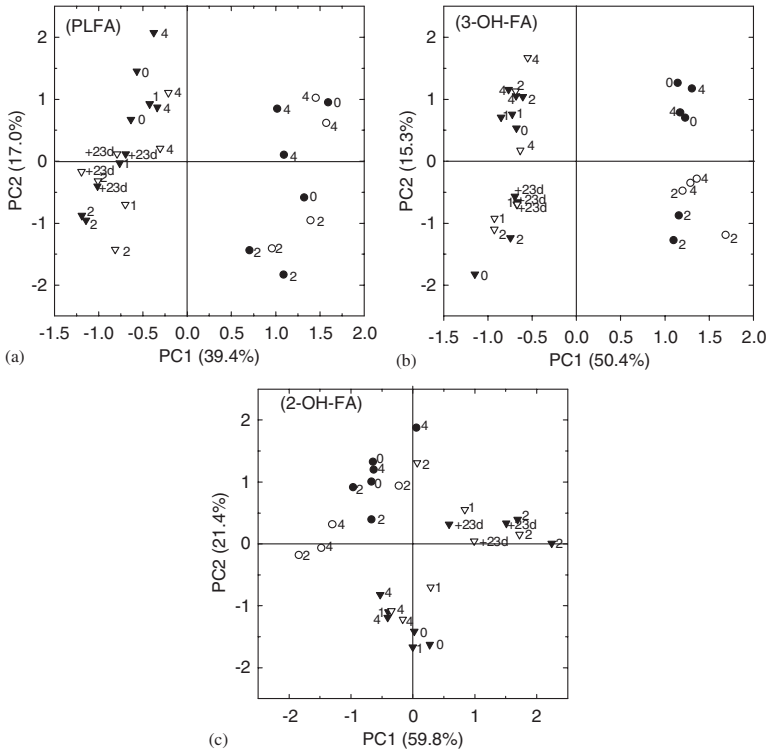

Fig. 2. Score plot of principal component analyses of phospholipid fatty acids (PLFA) (a), 3-hydroxy fatty acids (3-OH-FA) (b), and 2-hydroxy fatty acids (2-OH-FA) (c), for FTC treated peat (●), control peat (○), FTC treated loamy sand (▲) and control loamy sand (△). Freeze-thaw cycle is expressed by numbers (0 = FTC0, 1 = FTC1, 2 = FTC2, 4 = FTC4 and +23 = FTC4 + 23 d).

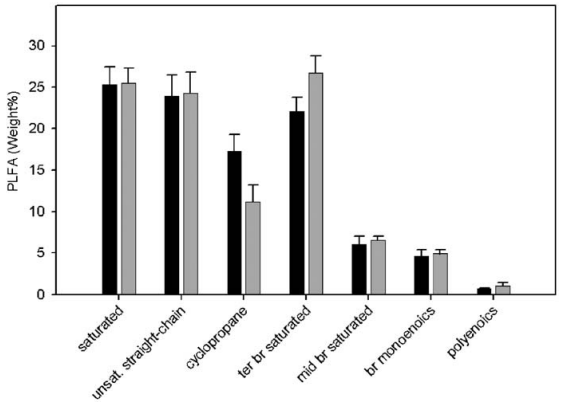

Fig. 3. Phospholipid fatty acids (PLFA) profiles of peat (black bar) and loamy sand (grey bar), with standard errors. Appreviations: unsat., unsaturated; ter br, terminally branched; mid br, middle branched; br, branched.

4. Discussion

Organic agricultural soils have been considered to be a potential source of N_2O (Martikainen et al., 1993, Kasimir-Klemedtsson et al., 1997). However, in this study the average N_2O emission from the peat site was extremely low. In peat, NO_3–N content increased during the incubation, while NH_4–N content first increased and then decreased. This indicates that nitrifying bacteria remained active during the experiments and produced ammonium was oxidized to nitrate by nitrifiers. There was nitrate for denitrification in the peat soil, and the low N_2O emission probably resulted from good aeration of the soil. The peat soil had a higher capacity to denitrify than the loamy sand, as shown by the anaerobic incubation experiments. Pihlatie et al. (2004) showed with the same soils that, with the soil moisture conditions of the present study, the main production mechanism for N_2O in peat (60% WFPS) is nitrification (76% of N_2O–N production), while in loamy sand (80% WFPS) denitrification and nitrification are almost equally important in N_2O production.

In this study, denitrification activity in vitro did not show any clear changes due to FTCs. Together with the denitrification measurements, we could also measure anaerobic respiration from the experimental flasks. Soil freeze-thaw enhanced anaerobic respiration in both soils during the first 3–4 FTCs, the increase being more evident for the peat soil. Soil respiration, measured from the soil

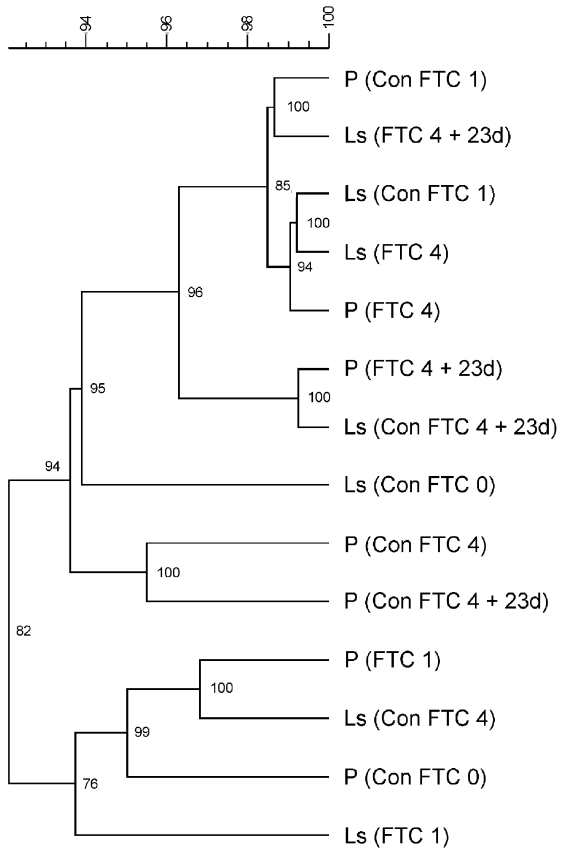

Fig. 4. Cluster analysis of the microbial community fingerprint data, based on curves deduced from normalized TGGE image. Clustering was calculated with the UPGMA method, using Pearson coefficient. Cophenetic correlations for the dendrogram are given in the nodes. The scale bar represents percent similarity. Ls: loamy sand, P: peat, Con.: control ($+6.6\pm2.0\,^{\circ}C$).

cores, did not show a similar trend in mineral soil, but the first FTC resulted in the highest respiration in peat soil. Respiration in soil cores is mainly aerobic. It is suggested in earlier studies that denitrification benefits more from soil freezing and thawing than does general heterotrophic microbial activity (Koponen et al., 2004). The results of the anaerobic flask experiments here reflect the same phenomenon. The anaerobic and/or facultative anaerobic bacteria might thus benefit especially from soil freezing and thawing possibly due to their better capacity to utilize extra substrates released during temperature stress. Several studies have addressed the potential importance of unfrozen microsites on N_2O production at low temperatures (e.g. Stähli and Stadler, 1997, Teepe et al., 2001, Koponen et al., 2004). Sehy et al. (2004) concluded that both the quantity and quality of available C has a crucial role in freeze-thaw related N_2O emissions. The FTCs might induce the release of extra substrates to the anaerobic microsites of soil where denitrifying bacteria can be active.

Soil FTC have been suggested to increase the nutrient concentration by destroying microbes, resulting in increased activity of the surviving microbes (Christensen and Tiedje, 1990). The viable microbial communities (PLFA biomass and profile) were unaffected by the FTC. Also, the fatty acid analyses did not reveal any changes in the physiological state of microbes. Microbes have been shown to adapt to temperatures using several strategies, i.e. there are changes in carbon chain length and the saturation of fatty acids, and changes in the ratio of *iso*- and *anteiso*-branched fatty acids (Suutari and Laakso, 1994), which could have been expected in our samples if the FTC had had any effects on microbes. On the other hand microbes here might have had some adaptation to low temperatures already during the storage period. The ratio of cyclopropane PL fatty acids to their $\omega7$ counterparts was closer to 2.5 than to 0.05, showing stationary growth phase of microbes probably due to the environmental stress of gram-negative bacteria in both soils (Guckert et al., 1986, White et al., 1996). Similarly, the 2-OH-FA profile not originating from gram-negative bacteria but mostly from fungi, mycobacteria and dead plant material (Brennan, 1988, Lösel, 1988 Rattray, 1988) showed no changes due to the freeze-thaw treatment. In the inner structure of gram-negative bacteria (3-OH-FA profile, Wilkinson, 1988), the only difference due to the FTC was seen in peat after the fourth FTC. However, all the fatty acids profiles within the same soil were rather similar, proving that differences due to the freeze-thaw treatment were minor. A part of microbes might have died during the freeze and thaw cycles but it would take longer to detect these changes in community structures or biomass. Raneklev and Bååth (2003) found that lipids from thermophilic organisms degraded very slowly at $+5\,^{\circ}C$, compared to much faster degradation at $+15\,^{\circ}C$. On the other hand, the viable microbial communities differed between the soils, with peat soil containing more cyclopropane PLFAs characteristic of gram-negative bacteria (Wilkinson, 1988), whereas loamy sand contained more terminally branched PLFAs characteristic of gram-positive bacteria (O′Leary and Wilkinson, 1988). The amount of eucaryotic cells such as fungi estimated on the basis of polyenoic PL fatty acids was very low. It is probable that the agricultural soils studied had a naturally low amount of fungi. On the other hand, fungal hyphae might have been damaged by the sieving before the experiment (Petersen and Klug, 1994).

There were no changes in microbial biomass-C or viable microbial biomass due to soil FTC, determined either by the SIR or PLFA methods. This result is in good agreement with the results of Lipson et al. (2000), where soil microbial biomass in alpine dry meadows was not affected by a single freeze-thaw event. However, Larsen et al. (2002) reported that microbial biomass C in subarctic heath decreased after 18 FTCs.

On the basis of 3-OH-FAs, peat soil contained more biomass of gram-negative bacteria than did loamy sand. The same was also true for fungi, mycobacteria and dead plant material (2-OH-FAs). Some of the 3-OH-FAs originated from dead gram-negative bacterial material, especially in peat, since there was quantitatively more 3-OH-FAs than PLFAs. Similarly, 2-OH-FAs originating from not only alive cells and not only from bacteria as the profile was dominated with 2-OH with more than 20 carbon atoms in carbon chain and the quantitative amount of 2-OH-FA was higher than that of PLFA, respectively.

According to the cluster analysis of the molecular population fingerprinting (TGGE) patterns, the soil microbial communities showed no response to FTC-treatment (Fig. 4). This is in line with the results of the lipid biomarker analysis. It has been shown that in agricultural soil, the freeze-thaw stress leads to lower DNA content, but has no effect on eubacterial community structure (Pesaro et al., 2003). Also, there was no clear difference between the microbial populations of the two soil types studied here (Fig. 4). Bacteria can grow in subzero temperatures (Junge et al., 2002) and may remain active even in extreme (−17 °C) coldness (Carpenter et al., 2000). The rather long cold storage of the soil material prior to the freeze-thaw experiment may have created selective pressure, leading to the enrichment of the cold tolerant microbial pool, and thus unifying the microbial population structures of the two soil types studied. A six-month storage of soil samples in +7 °C has been shown to alter the molecular community profile (Eriksson et al., 2001). However this does not change the fact that the FTC treatment had no effect on community profile when compared to start point (FTC0) and control profiles. The sample size (0.25 g) was rather small, and this has to be taken into account when interpreting the results of the TGGE fingerprints. Reproduction of the TGGE profiling and cluster analysis with replicate sample series, however, gave similar results (data not shown), suggesting rather high similarity between the microbial community profiles of the two soil types, and giving no indications of community level responses to the FTC-treatment in either of the soils.

It is currently not possible by any method to measure precisely the composition of a microbial population. Although the molecular approach used here may have its flaws (e.g. Farrelly et al., 1995, Wintzingerode et al., 1997, Martin-Laurent et al., 2001, Kisand and Wikner, 2003, Lopez et al., 2003), it enables the comparison of the spatial and temporal differences between bacterial communities, and estimations of the bacterial diversity. The extraction and sequencing of the main TGGE fragments would have given more detailed taxonomic and possibly even species-level information of the bacteria present in the samples. Also, using RNA instead of DNA as the target in the TGGE analysis would have given more information about the active bacterial pool in the studied soils, but this was, however, beyond the scope of this study.

The results of the present study suggest that the changes in soil microbial biomass and community structure do not explain the increase in N_2O production associated with soil FTC. Most likely, the liberation of microbiologically available substrates from soil organic matter is the key to understanding the enhanced N_2O production, and the substrates in soil microsites in particular should be studied in more detail.

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CHAPTER VI

GENERAL DISCUSSION

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6.1 High N_2O at low temperature without soil freezing

One of the experiments (Chapter II) revealed that soil N_2O emissions can suddenly increase near 0ºC, without recent soil freezing-thawing history. To my knowledge this is the first report on such a phenomenon. This phenomenon was observed in 4 different agricultural soils (organic, clay, silt and loam) and was later confirmed in an experiment with organic soil having agricultural history (Chapter V). Schimel and Milkan (2005) observed in labelling experiments with Arctic tundra soil that the proportion of respired CO_2 from microbial biomass and products increases at temperatures close to 0ºC. Dissolved material and recycled microbial biomass have lower C/N ratio than the plant detritus pool and a temperature induced shift in microbial substrate use pattern could explain higher N availability at low temperatures (Schimel and Milkan, 2005).

Kandror et al. (2004) introduced the term "near-freezing proteins" to describe the survival and functioning of microbes at low temperatures. They concluded that the increased production of disaccharide trehalose, due to induction of trehalose-synthesizing enzymes and specific heat shock proteins at low temperatures is an important component for viability of microbes at 0ºC or upon freezing.

High N_2O production at temperatures close to 0ºC without soil freezing is difficult to explain. Soil physical-chemical characteristics do not change dramatically from 5ºC to 1.5ºC, but still we observed changes in N_2O emission rates (Chapter II). Changes in microbial activities are the probable explanation here. At temperatures just above 0ºC, the general microbial activity is rather low. It could be that some of the denitrifiers are psychrophilic and thus could benefit from a lowered competition for substrates at temperatures unfavourable for general microbial activity. Also, since vegetation is dormant, there is no competition for inorganic nitrogen between roots and microbes (Papen and Butterbach-Bahl, 2001).

High N_2O emissions at temperatures close to 0ºC may be supported by lower N_2O reductase activity as compared to N_2O producing activity (e.g. Keeney et al., 1979, Melin and Nômmik, 1983, Maag and Vinther, 1996, Müller et al., 2003.) Holtan-Hartwin et al. (2002) suggested that this change in N_2O reductase activity is community dependent, which could explain the variable responses to temperature in different soils. The temperature sensitivity of the N_2O reductase was not studied in this thesis. However, a 150 fold higher N_2O emission at around 0ºC as compared to +15ºC was observed (Chapter II). It would be rather unrealistic to explain this emission difference by temperature-induced change in N_2O reductase activity alone. There must be other mechanisms, including increased denitrification activity to explain high N_2O emissions at low temperatures without previous FTC.

6.2 N_2O emissions from frozen soil

In boreal regions soil is covered by snow for several months. This snow cover is an effective insulator keeping soil temperatures just above or below 0ºC, even when air temperature is far below zero. The insulating snow also protects the soil from fluctuations in air temperature and therefore soil temperature under snow is rather stable. This, together with relatively constant moisture status during severe frost creates stable conditions for microbes (Schürmann et al., 2002).

N_2O emissions at sub-zero temperatures are closely connected to the presence of free

water. Free water can exist at temperatures below 0ºC (Konrad and Duquennoi, 1993) and water films on the surfaces of soil particles can remain unfrozen down to -20ºC (Rivkina et al., 2000). Dorland and Beauchamp (1991) demonstrated that denitrification activity continued at a temperature of -2ºC. In the present study, N_2O production at -6ºC was measured (Chapter II).

Measurable denitrification activity at sub-zero temperatures raises the important question as to how the physico-chemical conditions are close to the microbial cell surfaces at sub-zero temperatures. Limited diffusion of oxygen due to frost and snow cover together with O_2 consumption by active microbes is likely to create anaerobic conditions in soil microsites, as discussed by Teepe et al. (2001). Aggregate disruption by soil freezing and thawing together with slough-off from plant roots increases the availability of nutrients (Soulides and Allison, 1961, Christensen and Christensen, 1991, Denef et al., 2001). Due to partial freezing, dissolved material including NO_3^- and organic compounds concentrate in the non-frozen water film on the surfaces of the soil particles. This may result in high substrate levels for the active microrganisms at the same time as osmotic stress response is induced. Apparently, such micro-scale phenomena support transiently high microbial activities at temperatures suboptimal for microbial growth. Another important factor for sustaining microbial activities at low temperatures in soils seems to be the localization of freeze-thaw susceptible substrates within the soil matrix. Sehly et al. (2004) added soil solution from freeze-thaw treated soils to unfrozen soil column but could not find any measurable increase in N_2O emission. In contrast, freeze-thaw treatment of intact soil triggered N_2O emission. Obviously, it is the freeze-thaw susceptible organic material in the direct vicinity of active microorganisms (i.e close to the unfrozen water films) that stimulates N_2O formation rather than the bulk amount of easily degradable organic matter.

In the present study, no changes in the microbial biomass due to FTCs was detected (Chapter V), indicating that the possible increase in the carbon availability was not used to support the general growth of microbes but to enhance the activity of denitrifying bacteria. (See chapter 6.5). Together, the results suggest that especially denitrifiers benefit from the physical-chemical status in soil with temperature below zero (Chapters II, III, V).

At sub-zero temperatures, soil N_2O production capacity can be high. However, not all of the N_2O produced is emitted to the atmosphere immediately. High concentrations of N_2O can exist in soil air (Chapter II). Soil frost and snow cover act as a diffusion barrier resulting in high (percentage level) N_2O concentrations in soil air during winter (Goodroad and Keeney, 1984, Cates and Keeney, 1987, Dörsch et al., 2004, Maljanen et al., 2007). This phenomenon is mainly regulated by the soil moisture status during freezing (Chapters II and IV, Maljanen et al., 2007). From frozen soil, N_2O diffuses slowly to the atmosphere.

One important aspect is the dormant status of vegetation during the cold seasons. Schürmann et al. (2002) suggested that during winter micro-organisms experience less competition for nitrate due to dormant vegetation. This may be one of the mechanisms behind the high N_2O emissions at low temperatures, especially in NO_3^- limited environments. Also, stabilised N pools can become available during soil freezing and thawing as shown by Müller et al. (2002) in grassland soils, with ^{15}N labelling studies.

Nitrous oxide emissions at low temperature can also be related to changes in the processes responsible for N_2O production (Chapter V). Low temperature can affect

denitrification at the enzyme level. The effect of low temperature has been suggested to be stronger on the N_2O reductase enzyme than the N_2O producing enzymes (NO_3^-, NO_2^- and NO reductases) (Holtan-Hartwig et al., 2002) leading to an increase in N_2O production even though the total denitrification activity decreases. This inhibition mechanism may account for the results shown in the Chapter IV. With decreasing temperatures we observed an increase in the N_2O emission at -2.2ºC.

6.3 Soil thawing and the emissions of N_2O and NO

When boreal agricultural soil thaws, WFPS is usually rather high due to water from snow melting. High water content favours anaerobisis and thus denitrification. Denitrification has been suggested to be the major process for N_2O emissions during soil thawing (e.g. Röver et al., 1998; van Bochove et al., 2000, Müller et al., 2003, Ludwing et al., 2004) and was also assumed to dominate N_2O production in the experiments described in Chapter IV. Thawing and low temperature were found to have minor effects on nitric oxide production compared to that of N_2O (Chapter IV).

In Chapter V we tested the hypothesis that soil microbial biomass declines at critical temperatures near 0ºC, and that the extra nutrients released from lysed microbial biomass support the growth of surviving microbes. This theory is cited routinely, when discussing freeze-thaw related emissions (e.g. Skogland et al., 1988, Christensen and Tiedje, 1990; Christensen and Christensen, 1991, Herrmann and Witter, 2002). However, direct evidence for decreased viability of microorganisms due to FTC stress in soils is lacking. And indirect approaches reporting increased mineralisation from a labelled biomass pool are prone to overestimation due to unintended labelling of the microbial product pool. PLFA and SIR data presented in Chapter V did not give any evidence for cell lysis under the temperature conditions imposed in this work. The issue of biomass derived carbon for fuelling microbial activity at low temperatures will be discussed in more detail in chapter 6.5.

Soil thawing related N_2O emissions can be divided into two phases (Teepe and Ludwig 2004, Öquist et al., 2004, Syväsalo et al., 2006; Maljanen et al., 2007). In the first phase N_2O stored in the subsoil is released. In the second phase, N_2O is produced in situ at high rates in the thawing soil. These two processes can also occur at the same time and are dependent on soil texture, moisture content and other soil physical-chemical properties. The results from this study (Chapter IV) suggest that the changes in processes responsible for N_2O and NO production may play an important role during soil thawing (See Chapter 6.4).

Our study confirms earlier results (Papen and Butterbach-Bahl, 1999) on the importance of the freezing period for thawing-induced N_2O emissions. Not only the duration of freezing (Papen and Butterbach-Bahl, 1999) and soil moisture content (Teepe et al., 2000, Chapter III) but also the freezing temperature (Chapter III) has a great importance for the spring time N_2O emissions. In boreal regions, climate change may result in colder soils due to diminishing thickness of the snow pack in winter (Groffman et al., 2001). Our results (Chapter III) suggest that this can lead to higher N_2O emissions when soil is thawing. This conclusion was recently confirmed by snow-manipulation experiments (Maljanen et al., 2007).

6.4 Processes responsible for NO and N_2O production in soil at low temperatures

One of the key results in this study is that NO production in soil does not follow the same patterns as that of N_2O during soil

freezing and thawing. The dominant source for NO and N_2O is commonly estimated from the ratio of NO to N_2O produced in soil. If the ratio is less than one, the dominant process can be expected to be denitrification, while ratios > 1 indicate the dominance of nitrification (Anderson and Levine, 1986, Skiba et al., 1993) In Chapter IV, the nitrification process was shown to have a role for NO and N_2O emissions during temperature decline to near 0ºC. Syväsalo et al. (2004) also stressed the importance of nitrification during winter. Nitrification can play an important role as a bottle neck for N flow, thus also limiting denitrification via supplying NO_3^- (Öquist et al., 2004, Öquist et al., 2007). The possibility, that denitrification is the dominant process for N_2O during soil thawing was confirmed by calculating NO / N_2O ratios (Chapter IV). However, this was done only for two soil moisture contents (WFPS 71±7% and 78±1%) in organic soils. However, these moisture contents are realistic for thawing soils. The importance of the denitrification process for N_2O emissions at low temperatures has been suggested elsewhere (e.g. Christensen and Tiedje, 1990, Priemé and Christensen, 2001). It can be concluded that both nitrification and denitrification occur at low temperatures and that the dominant process for N_2O and NO production may be different in frozen soil than during soil thawing. This conclusion is in good agreement with the results from Papen and Butterbach-Bahl (1999), who showed both nitrification and denitrification activity at low temperatures (-1.5ºC).

The rate of NO production and consumption not only depends on the overall denitrification rate, but is also strongly dependent on factors affecting the proportion of produced NO relative to the production of N_2O and N_2. McKenney and Dury (1997) suggested that NO produced near the soil surface is readily emitted because of a short diffusive path and exposure to wind, while in greater depths the increased path length allows increased conversion of NO to N_2O. Although there may be formation of NO during winter, ice in soil decreases the diffusion rate allowing reduction of NO to N_2O by denitrifier nitric oxide reductase (NOR) resulting in low NO emissions from soils. Skiba et al. (1997) reported that only 13% of NO produced was actually emitted from the soil surface under anaerobic conditions. In boreal regions soil moisture content is typically high in autumn and spring, resulting in anaerobic conditions close to the soil surface. This may result in low NO emissions, although the production in soil is high.

6.5 CO_2 production, microbial biomass and composition at low temperatures

Schadt et al. (2003) found a shift from bacterial dominance in summer to fungal dominance in winter. There is also evidence for shifts in microbial community composition in response to freeze-thaw cycles (Larsen et al., 2002, Sharma et al., 2006). In contrast, there are studies where no effect on microbial biomass has been found (e.g. Walker et al., 2006, Grogan et al., 2004). Lipson et al. (2000) found no effect on soil microbial biomass and suggested that microorganisms in a cold ecosystem can tolerate moderate freeze-thaw events. In this study we found no changes in the amount of microbial biomass (Chapter V), when studying boreal agricultural soil.

Our results (Chapter V) are in good agreement with Stenberg et al. (1998), who found that soil microbes are resistant to freeze-thaw cycles and low temperatures. Active soil microbial populations may undergo a critical shift between winter and summer, suggesting that winter-adapted microbial communities would be intolerant to higher temperatures (Lipson et al., 2000, Schmidt and Lipson, 2004). However, in our study (Chapter V) we found no changes in microbial community structure**.** Shifts in the taxonomic composition of microbial

communities by freeze-thaw would require that freezing extinguishes a part of the microbial community thus giving space for re-growth which then could lead to change in community structure. There was no evidence for structural change in the present study (Chapter V) and it may be therefore concluded that frost killing of microbial biomass was small.

Mørkved et al. (2006) calculated that the carbon contained in 1% of the microbial biomass would be sufficient to explain increased respiration by microbially derived substrates after thawing. Herrmann and Witter (2002) calculated that the flush of carbon associated with freezing-thawing corresponded to 5% of the microbial biomass C. The number of freeze-thaw cycles can have different effect on microbial biomass (Winter et al., 1994, Walker et al., 2006). The conclusion from this is, that lysis of a minor part of the microbial biomass may significantly contribute to fuelling post-thaw microbial activity and possibly N_2O formation without inducing any sustainable compositional change in the re-growing community. As already discussed in chapter 6.3, microbial lysis in soils is difficult to quantify, preventing a final evaluation of the importance of this mechanisms for FTC related N_2O production at that stage.

In this study, no effect of FTC on denitrification activity *in vitro* was shown (Chapter V), which supports the notion that freeze-thaw does not lead to major shifts in microbial community structure. Freeze-thaw may, however, increase the activity of the denitrifying community as shown by Sharma et al. (2006). They found a 5 fold increase in the transcript level of napA (periplasmic and membrane-bound nitrate reductase gene) and 10-fold increase in nirS (genes encoding cytochrome cd_1) during soil thawing, suggesting changes in denitrification activity after freezing thawing.

Soil respiration has been reported to be highly sensible to temperatures below 0 ºC, suggesting indirect control through physical factors (Milkan et al., 2002). Heterotrophic respiration occurs at temperatures below 0 ºC (Brooks et al., 2005). Soil respiration causes oxygen consumption; the higher the respiration, the higher is the oxygen depletion in soil (Mørkved et al., 2006). This can have great importance for N-fluxes from denitrification during soil thawing.

In soils, denitrifiers are thought to reside in microsites characterised by low oxygen pressures and high C and NO_3^- availabilty (Sexton et al., 1985). If freeze-thaw targets these microsites, the increased energy (carbon) and/or nutrients may greatly stimulate N_2O production although the change in the total energy flow can be small.

One of the main conclusions of this work is the importance of micro-scale phenomena. Various functional microbial groups inhabiting different microhabitats in soil can have variable benefit from the extra substrates becoming available at low temperatures. As discussed in chapter 6.2, especially denitrifiers can benefit from the extra substrates, more than the heterotrophic microbes in general. This may be due to lack of oxygen, or due to a more psycrophilic nature of denitrifiers.

6.6 The effect of soil type on N_2O emissions

Soil type affects soil N_2O emissions. In coarse-textured soils (with low bulk density and high air-filled pore space) gas diffusion is higher than in heavy-textured soils. As air-filled pore space decreases, diffusion becomes increasingly restricted (Amundson and Davidson, 1990) affecting both oxygen diffusion into the soil and N_2O diffusion out of the soil. In most of our studies, soil WFPS was near or above 60%, a WFPS where denitrification is the dominant N_2O source (Davidson et al., 1991). The

temperature manipulations during soil thawing were also rather similar in Chapters II, III and IV including frost at temperatures from -5.9 to -1.5°C and soils were allowed to thaw at 4 to 5.5°C. There was one experiment (Chapter V) in which -15°C was used as freezing temperature.

Highest N_2O emission was observed with all soil types during soil thawing, with the exception of loamy soil (Chapter II). In this soil, soil moisture content was low (WFPS 42%). Thawing related N_2O emissions were highest from organic soil. This result was expected since organic soils contain high amounts of FTC susceptible organic carbon and nitrogen. An interesting observation was the large variation in N_2O emission rates in the organic soil, even within the same field (Chapters II and IV). In the experiments of Chapter II, organic soil had thawing related N_2O emissions 500 times higher than the emission in the experiments of Chapter IV. This difference may be due to different temperature histories of the soils. In Chapter II, soil cores underwent long temperature manipulation prior to rapid thawing, while in Chapter IV soil thawing took place after 5 days at -1.5°C. In nature, thawing related N_2O emissions can occur also during autumn, when soil freezes during the night and thaws during daytime (Regina et al., 2004).

Also mineral soils can produce significant amounts of N_2O during soil thawing as shown here and also in previous studies (e.g. Röver et al., 1998, Wagner-Riddle and Thurtell, 1998, Kaiser et al., 1998, van Bochove et al., 2000). Müller et al. (2002) concluded that an important soil characteristic associated with increased N_2O emissions at thawing is the soil's capacity to fix NH_4^+ by clay minerals and to immobilize NO_3^- and NH_4^+ shortly after N fertilization. This capacity apparently enables high N release during thawing.

6.7 Methods

Firstly, attention must be paid to the experimental setup. Soils used in microcosms were sampled from various agricultural fields and temperature and moisture manipulations were done in laboratory. This method allowed a precise control of temperature during incubations, giving valuable information on soil processes. Our cores had a rather wide diameter (10-19cm) which diminished boundary effects.

Secondly, soil homogenization and repacking as used in this study has both advantages and disadvantages. Homogenization of agricultural soil resembles agricultural practices such as ploughing and harrowing and may have similar effects on soil structure and substrate availability. In laboratory studies, homogenization ensures an even distribution of soil material and nutrients. Homogenization is necessary, especially if soil volume in the experiments is small (Chapter V). However, homogenization changes the soil structure, creating unnatural conditions which may have an impact on the soil processes. In the experiments of Chapter III, soil homogenization and repacking affected soil bulk density, and thus soil WFPS, a soil characteristic affecting microbial activity. However, the key aims of this experiment were to compare the effect of different temperature treatments on soil biological variables which are notoriously variable in space. We therefore used homogenised soil samples to reduce this variability. The observed lack of significant temperature effects on microbial biomass and composition observed may therefore represent a conservative estimate under conditions of artificially low variability.

Thirdly, the manipulation of soil temperature in laboratory mimicking natural conditions is not an easy task. In the experiments of Chapters II, III and V, soil temperature manipulation allowed heat

transport almost equally from every side of the soil cores. This does not equal freezing and thawing conditions in soil profiles *in situ* where heat loss takes place mainly via the soil surface. The more rapid temperature change in the whole soil profile in the laboratory may affect the results (Henry, 2007). In the experiments of Chapter IV, a more sophisticated method was applied: the bottom and sides of the cores were covered with insulating material (Styrofoam). This method allows the main heat transfer to occur via the soil surface and thawing was started just from the soil surface similarly to *in situ* conditions. This method allows lower thawing rates and hence mimics natural soil conditions when studying thawing related N_2O fluxes (Hu et al., 2006).

6.8 Conclusions

- The N_2O production rates near 0^oC can be very high, even exceeding the rates at +10ºC.
- Decrease in temperature to low plus degrees can result in high N_2O production.
- At sub-zero ºC, soil can produce and store significant amounts of N_2O. Stored N_2O is released when the soil thaws.
- High soil moisture status at freezing increases thawing related N_2O emissions.
- Soil freezing temperature affects thawing related N_2O emissions; more severe frost induces higher thawing-related N_2O emissions
- Nitric oxide production is regulated more directly by temperature than nitrous oxide production.
- Denitrifying bacteria benefit more from freeze-thaw cycle related changes in soil chemistry and physics than general heterotrophic microbial activity. This stresses the importance of soil micro-environments in biogeochemistry.
- In boreal regions, soil microbes (biomass, community structure) are quite robust to freezing-thawing perturbation.

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