

Influence of Freeze-Thaw Stress on the Structure and Function of Microbial Communities and Denitrifying Populations in Soil†

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Microbial N₂O release during the course of thawing of soil was investigated in model experiment focusing on denitrification, since freeze-thaw has been shown to cause significant physical and biological changes in soil, including a surge of N₂O and CO₂. The origin of these is still controversially discussed. The increase in denitrification after thawing may be attributed to the diffusion of organic substrates newly available to denitrifiers from disrupted soil aggregates, leading to an increase in microbial activity. Laboratory experiments with upper soil layer of a grassland were conducted in microcosms for real-time gas measurements during the entire phase of freeze and thaw. Shifts in microbial communities were evident on resolution of 16S and 18S rRNA genes and transcripts by denaturing gradient gel electrophoresis (DGGE). Microbial expression profiles were compared by RNA-arbitrarily primed PCR technique and subsequent resolution of amplified products on acrylamide gels. Differences in expression levels of periplasmic nitrate reductase gene (*napA*) and cytochrome *cd*₁ nitrite reductase (*nirS*) were observed by most-probable-number–reverse transcription-PCR, with higher levels of expression occurring just after thawing began, followed by a decrease. *napA* and *nirS* DGGE profiles showed no change in banding patterns with fingerprints derived from DNA, whereas those derived from cDNA showed a clear succession of denitrifying bacteria, with the most complex pattern being observed at the end of the N₂O surge. This study provides insight into the structural community changes and expression dynamics of denitrifiers as a result of freeze-thaw stress. Also, the results presented here support the belief that the gas fluxes observed during thawing is a result of freezing initiated high microbial activity.

The main process that forms the trace gas nitrous oxide (N₂O) in agricultural soils is denitrification. It contributes significantly to the greenhouse effect. The concentration of N₂O in the troposphere has increased from 270 ppb in 1750 to concentration of 316 ppb in the year 2000 and continues to rise (15). Moreover, it is involved in the destruction of stratospheric ozone. Therefore, identifying sources of N₂O has been a subject of research over the last two decades. Several field studies in the temperate regions have indicated that N₂O emissions in winter and spring, due to freezing and thawing of agricultural soils, can reach between 20 and 70% of the annual budget (28, 35).

These high amounts of N₂O emissions are due to changes in the soil structure by the freeze-thaw events. These changes include the disruption of soil aggregates (4), the release of aggregate-protected organic C (8), and the death of microorganisms due to stress, resulting in increased availability of substrate and enhanced microbial activity in soils. An increased availability of substrates, resulting from freeze-thaw of soil, could stimulate the activity of denitrifiers. This view is supported by reports of large and often ephemeral fluxes of N₂O emission at spring thaw (5, 6, 16), whereas rates of N₂O production by nitrification tend to be smaller (20, 38).

Although microbial denitrification is believed to be the source of N₂O, very few reports have concentrated on microbial community analysis during this process. Most of the previous work has concentrated on gas and soil analysis. A couple of studies reported no change in the microbial biomass with the progress of single freeze-thaw in soil (12, 22). Schimel and Mikan (30) showed that in Arctic soils, microbes showed a shift in metabolism as soils cooled from 2 to 0.5°C with differences in substrate use patterns on thawing. The only report on microbial community structure during freeze-thaw event has been by Eriksson et al. (9), where a change in ribosomal internal spacer analysis patterns was observed in soil exposed to freeze-thaw using DNA extracted from soil. However, nothing is known thus far about the effects of freezing and thawing on the microbial gene and transcript pool with respect to denitrifying genes. Denitrification involves the reduction of nitrate, via nitrite and nitric oxide, to nitrous oxide or dinitrogen gas by a respiratory process under oxygen deficiency conditions. The functional genes involved in denitrification include periplasmic and membrane-bound nitrate reductase genes (*napA* and *narG*), genes encoding for cytochrome *cd*₁ and copper-containing nitrite reductases (*nirS* and *nirK*, respectively), nitric oxide genes encoding the quinol-oxidizing single-subunit class and cytochrome *bc*-type complex (*qnorB* and *cnorB*, respectively), and a gene encoding for nitrous oxide reductase (*nosZ*). Therefore, in our study we concentrated on the effects of freeze-thawing on the microbial denitrifying potential (gene pool analysis) and the expression of denitrifying genes (transcript analysis) *napA*, *nirK*, *nirS*, and *nosZ*.

We hypothesized that soil freezing and thawing could greatly affect both the structure and function of soil microbial popu-

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lation. Hence, the objectives of the present study were (i) to analyze shifts in the microbial communities with the progress of N₂O emission at thawing event, (ii) to compare the total microbial population with its active counterpart, and (iii) to study the effect of freeze-thaw on transcription dynamics and diversity of genes encoding the nitrate and nitrite reductases.

MATERIALS AND METHODS

Soil samples. Experiments were performed with soil collected from the research farm of Forschungsverbund Agrarökosysteme München (FAM) in Southern Germany (latitude, 48°24' to 48°36'N; longitude, 11°20' to 11°40'E), approximately 45 km north of Munich in the Bavarian tertiary hills. The mean annual air temperature is 7.4°C, with a minimum in January (−4°C daily mean temperature) and a maximum in August (22°C daily mean temperature). The annual precipitation is 833 mm (60% in the summer months). This area experiences repeated freeze-thaws in winter. Sampling of upper soil horizon was performed in early May 2003 from a grassland soil classified as Dystric Eutrochrept (U.S. Department of Agriculture taxonomy). The air temperature was recorded to be 8°C at the time of soil sampling. Soil was homogenized, sieved (2-mm pore size), and maintained at a water-filled pore space of 60%. It was stored at 7°C in the dark till the start of the experiment. Thus, it was maintained at its natural state as in spring. The soil had an ammonium concentration of 1.2 g kg of dry soil^{−1}, a nitrate concentration of 11.49 mg kg of dry soil^{−1}, and a biomass carbon of 182.20 mg of C kg of dry soil^{−1}.

Microcosm experiment. The freeze-thaw experiment was performed in a microcosm system, which allowed an automated sampling of air and analysis of the fluxes of N₂O and CO₂. Each microcosm (inner diameter, 0.14 m; height, 0.35 m) was filled with 5.5 kg of the homogenized soil maintaining the bulk density at 1.2 g/cm³. Nine soil-filled microcosms were placed in freezers at −20°C for 1 day, followed by thawing with a change in temperature to 10°C. The headspace (volume of 883.6 cm³) of each soil column was continuously flushed with a controlled flow of fresh air. The concentrations of N₂O and CO₂ in the air space in every microcosm were determined every 2 h from the start of thawing on an automated gas chromatograph system (Shimadzu 14B with detectors: ⁶³Ni-ECD for CO₂ and N₂O). Nondestructive sampling of only the upper 5 cm was performed from three different microcosms in triplicates (3 × 3 = 9 samples for each time point, 27 samples in total) at three different time points as deduced from evolution of N₂O (start, exponential rise and end): day 2 (microcosms 1, 4, and 7), day 3 (microcosms 2, 5, and 8), and day 9 (microcosms 3, 6, and 9). Thus, effectively, one microcosm was sampled only once in the experiment to minimize disturbance. Samples were shock frozen for molecular analysis. Besides the three time points, three replicate soil samples were also taken just before starting the experiment from original soil (before exposing to freeze-thaw treatment), which was termed the control soil (C). Replicates were further analyzed separately.

Microbial biomass carbon measurement and nucleic acid extraction. Microbial biomass carbon was measured according to the protocol described by Brookes et al. (3). Nucleic acid extraction from the soil material was performed by using the method of coextraction of DNA and RNA described by Griffiths et al. (11). The method principally involved bead beating and solvent extraction of the nucleic acids. To prevent the degradation of RNA, all incubations were performed on ice. To obtain pure DNA, incubation at 37°C with RNase A (Sigma, Munich, Germany) at a final concentration of 100 µg ml^{−1} for 10 min was performed. Quantification and purity of DNA and RNA was measured by using a fluorospectrometer (NanoDrop, Wilmington, DE). To obtain pure RNA, DNA was removed from RNA by treatment with DNase (1 U µl^{−1}; RNase-free; Promega) according to the manufacturer's instructions.

cDNA synthesis. RNA was reverse transcribed by using the Omniscript RT Kit (QIAGEN GmbH, Hilden, Germany). Two microliters of total-RNA sample was added to an 18-µl reverse transcriptase (RT) mixture containing 20 pmol of random hexamers (the RT mixture was prepared according to the manufacturer's instructions). The reaction was incubated at 37°C for 90 min and then heated at 93°C for 5 min for enzyme inactivation, followed by rapid cooling on ice. The synthesized cDNA was stored at −20°C.

AP-PCR and RAP-PCR. DNA and RNA fingerprints for the soil samples were generated by using arbitrarily primed (AP-PCR) and RNA arbitrarily primed PCR (RAP-PCR) methods. A 10-mer primer, 5'-TCACGATGCA-3', described by Williams et al. (37) was used to generate fingerprints. Amplification reactions were performed as described by Sharma et al. (31). Controls to rule out the possibility of DNA contamination in the RNA preparation were performed by using DNase-treated but not reverse-transcribed (DNase⁺ RT[−]) nucleic acids in the assays.

Polyacrylamide gel electrophoresis. Nondenaturing polyacrylamide gels (8% with a 29:1 ratio of acrylamide to bisacrylamide) were prepared as described by Sambrook et al. (29). Appropriate volumes containing about 2 µg of AP-PCR and RAP-PCR products, measured by determining the absorbance at 260 nm, were loaded. The gels were electrophoresed at 50 V for 17 h by using the D-Gene system (Bio-Rad Laboratories, Munich, Germany) and silver stained according to the protocol of Heukeshoven and Dernick (14).

PCR and RT-PCR amplification for rRNA. Primer pair 968f-GC and 1401r (13), with an annealing temperature of 54°C, was used in specific PCR and RT-PCR analyses targeting bacteria. For fungi, primer pair NS1 and NS2-GC at an annealing temperature of 52°C (36) was used in the reaction. Then, 1 µl of template was used in a 48-µl PCR, which consisted of 5 µl of 10× reaction buffer, 5 µl of 3% bovine serum albumin, 2.5 µl of dimethyl sulfoxide, 3 µl of 25 mM MgCl₂ for bacteria (for fungi, 5 µl was added), 5 µl of 2 mM deoxyribonucleoside triphosphate mixture, and 1 µl each of a 10 µM concentration of the primer pair. The reaction involved hot start at 95°C for 10 min, followed by the addition of 2.5 U of *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany). The cycling parameters were 94°C for 1 min, followed by annealing for 1 min and 72°C for 1 min for 30 cycles, followed by a final extension at 72°C for 10 min. PCR and RT-PCR products were purified by using a QIAquick PCR purification kit (QIAGEN). DNase⁺ RT[−] nucleic acids were used as controls in RT-PCR to check for residual DNA in RNA preparations.

MPN-PCR and RT-PCR for denitrifying genes and transcripts. PCR and RT-PCR targeting *napA*, using the primers and protocol described by Flanagan et al. (10) and *nirS*, *nirK*, and *nosZ*, using primers described by Thröback et al. (34), were used to check for the presence of the gene and its expression. Serial dilution of the template was performed. PCR and RT-PCR, with different dilutions of template, were set in triplicates. Amplification products were resolved on 1.5% agarose gels in 1× TAE buffer and detected by ethidium bromide staining. The most-probable-number (MPN) value was calculated by using the MPN calculator available online (<http://www.i2workout.com/mcuriale/mpn/index.html>), recording the number of tubes positive for amplification for each dilution, and calculating the 95% confidence limits. The set up was repeated for the three replicates from different microcosms for each time point, standard deviations were calculated ($n = 3$), and the Student *t* test was applied.

DGGE. Denaturing gradient gel electrophoresis (DGGE) for amplicons was performed by using 6% polyacrylamide gels (ratio of acrylamide to bisacrylamide of 37:1) with 50 to 58% denaturant for 16S, 30 to 38% denaturant for 18S, and 80 to 87% denaturant for *napA* and 75 to 82% denaturant for *nirS*. We defined 100% denaturant as 7 M urea plus 40% formamide (1). Appropriate volumes containing about 2 µg of the purified PCR and RT-PCR products, measured by determining the absorbance at 260 nm, were loaded. The gels were electrophoresed at 60°C (50 V for 16S and 18S profiles and 110 V for *napA* and *nirS* profiles) for 17 h by using the D-Gene system (Bio-Rad Laboratories, Munich, Germany) and silver stained as described for polyacrylamide gels.

Dried acrylamide and DGGE gels were scanned by using an HP Scanjet 7400c. The profiles obtained were analyzed by clustering via the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) by using GelCompar II Software (Applied Maths, Kortrijk, Belgium). The position tolerance was set at 1%, and background subtraction was applied. Both strong and weak bands were included in the analysis, thus taking into account the presence or absence of bands at specific positions. Cophenetic correlations were calculated by using the same software.

RESULTS

Gas emissions. Figure 1 shows the mean concentrations of N₂O and CO₂ at the air space above the soil columns in nine microcosms after soil thawing. Emission of both of the gases started to increase immediately on exposure to thawing, although the increase was gradual on the first day and N₂O concentration was steeper than CO₂. The increase in N₂O concentration reached a peak (6,233 ppb) after 5 days of the start of thawing, whereas the CO₂ concentration still kept increasing. Sampling of soil was performed at three time points: at day 2 when the concentration of N₂O started increasing linearly, at day 3 during the exponential rise of the N₂O concentration, and at day 9 when the N₂O concentration had declined.

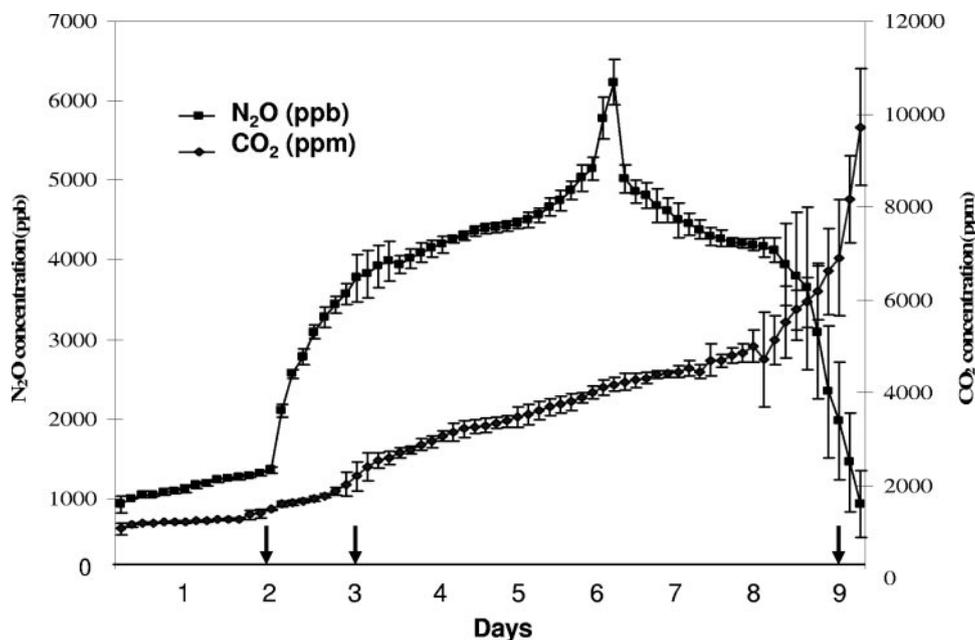


FIG. 1. Concentrations of N_2O and CO_2 in the air space above soil core in the microcosms. Arrows mark the points of sampling. Error bars represent standard deviation ($n = 9$).

Microbial biomass carbon and nucleic acid yield. Microbial biomass carbon levels were not significantly different between the three time points, with 225, 190, and 229 mg of C kg of dry soil⁻¹ at days 2, 3, and 9, respectively. Nucleic acid extraction efficiency was not affected by the freeze-thaw treatment since samples at all of the time points yielded a DNA concentration of approximately 250 ng/ μ l (± 20 ng/ μ l) and an RNA yield of approximately 210 ng/ μ l (± 12 ng/ μ l). No significant differences were observed between the replicates for each sampling.

AP-PCR and RAP-PCR profiles. Fingerprints of AP-PCR and RAP-PCR performed with a 10-mer primer revealed maximum number of bands in control soil compared to the other sampling points (Fig. 2). The numbers of bands for the other three time points were about half of those present in the control soil. Profiles derived from the nine replicates for each sampling point were more than 98% identical (data not shown). When the profiles were compared, two distinct clusters could be observed: one was formed by profiles derived from DNA, while the other was derived from cDNA, with only the profile derived from DNA of day 9 being separate from the two clusters. With both DNA- and cDNA-derived profiles, the second- and third-day profiles were more similar to each other (>80%). The control soil was related to the first two points by a similarity level of >65%. The ninth day was the most distant with respect to its similarity with other time points. This provided the initial evidence of differences in microbial community structure and function during the progress of freeze-thaw event in soil.

Bacterial and fungal community analysis. To look specifically at bacterial and fungal members, universal primers were used for amplification of rRNA genes and transcripts. The expected band could be obtained for all replicates. 16S and 18S amplicons derived from both DNA and cDNA were resolved on denaturing gradients of 50 to 58% and 30 to 38%, respec-

tively. High reproducibility (>95%) could be observed with nine replicate samples and also multiple nucleic acid extractions (data not shown) for both 16S and 18S profiles. The clustering of DNA-derived profiles was separate from those derived from cDNA in 16S and 18S fingerprints (Fig. 3 and 4). The cDNA-derived profiles were a subset of bands present in DNA-derived fingerprints. Although the number of bands was reduced in the cDNA profiles, major differences could be observed by the appearance of new bands in the same. The second- and third-day findings were >85% similar in 16S and 92% similar in 18S rRNA gene profiles. Trend remained the same with cDNA-derived fingerprints, although the levels of similarity were higher for eukaryotic profiles. The profiles derived from the ninth day were the most distinct and had a similarity level of only 70% to the other bacterial profiles. In contrast, for the 18S profiles the ninth day was >80% similar when DNA-derived profiles were compared and >90% similar with the fingerprints of the second and third days when we compared the cDNA-derived profiles. At the ninth day, a couple of new bands could be observed in both 16S and 18S profiles.

***napA* and *nirS* expression levels and diversity.** Desired products were obtained with both DNA and cDNA for *napA* and *nirS* with all of the replicate samples, but no RT-PCR product could be observed for *nirK* and *nosZ*. The MPN of *napA* genes per nanogram of DNA (as calculated by MPN-PCR) was not significantly different at the four sampling points, ranging from 3,400 gene copies/ng of DNA at the 9th day to 5,000 gene copies/ng of DNA in the control soil sample. For *nirS* the values ranged from 7,000 gene copies/ng of DNA at the ninth day to 10,000 gene copies/ng of DNA in the control soil sample, but these differences were not significant. However, MPN-PCR with cDNA (MPN-RT-PCR) revealed an increase in the number of transcripts of both *napA* and *nirS* at the day 2. In the

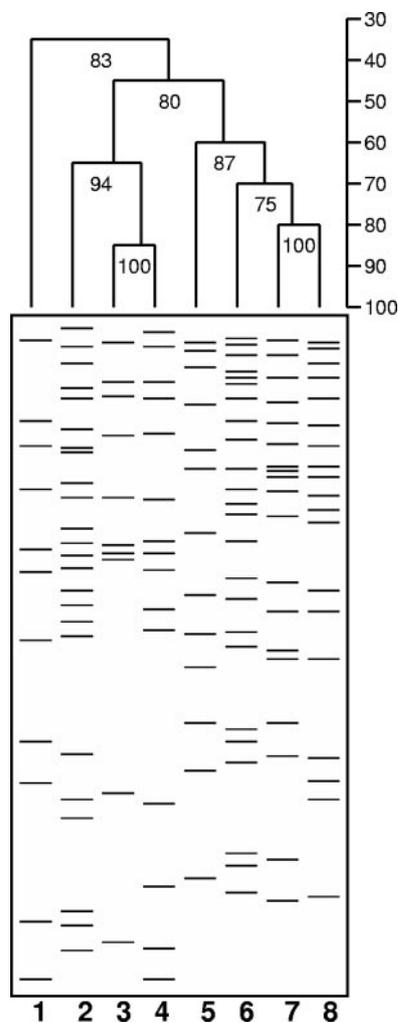


FIG. 2. Digitalized image of AP-PCR and RAP-PCR products resolved on nondenaturing polyacrylamide gel. Lanes: 1, 2, 3, and 4, DNA fingerprints of the time points 9 days, control, 2 days, and 3 days, respectively; 5, 6, 7, and 8, cDNA fingerprints of the time points 9 days, control, 2 days, and 3 days, respectively. A UPGMA tree representing the similarity of the microbial community structure and expression profiles of different time points obtained by AP-PCR and RAP-PCR using a 10-mer primer is shown. The scale represents the percent similarity. The numbers at the branch points indicate the cophenetic correlation value, which is a parameter that expresses the consistency of a cluster. See the supplemental material.

control soil, the MPNs of *napA* and *nirS* transcripts were about 6/ng of RNA and 4/ng of RNA, respectively. At day 2 the values showed 5- and 10-fold increases in the levels of transcripts of *napA* and *nirS*, with values of 31 and 41/ng of RNA, respectively. Again, at the third and ninth days the transcript levels decreased to values at an intermediate level between those of the control soil and the second day. There were no significant differences between the values of third and ninth days.

napA amplicons were resolved on DGGE gradient of 80 to 87%. With the DNA-derived profiles the bands initially decreased (at the second and third days) in intensity but at the ninth day two distinct bands could be observed (Fig. 5). With cDNA-derived patterns there was a decrease in complexity, during the process of thawing, that was observed by the reduc-

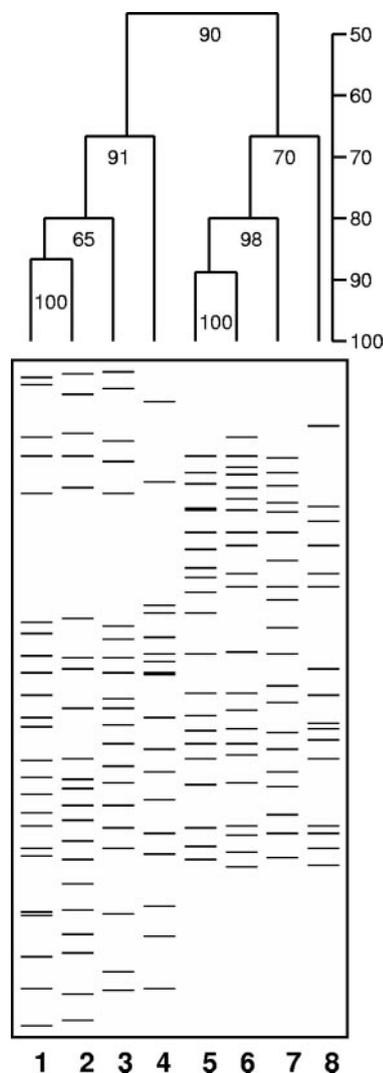


FIG. 3. Digitalized DGGE profiles of 16S rRNA genes and transcripts amplified from different time points. Lanes: 1, 2, 3, and 4, DNA fingerprints of the time points 2 days, 3 days, control, and 9 days, respectively; 5, 6, 7, and 8, cDNA fingerprints of the time points 2 days, 3 days, control, and 9 days, respectively. A UPGMA tree representing the similarity of the microbial community profiles obtained by amplification of 16S rRNA genes and transcripts from different time points is shown. The scale represents the percent similarity. The numbers at the branch points indicate the cophenetic correlation value, which is a parameter that expresses the consistency of a cluster. See the supplemental material.

tion in the number of bands from four in the control soil to two at the ninth day. The positions of the bands in the cDNA-derived profiles were similar to those in the DNA-derived ones. To analyze the *nirS* amplicons, a gradient of 75 to 82% was used. High reproducibility of fingerprints could be observed for the nine replicates of each sampling time for both *napA* and *nirS* (data not shown). The profiles derived from DNA were similar at all of the four time points, with only two bands (Fig. 5). However, the fingerprints of *nirS* derived from cDNA showed up to a maximum of 12 bands, with the bands common to those present in DNA-derived profiles coming up only at the ninth day. Unlike the *napA* profiles, in this case

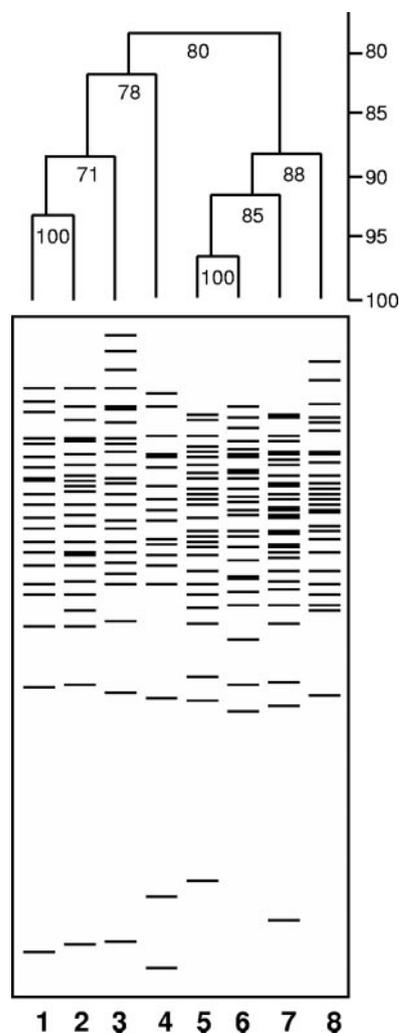


FIG. 4. Digitalized DGGE profiles of 18S rRNA genes and transcripts amplified from different time points. Lanes: 1, 2, 3, and 4, DNA fingerprints of the time points 2 days, 3 days, control, and 9 days, respectively; 5, 6, 7, and 8, cDNA fingerprints of the time points 2 days, 3 days, 9 days, and control, respectively. A UPGMA tree representing the similarity of the microbial community profiles obtained by amplification of 18S rRNA genes and transcripts from different time points is shown. The scale represents the percent similarity. The numbers at the branch points indicate the cophenetic correlation value, which is a parameter to express the consistency of a cluster. See the supplemental material.

complexity (according to the number of bands in the profiles) increased in the cDNA-derived patterns from the control soil to the ninth day. The profile from the control soil had only eight bands. As N_2O emission continued, the number of intense bands increased, and at the ninth day 12 bands could be observed.

DISCUSSION

The exponential rise in N_2O emission on thawing, after a brief period of slow increase, has been a regular observation in other studies even if undisturbed soil cores were used (18, 33). This observation confirms the fact that soil homogenization, as done in the present study, does not affect the process of N_2O

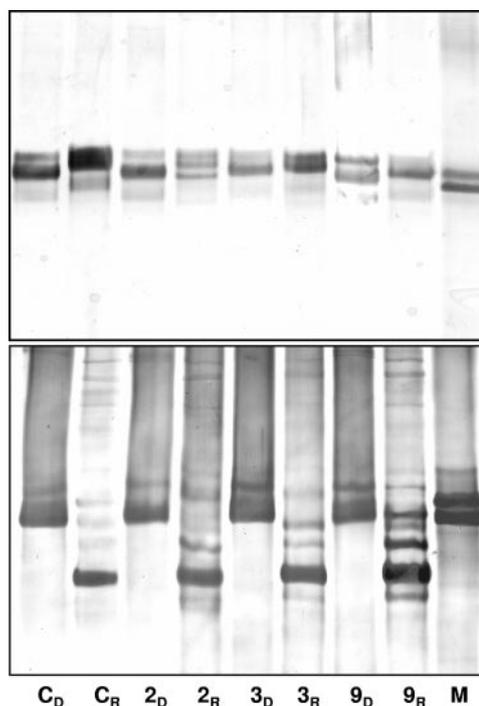


FIG. 5. DGGE profiles showing the diversity of *napA* (above) and *nirS* (below) at the four time points. The 2, 3, and 9 stand for the sampling days as in Fig. 1, and the C stands for control soil. D and R, profiles derived from DNA and RNA, respectively. M stands for the reference lane with genes amplified from pure cultures.

emission when frozen soil thaws. Even when the N_2O concentration started declining, the CO_2 concentration kept rising, indicating increased microbial activity. Koponen et al. (18) showed that this trend is independent of soil type.

Only a couple of studies have analyzed microbial community dynamics during the freeze-thawing of soil. This has been restricted mainly to the measurement of microbial biomass. Although some reports mention a decrease in microbial biomass or cell numbers after thawing (17, 19, 25), we observed no such decrease after sample exposure to freeze-thawing. Also, no significant difference was observed between the three time points. In fact, an increasing number of reports have indicated that no effect on microbial biomass could be observed after this process (12, 22). Although the microbial biomass remained unaffected, changes in microbial community structure could be observed. Fewer bands were obtained for the three sampling points compared to control soil in both RAP and DGGE fingerprints. This could be attributed to a decrease in microbial members that is counterbalanced by the proliferation of survivors. As observed by both 16S and 18S fingerprinting, it was the ninth day that was distinct from the other three. This points to an ongoing selection process, as a result of freeze-thawing, on the microbial communities. Schimel and Mikan (30) reported a change in substrate use representing a shift from processing N-poor detritus to N-rich microbial products, causing N that is available for either plant uptake or leaching to be at the highest level when soils are near $0^\circ C$ in Arctic soils. Using ribosomal intergenic spacer length analysis, Eriksson et al. (9) reported differences in the banding pattern of soil samples

exposed to freeze-thaw, while other soil samples that were continuously incubated at different constant temperatures showed profiles similar to each other. In the present study, fingerprinting revealed that the stability of microbial communities is in the following order: fungi > bacteria > total DNA and RNA. The high stability of the fungal community could be due to (i) fungal tolerance to temperature change; (ii) less diversity of fungal members, which did not reveal a big community shift; and/or (iii) the dominance of bacteria due to their ability to metabolize the newly mobilized organic substrates as a result of the freeze-thaw. The highest variability was observed in the profiles derived from total DNA and RNA (45% similarity between the DNA- and cDNA-derived clusters; 35% similarity of the profile derived from DNA of the ninth day to the main cluster). This can be accounted for by the fact that the total nucleic acid fingerprinting is not restricted to genes for rRNA only. In fact, it has been shown that ca. 20% of the RAP-PCR products originate from mRNA (2) and are more sensitive to changes in the microbial communities.

Various studies have reported the emission of N₂O by soil thawing to be microbial in origin. Öquist et al. (24) reported the source of N₂O to be denitrification occurring in anoxic microsites in frozen soil, before thawing. However, there have been no reports correlating gas emissions to the diversity and quantity of denitrifying genes and transcript during the freeze-thaw process. We observed 5- and 10-fold increases in the transcript levels of *napA* and *nirS*, respectively, 1 day after soil thawing. This was the time point where N₂O emission had just started to rise exponentially. Ludwig et al. (20) estimated that immediately after the beginning of thawing, denitrification contributed to 83% of N₂O emission. The transcript levels started decreasing at the third and ninth days. Although no housekeeping gene was evaluated to confirm that the changes in the transcripts were because of changed expression levels, similar nucleic acid yield and gene copy numbers observed by MPN-PCR support an effect on the expression of the denitrifying genes. In the present study, sampling was performed only from the top 0 to 5 cm of the soil column since this is the zone most prone to changes in the environment.

The presence of fewer bands in DNA-derived profiles compared to the cDNA-derived ones indicates that the most dominant denitrifiers are not the most active ones. More bands could be observed at the ninth day compared to the other three sampling points, which can be explained by the growth of surviving members. Thawing led to changes both in microbial community structure (as evidenced by changes in the AP-PCR of total DNA and the DGGE profiles of 16S and 18S rRNA) and in function (as evidenced by differences in the profiles and transcript copies of *napA* and *nirS*).

nirK, which has been found to be abundant in soil (26, 27, 34), could be detected in the present study only at the level of DNA. Although there have been reports on detection of the expression of *nirK* (23, 32), these studies have been performed on high microbial activity zones, e.g., sediment from estuary and rhizospheres. The system used here is also a site of high microbial activity, but the difference in habitat could have resulted in either a low level of expression that is below the detection limit by RT-PCR or in no expression at all. No *nosZ* (coding for nitrous oxide reductase) transcript could be detected in the system, although amplification with DNA as a

template yielded the expected amplicon. The accumulation of N₂O in the system indicates either a very low or no expression of *nosZ*, which is needed for further conversion of N₂O to N₂, even though it is present in the gene pool. Dendooven and Anderson (7) reported a low persistence of N₂O reductase in combination with its retarded derepression, resulting in a high N₂O-to-N₂ ratio when anaerobic conditions are rapidly induced. It is known that in the presence of labile organic carbon more N₂O is produced than N₂ (21).

The present study is the first report linking the commonly observed phenomenon of N₂O and CO₂ emission, after the thawing of frozen soil, to the structural and functional diversity of microbial communities in the freshly thawed soil zone. It is clear from our study that freeze-thawing increased microbial activity (as evidenced by the increasing CO₂ production) and the expression of denitrifying genes, which lead to the release of N₂O gas. This supports the view that the flush of N₂O and CO₂ is a result of increased microbial activity during the dynamic event of freeze and thaw (5, 6, 16, 24, 33), instead of mere entrapment of gases due to the freeze barrier. However, this was a microcosm experiment under controlled conditions, and the freeze-thaw process was laboratory simulated. As such, this process does not resemble the natural process of gradual freezing and thawing from top to depth, and so similar studies need to be conducted under natural conditions. Also, more frequent sampling during the course of N₂O emission would further clarify the change in expression of the denitrifying genes.

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