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ORIGINAL ARTICLE

Contrasting denitrifier communities relate to contrasting N_2O emission patterns from acidic peat soils in arctic tundra

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Cryoturbated peat circles (that is, bare surface soil mixed by frost action; pH 3–4) in the Russian discontinuous permafrost tundra are nitrate-rich 'hotspots' of nitrous oxide (N_2O) emissions in arctic ecosystems, whereas adjacent unturbated peat areas are not. N_2O was produced and subsequently consumed at pH 4 in unsupplemented anoxic microcosms with cryoturbated but not in those with unturbated peat soil. Nitrate, nitrite and acetylene stimulated net N_2O production of both soils in anoxic microcosms, indicating denitrification as the source of N_2O. Up to 500 and 10 μM nitrate stimulated denitrification in cryoturbated and unturbated peat soils, respectively. Apparent maximal reaction velocities of nitrite-dependent denitrification were 28 and 18 nmol $N_2O\ g_{DW}^{-1}\ h^{-1}$, for cryoturbated and unturbated peat soils, respectively. Barcoded amplicon pyrosequencing of *narG*, *nirK/nirS* and *nosZ* (encoding nitrate, nitrite and N_2O reductases, respectively) yielded ≈49 000 quality-filtered sequences with an average sequence length of 444 bp. Up to 19 species-level operational taxonomic units were detected per soil and gene, many of which were distantly related to cultured denitrifiers or environmental sequences. Denitrification-associated gene diversity in cryoturbated and in unturbated peat soils differed. Quantitative PCR (inhibition-corrected per DNA extract) revealed higher copy numbers of *narG* in cryoturbated than in unturbated peat soil. Copy numbers of *nirS* were up to 1000 × higher than those of *nirK* in both soils, and *nirS nirK*$^{-1}$ copy number ratios in cryoturbated and unturbated peat soils differed. The collective data indicate that the contrasting N_2O emission patterns of cryoturbated and unturbated peat soils are associated with contrasting denitrifier communities.
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Introduction

Nitrous oxide (N_2O) is a major ozone-depleting substance in the atmosphere and the third most important greenhouse gas on earth (Forster *et al.*, 2007; Ravishankara *et al.*, 2009). The global warming potential of N_2O is 300-fold higher than that of CO_2 on a 100-year basis, and the atmospheric concentration of N_2O increased from 270 to 319 ppb from 1750 to 2005 (Forster *et al.*, 2007). Agricultural and pristine tropical soils are well-recognized major sources of N_2O, whereas the importance of arctic peatlands as sources of N_2O is just emerging (Denman *et al.*, 2007; Repo *et al.*, 2009; Marushchak *et al.*, 2011).

Areas of bare surface soil mixed by frost action in acidic tundra (pH 3–4) are termed 'cryoturbated peat circles', and emit N_2O at rates documented for tropical and agricultural soils (Maljanen *et al.*, 2007; Werner *et al.*, 2007; Repo *et al.*, 2009). The estimated global N_2O emission from cryoturbated peat circles is ∼0.1 Tg N_2O per year, which is equivalent to 4% of the global warming potential of the arctic methane emissions and to 0.6% of the total global annual N_2O emission (Christensen, 1993; Denman *et al.*, 2007; Repo *et al.*, 2009). Vegetation is absent from ∼12% of the area in the arctic, including cryoturbated peat circles (Walker *et al.*, 2005). Nitrate concentrations approximate 2 mM in the pore water of such unvegetated cryoturbated peat soil, and are ∼1000 × higher than in adjacent vegetated unturbated peat areas where N_2O emissions are negligible (Repo *et al.*, 2009). Repeated freezing and thawing of the cryoturbated soil leads to breakdown of soil aggregates, renders decomposable organic carbon more easily accessible to microbes and may thereby activate the microbial

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community including N_2O producers (Mørkved *et al*., 2006; Sharma *et al*., 2006). Thus, cryoturbated peat circles represent acidic 'hotspots' of microbial N_2O emission in the tundra (Repo *et al*., 2009; Marushchak *et al*., 2011).

The main source of N_2O in water-logged anoxic soils including peatlands is denitrification (Conrad, 1996; Pihlatie *et al*., 2004; Palmer *et al*., 2010). Complete denitrification is the sequential reduction of nitrate or nitrite to dinitrogen (N_2) through nitric oxide (NO) and N_2O; nitrite is likewise an intermediate when nitrate is used (Zumft, 1997). The oxidoreductases involved in denitrification include dissimilatory nitrate reductases encoded by *narG* or *napA*, copper- and cytochrome cd_1-containing nitrite reductases (encoded by *nirK* and *nirS*, respectively), NO reductases encoded by *norBC* and N_2O reductases encoded by *nosZ* (Zumft, 1997). Nitrate reductases likewise occur in dissimilatory nitrate reducers (Stolz and Basu, 2002). NirK and NirS are structurally different but functionally equivalent (Jones *et al*., 2008). Organisms hosting both types of nitrite reductase are unknown to date (Heylen *et al*., 2006). The genes coding for the above-named oxidoreductases are commonly used as structural gene markers for the analysis of nitrate reducer and denitrifier communities (Braker *et al*., 2000; Philippot *et al*., 2002; Prieme *et al*., 2002; Rich *et al*., 2003; Horn *et al*., 2006; Enwall *et al*., 2010; Jones and Hallin, 2010; Palmer *et al*., 2010; Bru *et al*., 2011). The main products of denitrification that are released into the atmosphere are N_2 or N_2O. Denitrifiers might lack nitrate reductases and/or N_2O reductases, and occupy diverse ecological niches (Tiedje, 1988; Zumft, 1997; Shapleigh, 2006). Denitrification rates and the product ratio of N_2O to N_2 are regulated by the denitrifying community and *in situ* conditions (for example, pH, temperature, C-to-N-ratio, as well as the availability of substrates and electron acceptors; van Cleemput, 1998). Acidic $\mathrm{pH} < 5$ impairs denitrification and increases the product ratio of N_2O to N_2 (Simek and Cooper, 2002; Cuhel *et al*., 2010). The increased product ratio of N_2O to N_2 is likely caused by post-transcriptional effects of low pH on N_2O reductase formation (Liu *et al*., 2010). However, information on denitrifier communities that thrive at $\mathrm{pH} < 5$ in peatlands is scarce (Palmer *et al*., 2010).

Denitrifier communities in permafrost-affected acidic tundra soils are unresolved to date, despite the fact that such soils are prone to react sensitively to global warming, which might accelerate cryoturbation and in turn increase N_2O emissions (Bockheim, 2007; Repo *et al*., 2009). It is hypothesized that the observed contrasting N_2O emission patterns of cryoturbated and unturbated acidic peat soil are associated with contrasting denitrifier communities. The main objectives of this study were (1) to compare ecophysiological traits (that is, capacities) of acid-tolerant denitrifier communities in cryoturbated and unturbated peat soils, (2) to develop pyrosequencing-based strategies for in-depth analysis of denitrifier communities by parallel analysis of multiple denitrification-associated genes, (3) to determine whether contrasting and new denitrifier communities occur in cryoturbated and unturbated peat soils by such pyrosequencing-based strategies and quantitative PCR and (4) thus to identify potential microbial catalysts of the exceptionally high N_2O emissions from cryoturbated peat soil.

Materials and methods

Site description and soil sampling

The sampling area is located in the Russian discontinuous permafrost zone (62°57′E, 67°03′N) and was described previously (Repo *et al*., 2009; Supplementary Materials and methods). Cumulative N_2O emissions in the field from the cryoturbated soil are $1.2 \pm 0.3\,\mathrm{g\ N_2O\,m^{-2}}$, whereas those of the unturbated soil are negligible ($< 0.006\,\mathrm{g\ N_2O\,m^{-2}}$). Topsoil was identified as the site of highest N_2O production in the peat profile (data not shown), and the upper 5 cm was sampled from three cryoturbated peat circles and three adjacent, unturbated areas in September 2010. Roots were removed from unturbated soil, and soil for microcosm studies was stored at 4 °C until further processing. Soil for DNA extraction was suspended in RNAlater (Qiagen, Hilden, Germany) immediately after sampling to avoid decomposition of nucleic acids, and stored at −20 °C upon arrival at the laboratory. Experiments were conducted within 2 months after sampling. Moisture content was determined by weighing the soil before and after drying at 60 °C for 3 days and was 71% and 81% in cryoturbated and unturbated peat soils, respectively.

Assessment of denitrification in soil microcosms

Soil of the three replicate sampling sites was homogenized and pooled before microcosm experiments. Soil slurries at *in situ* $\mathrm{pH_{H2O}}$ of ~ 4 were prepared with 4–5 g of soil and 3 volumes of deionized water in 125-ml infusion flasks, and sealed using gas-tight rubber stoppers. The gasphase was 100% argon. Microcosms were incubated at 20 °C in the dark and performed in triplicate unless stated otherwise.

Acetylene blocks N_2O reductases and thus the reduction of N_2O to N_2 (Yoshinari and Knowles, 1976). Parallel microcosms with and without acetylene (15% (vol/vol) in headspace) were used to differentiate between total denitrification and N_2O-production potentials. Total denitrification stopped after ~ 4 days (90 h) in unsupplemented microcosms with cryoturbated peat soil and acetylene, indicating that internal nitrate and nitrite were depleted (Figure 1a).

For apparent Michaelis–Menten kinetics, soil was pre-incubated for 7 days under anoxic conditions to

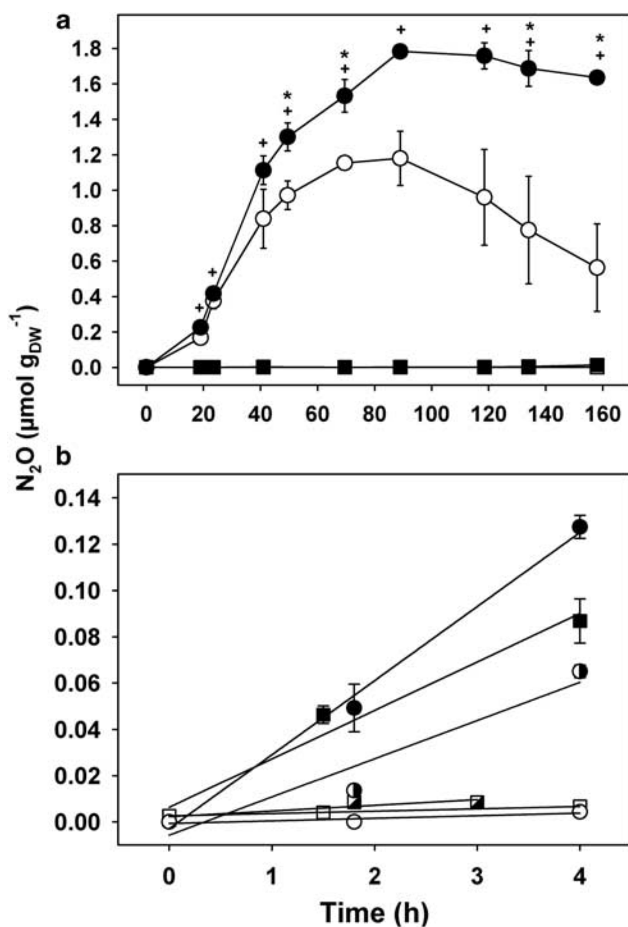


Figure 1 (**a**) Denitrification and effect of acetylene on the production and consumption of N_2O in anoxic microcosms with unsupplemented peat soil. Squares and circles represent unturbated and cryoturbated peat soils, respectively. Closed and open symbols represent microcosms with and without acetylene, respectively. Time points at which N_2O concentrations in cryoturbated peat soil with acetylene differed significantly ($P<0.05$) from N_2O concentrations in cryoturbated peat soil without acetylene or in unturbated peat soil with acetylene are indicated with (*) or (+), respectively. (**b**) Effect of 100 µM nitrite or nitrate on the production of N_2O in anoxic microcosms with nitrate-depleted peat soil in the presence of acetylene. Squares and circles represent unturbated and cryoturbated peat soils, respectively. Closed symbols represent microcosms supplemented with nitrite, half-filled symbols represent microcosms supplemented with nitrate, and open symbols represent unsupplemented controls. Solid lines represent linear regression curves ($R^2 = 0.8$–0.99). Mean values and s.e. of three replicate microcosms are shown in panels **a** and **b**.

deplete internal nitrate and nitrite. Such soil was supplemented with 0–500 µM $NaNO_3$ or $NaNO_2$. N_2O did not accumulate in anoxic microcosms containing 1 mM nitrite in sterile water at pH 4 within 2 days (data not shown). Apparent Michaelis–Menten kinetics were based on the production of N_2O in the presence of acetylene as described previously (Segel, 1993; Palmer *et al.*, 2010; Supplementary Materials and methods). Soil that was pre-incubated under anoxic conditions for 9 days was used to study the effects of the electron donors acetate, ethanol, formate, propionate, butyrate and lactate on denitrification in microcosms supplemented with 1 mM nitrite and 0.5 mM electron donors in the presence of acetylene. After another 47 days of anoxic incubation, 1 mM nitrite and 2 mM electron donors (0.5 mM for propionate only) were resupplied. N_2O production, nitrite and electron donors were determined regularly after initial supplementation and after resupplementation. N_2O production rates were calculated from 3 to 4 data points determined within 8–25 h after addition of substrates (nitrite and/or electron donors) when N_2O production was linear. R^2-values of the linear regressions were always >0.88.

Concentrations of electron donors were assessed by high-performance liquid chromatography, and nitrate as well as nitrite by ion chromatography (Palmer *et al.*, 2010; Supplementary Materials and methods).

Extraction of nucleic acids, and amplification of narG*,* nirK*,* nirS *and* nosZ

Nucleic acids were extracted from triplicate cryoturbated and unturbated peat soil samples to account for lateral heterogeneity in microbial communities. A bead-beating protocol tailored for the efficient removal of PCR-inhibiting humic acids by aluminum sulfate precipitation before cell lysis was applied (Peršoh *et al.*, 2008; Supplemental Materials and methods).

narG, *nirK*, *nirS* and *nosZ* were amplified using the primer pairs narG1960f (TAYGTSGGSCARGARAA)/narG2650r (TTYTCRTACCABGTBGC; Philippot *et al.*, 2002), F1aCu (ATCATGGTSCTGCCGCG)/R3Cu (GCCTCGATCAGRTTGTGGTT; Throbäck *et al.*, 2004), cd3aF (GTSAACGTSAAGGARACSGG)/R3cd (GASTTCGGRTGSGTCTTGA; Throbäck *et al.*, 2004) and nosZF (CGCTGTTCITCGACAGYCAG)/nosZR (ATGTGCAKIGCRTGGCAGAA; Rich *et al.*, 2003), respectively. Each primer was preceeded by a 6-bp-long barcode (AGCGTC for unturbated and ATATAC for cryoturbated soil samples) to separate sequences after pyrosequencing. In all, 8 replicate 25 µl PCR reactions per target gene were performed at 8 different annealing temperatures from 54.7 to 63.6 °C to maximize the likelihood of detecting a high diversity of target genes. All replicate PCR reactions that yielded products (that is, amplicons) of the correct size were pooled before subsequent analyses. For detailed PCR protocols, refer to Supplementary Materials and methods.

Barcoded amplicon pyrosequencing of structural genes

Previously published amplicon pyrosequencing strategies (Huber *et al.*, 2007; Iwai *et al.*, 2010; Will *et al.*, 2010) were modified to maximize the likelyhood of specific amplification of denitrification-associated structural genes during amplicon generation. Pyrosequencing requires amplicons

fused with sequencing adaptors. Published strategies use target gene-specific primers fused with a barcode and an ∼30-bp-long sequencing adaptor, resulting in primers with >50% of the sequence being not complementary to the target genes, and thus allowing for unspecific amplifications. In this study, amplicons were generated during PCR with target gene-specific primers fused with the barcode only (see above) rather than using primers that contain barcode and sequencing adaptors. Sequencing adaptors were ligated after PCR to gel-purified amplicons.

Amplicons of similar lengths of both soil types were combined in equal amounts (that is, *narG* and *nosZ* amplicons were pooled, as well as *nirK* and *nirS*). Amplicon mixtures were treated with PreCR Repair Mix (New England Biolabs, Frankfurt am Main, Germany) to eliminate possible PCR-blocking DNA damage that might have occurred during gel purification or storage of amplicons, and purified through isopropanol precipitation. Sequencing from 5′ (forward) and 3′ (reverse) ends of amplicons was performed after ligation of A (CGTATCGCCTCCCTC GCGCCATCAG) and B (CTATGCGCCTTGCCAGCCC GCTCAG) sequencing adaptors at the Göttingen Genomics Laboratory using the Roche GS-FLX 454 pyrosequencer and GS FLX Titanium series reagents (Roche, Mannheim, Germany) according to the manufacturer's instructions.

Sequence filtering and analysis
Sequences with ambiguities, and those with incorrect primer or barcode sequences were discarded. *narG* and *nosZ* sequences shorter than 350 bp, and *nirK* as well as *nirS* sequences shorter than 300 bp were likewise excluded from further analyses. Amplicon sequences were sorted according to their barcodes and primers, and combined subsets of sequences for each structural gene (that is, containing sequences from both cryoturbated and unturbated peat soils) were clustered (that is, assigned to operational taxonomic units (OTUs)) at species-level threshold distances of 33% (*narG* (Palmer *et al*., 2009)), 17% (*nirK* (PS Depkat-Jakob, HL Drake, MA Horn, personal communication)), 18% (*nirS* (PS Depkat-Jakob, HL Drake, MA Horn, personal communication)) or 20% (*nosZ* (Palmer *et al*., 2009)) based on DNA sequences using the JAguc2 pipeline (http://wwwagak.informatik.uni-kl.de/research/JAguc/; Nebel *et al*., 2011; Supplementary Figure S1). In brief, JAguc2 generates a pairwise sequence alignment before calculation of a distance matrix and clustering with the average similarity method. This approach is more reliable (that is, less sensitive to PCR and pyrosequencing noise, and thus less sensitive to an artifical inflation of diversity (Kunin *et al*., 2010)) than multiple alignments and/or clustering with complete linkage algorithms (Quince *et al*., 2009; Sun *et al*., 2009; Huse *et al*., 2010). Amplicon sequences obtained by pyrosequencing from defined template mixtures were essentially at most 10% dissimilar to template sequences due to PCR and pyrosequencing noise (Behnke *et al*., 2011; Quince *et al*., 2011). The threshold distances used to call OTUs in this study were 17–33%, which is substantially greater than the above-reported maximal PCR and pyrosequencing noise (Supplementary Figure S1). Thus, our approach was rather unaffected by PCR and pyrosequencing noise, although we did not apply flowgram-based
sequence correction algorithms for pyrosequencing (as implemented in for example, AmpliconNoise; Quince *et al*., 2011). Clustering can be easily redone with JAguc2 at different threshold distances without the need for time-consuming re-calculation of the distance matrix to test the effect of threshold distance on the number of OTUs (Supplementary Figure S1). Rarefaction curves were generated for each sequence set using aRarefact (http://www.huntmountainsoftware.com/html/rarefaction.html) as part of a strategy to further minimize the effect of pyrosequencing noise on comparative diversity analyses (Dickie, 2010). The closest relatives of OTU representatives were determined using BLAST (Altschul *et al*., 1990). OTU representatives were exported from JAguc2, edited, translated *in silico* and aligned with reference sequences using the ClustalW algorithm implemented in MEGA 5.0 (Kumar *et al*., 2008). The alignments were refined manually, and phylogenetic trees were constructed with the neighbor-joining algorithm using *p*-distances from *in silico*-translated sequences with MEGA 5.0. The stability of tree topologies was assessed by calculating 10 000 bootstrap replicates (Saitou and Nei, 1987). Diversity measures with 95% confidence intervals were calculated as described previously (Sørensen, 1948; Bray and Curtis, 1957; Hill *et al*., 2003; Zaprasis *et al*., 2010). Normalized weighted UniFrac significance was calculated to evaluate differences between the communities of *narG*, *nirK*, *nirS* and *nosZ* based on phylogenetic information (Lozupone and Knight, 2005; Lozupone *et al*., 2007).

Quantification of narG*,* nirK*,* nirS*,* nosZ *and 16S rRNA genes in soil*
Quantitative kinetic real-time PCRs were performed as described with DNA extracts from three replicate cryoturbated and three unturbated sites in six technical replicates per DNA extract (Zaprasis *et al*., 2010; Supplementary Materials and methods). Thermal protocols and primers were as described previously (Supplementary Materials and methods, Table 1). Melting-curve analyses, agarose gel electrophoresis and sequencing of amplicons generated with the same primers indicated that the amplification was specific. The lower limits of quantification were $\leqslant 10^1$ gene copy numbers μl^{-1} of DNA extract. 16S rRNA gene copy numbers were determined concomitantly for all environmental samples to

Table 1 Thermal protocols for qPCR of *narG*, *nirK*, *nirS*, *nosZ* and 16S rRNA genes

Primer set	*Temperature (°C)/time (min)*				
	narG1960f/ narG2650r[a]	*F1aCu/ R3Cu*[b]	*cd3aF/ R3cd*[b]	*nosZF/ nosZR*[c]	*Eub341F/ Eub534R*[d]
Initial denaturation	95/10	95/10	95/10	95/10	95/10
Denaturation	95/0.75	95/1	95/0.5	95/0.5	95/0.5
Annealing	64/0.75	55/1	58.5/0.5	63/0.5	55.7/0.4
Elongation	72/1.3	72/1.7	72/0.5	72/0.75	72/0.4
Recording of fluorescence	80/0.3	83.5/0.3	80/0.3	(72)[e]	(72)[e]
No. of cycles	40	50	40	35	35
Final elongation	72/5	72/5	72/5	72/5	72/5

Abbreviation: qPCR, quantitative kinetic real-time PCR.
[a]Philippot *et al.*, 2002.
[b]Throbäck *et al.*, 2004.
[c]Rich *et al.*, 2003.
[d]Muyzer *et al.*, 1993.
[e]Fluorescence recorded during elongation step.

quantify microorganisms that harbor genes associated with denitrification in soils relative to the total bacterial population (Muyzer *et al.*, 1993; Zaprasis *et al.*, 2010). Inhibition of quantitative kinetic real-time PCR was assessed per individual DNA extract according to Zaprasis *et al.* (2010) by spiking soil DNA with pure standard DNA. Please refer to Supplementary Materials and methods for further details.

Statistical analyses
Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Mean differences between cryoturbated and unturbated peat soils and differences in slopes of linear regression curves were tested using a two-tailed *t*-test. The correlation between N_2O to total N gases was analyzed with Spearman's rank correlation. Non-linear regressions for apparent Michaelis–Menten kinetics and the resulting v_{max} were compared with a sum-of-squares F test.

Nucleotide sequence accession numbers
The OTU representatives of *narG*, *nirK*, *nirS* and *nosZ* gene sequences derived from barcoded amplicon pyrosequencing were deposited in EMBL under accession numbers FR865777 to FR865864. Complete amplicon sequence libraries were deposited in the ENA Short Read Archive under submission number ERA062401.

Results

Denitrification activities in cryoturbated and unturbated peat soils
Unsupplemented cryoturbated peat soil but not unturbated peat soil produced N_2O under anoxic conditions without apparent delay (Figure 1a). pH approximated 4 in microcosms with cryoturbated and unturbated peat soils. After 49.5 h of incubation, N_2O concentrations were significantly higher ($P<0.04$) in anoxic microcosms with cryoturbated peat soil when N_2O-reductase was blocked by acetylene than in those without acetylene (Figure 1a). Approximately 1.8 μmol $N_2O\ g_{DW}^{-1}$ accumulated and remained constant after 90 h in acetylene-treated microcosms with cryoturbated peat soil, indicating that soil endogeneous nitrate and nitrite were depleted (Figure 1a). Cryoturbated peat soil contained 4.8 μmol $NO_3^-\ g_{DW}^{-1}$ before anoxic incubation. Nitrate was below the detection limit (that is, <1.5 μmol $NO_3^-\ g_{DW}^{-1}$) after 90 h of incubation, and 75% of the initially present NO_3^--N was recovered in N_2O. In cryoturbated peat soil microcosms without acetylene, up to 1.2 μmol $N_2O\ g_{DW}^{-1}$ accumulated within the first 90 h (Figure 1a); N_2O decreased linearly to 0.54 μmol g_{DW}^{-1} within the next 70 h. Nitrate was below the detection limit in unturbated peat soil. N_2O did not accumulate in anoxic unturbated peat soil microcosms without acetylene, and only minor amounts of N_2O accumulated in the presence of acetylene (<0.014 μmol g_{DW}^{-1} within 160 h; Figure 1a).

Effect of supplemental nitrate and nitrite on denitrification
Supplemental nitrate (10 μM) significantly stimulated the production of N_2O in anoxic microcosms with unturbated (4.5×10^{-4} and 2.1×10^{-2} μmol $N_2O\ g_{DW}^{-1}\ h^{-1}$ for unsupplemented and nitrate supplemented unturbated peat soils; $P=0.001$) but essentially not with cryoturbated peat soil, indicating that denitrifiers in cryoturbated peat soil were apparently saturated with soil endogenous nitrate (data not shown). However, in anoxic microcosms with nitrate-depleted peat soils, 100 μM of supplemental nitrate significantly stimulated the production of N_2O by cryoturbated peat soil without apparent delay ($P=0.04$ when N_2O production rates were compared; Figure 1b), whereas stimulation of N_2O production by 100 μM of nitrate was unexpectedly not significant in microcosms with unturbated

peat soil ($P = 0.273$). The latter finding provides first evidence for substrate inhibition of unturbated peat soil denitrifiers by high nitrate concentrations (see below and Figure 2). Nitrite significantly stimulated the production of N_2O without apparent delay in microcosms with cryoturbated ($P = 0.02$) and unturbated ($P = 0.03$) peat soils (Figure 1b). Stimulation of N_2O production was higher with nitrite than with nitrate in cryoturbated ($P = 0.04$) and unturbated peat soils ($P = 0.06$) when N_2O production rates were compared. N_2O concentrations were similar after 1–2 h of incubation in anoxic cryoturbated and unturbated peat soil microcosms containing the same supplement, demonstrating similar denitrification potentials in both soils when 100 μM nitrite as electron acceptor for denitrification was supplied (Figure 1b).

Initial nitrite-dependent N_2O production rates of microcosms with cryoturbated and unturbated peat soils and nitrate-dependent N_2O production rates of microcosms with cryoturbated peat soil followed apparent Michaelis–Menten kinetics, whereas nitrate-dependent N_2O production rates of microcosms with unturbated peat soil did not (Figure 2). N_2O production rates were up to 4 times greater in microcosms with unturbated peat soil containing 10 μM supplemental nitrate than in those containing 20–500 μM supplemental nitrate (pairwise *t*-test of N_2O production rates for 10 μM and rates for 0, 20, 50, 100 and 500 μM nitrate yielded *P*-values of <0.001, 0.001, 0.001, <0.001 and <0.001, respectively), suggesting that unturbated peat soil denitrifiers are saturated with 10 μM nitrate and subjected to substrate inhibition by higher nitrate concentrations (Figure 2). In contrast, N_2O production plateaued out in microcosms with cryoturbated peat soil when supplemental nitrate concentrations were $\geqslant 50$ μM (Figure 2). Apparent maximal reaction velocities (v_{max}) were higher for nitrite than for nitrate in cryoturbated peat soil microcosms ($P < 0.001$), and higher in cryoturbated than in unturbated peat soil microcosms ($P = 0.001$; Table 2). Apparent Michaelis–Menten constants (K_M) for nitrite were lower in cryoturbated than in unturbated peat soil microcosms ($P = 0.05$; Table 2).

The ratio of N_2O to total N gases (that is, N_2 plus N_2O) after 8 h of anoxic incubation was below 30% for 10 μM nitrate or nitrite in microcosms with cryoturbated peat soil and increased with increasing concentrations of nitrate and nitrite (Spearman's correlation coefficients of 1.0 and 0.9, respectively; Supplementary Figure S2). In unturbated peat soil microcosms, the ratio of N_2O to total N gases approximated 100% for all supplied concentrations of nitrate or nitrite. Such data suggest a higher N_2O consumption potential of cryoturbated relative to unturbated peat soil denitrifiers for low concentrations of electron acceptors.

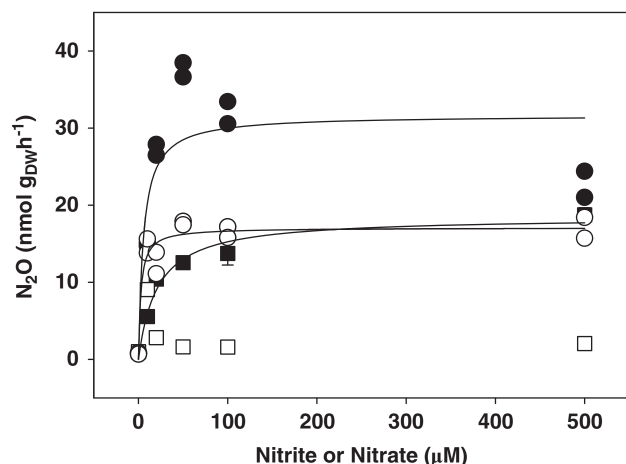


Figure 2 Apparent Michaelis–Menten kinetics of nitrate- and nitrite-dependent denitrification in anoxic microcosms with peat soil in the presence of acetylene. Squares and circles represent unturbated and cryoturbated peat soils, respectively. Closed and open symbols represent microcosms supplemented with nitrite and nitrate, respectively. Mean values and s.e. of three replicate microcosms are shown for unturbated peat soil; individual values of duplicate microcosms are shown for cryoturbated peat soil. Solid lines indicate Michaelis–Menten curves fitted to the data.

Anaerobic fermentation activities and trophic links to denitrifiers

Organic acids were not detectable in cryoturbated peat soil after anoxic pre-incubation to deplete nitrate and nitrite (0 h, Supplementary Figure S3a). In unsupplemented, nitrate-depleted anoxic microcosms with cryoturbated peat soil, only trace amounts of formate were transiently produced, and up to 0.1 mM of lactate accumulated within 58 days of incubation (that is, 9 days of preincubation plus 49 days of treatment; Supplementary Figure S3a). In contrast, ~0.7 mM of acetate and trace amounts of formate, propionate, butyrate and lactate were produced in unsupplemented anoxic microcosms with unturbated peat soil during the 9 days of pre-incubation (0 h, Supplementary Figure S3a). Up to 1.7 mM of acetate, and 0.1 to 0.2 mM of propionate, butyrate and lactate accumulated within 58 days of incubation (that is, 9 days of preincubation plus 49 days of treatment; Supplementary Figure S3a). Formate was below the detection limit after 58 days of incubation, indicating formate consumption.

Table 2 Kinetic parameters of apparent Michaelis–Menten kinetics of nitrate- and nitrite-dependent denitrification in anoxic microcosms with peat soil in the presence of acetylene

Soil	*Nitrate amended*		*Nitrite amended*	
	v_{max}[a] (nmol g_{DW}^{-1} h^{-1})	K_M[a] (μM)	v_{max}[a] (nmol g_{DW}^{-1} h^{-1})	K_M[a] (μM)
Unturbated	NA	NA	18 ± 1	21 ± 5
Cryoturbated	17 ± 1	3 ± 1	32 ± 3	6 ± 4

Abbreviation: NA, not applicable.
[a]Kinetic parameters (calculated from Figure 2) ± s.e.

Such data suggest that fermentation potentials are lower in cryoturbated than in unturbated peat soils.

Six low-molecular-weight organic electron donors were tested for their potential to stimulate denitrification in anoxic peat soil microcosms supplied with nitrite and acetylene to identify putative substrates of acid-tolerant permafrost denitrifiers (Table 3; Supplementary Results). Nitrite was consumed in all treatments (data not shown). Supplemental acetate tended to stimulate N_2O production after the first pulse, and when resupplied after 47 days of anoxic incubation (second pulse) in microcosms with cryoturbated peat soil (Table 3). Supplemental acetate, formate and propionate tended to stimulate initial N_2O production after the first pulse (that is, when electron donors and nitrite were supplemented to the microcosms for the first time; Table 3). Such data suggest that acid-tolerant denitrifiers in cryoturbated and unturbated peat soils are capable of acetate consumption.

Phylogenetic analysis of denitrifiers by structural gene targeted amplicon pyrosequencing

In total, 48 917 quality-filtered sequences of the structural gene markers *narG*, *nirK*, *nirS* and *nosZ* were analyzed. On average, 6115 ± 1447 sequences per gene marker and soil with an average sequence length of 444 ± 19 bp were obtained (Supplementary Figure S4). As forward and reverse reads of *nirK* and *nirS* amplicons overlapped almost completely (~470 and 410 bp average read length, respectively), forward and reverse reads of *nirK*, and forward and reverse reads of *nirS* were analyzed together. Overlaps of forward and reverse reads were not sufficient for *narG* and *nosZ* amplicons (~670 and 700 bp, respectively). Thus, forward and reverse reads of both *narG* and *nosZ* were analyzed separately (Table 4). Only few non-target sequences occurred and were excluded from further analyses; 95 ± 7% of sequences generated from amplicons obtained with a certain structural gene-specific (that is, *narG*, *nirK*, *nirS* or *nosZ*) primer set were related to publicly available target genes of that primer set. Such amplification specificity is above or in the range of values obtained for other structural gene marker analyses (for example, average of 87 ± 11% for *narG*, *dsrAB*, [FeFe]-hydrogenase and dioxygenase genes; Philippot *et al.*, 2002; Loy *et al.*, 2004; Iwai *et al.*, 2010; Schmidt *et al.*, 2010).

Sequences were assigned to OTUs on the basis of threshold distances. The number of OTUs decreased rapidly with increasing threshold distance from 0 to 10%, which might be attributed to inaccuracies during PCR and pyrosequencing (Supplementary Figure S1; Behnke *et al.*, 2011; Quince *et al.*, 2011). However, the number of OTUs stabilized from 10 to 30% of threshold distance, indicating that the threshold distances of 17–33% used for diversity analyses in this study rendered the analysis insensitive to such potential inaccuracies (Supplementary Figure S1; see the 'Materials and methods' section for details). Coverages were always >98%, the number of taxa as estimated by Chao1 was essentially identical to those observed in the amplicon libraries (Table 4), and rarefaction curves essentially plateaued out for most genes analyzed (Supplementary Figure S5), indicating that the number of obtained sequences sufficed for the structural gene-based diversity analysis of denitrifiers.

Forward reads of *narG* amplicons yielded more OTUs than did reverse reads, although a similar number of sequences was obtained, indicating that the utility of forward reads of *narG* amplicons is higher for diversity analyses than reverse reads (Table 4, Supplementary Figure S5). Results obtained from reverse reads show similar overall

Table 3 Effect of supplemented electron donors on N_2O production in nitrite-amended anoxic peat soil microcosms

Treatment	*First pulse*[a]		*Second pulse*[b]	
	Unturbated	*Cryoturbated*	*Unturbated*	*Cryoturbated*
Control[c]	100 (98–102)[d,e]	100[f] (79–121)	100 (96–104)[g]	100 (77–123)[h]
Ethanol	103 (84–122)	70 (57–82)	93 (89–97)	123 (115–132)
Acetate	118 (111–124)	111 (103–120)	100 (88–113)	151 (143–159)
Formate	121 (114–128)	92 (83–100)	85 (82–87)	72 (72)
Propionate	118 (108–127)	86 (76–97)	78 (75–81)	95 (75–115)
Butyrate	89 (81–97)	82 (71–93)	70 (60–79)	51 (47–55)
Lactate	70 (68–74)	81 (78–84)	82 (80–84)	145 (127–163)

Percentage of N_2O production in each treatment as compared with microcosms supplemented with nitrite only (that is, control).
[a]Nitrite and electron donors added after 9 days of pre-incubation.
[b]Nitrite and electron donors resupplied 47 days after the first pulse.
[c]Microcosms supplemented with nitrite only.
[d]N_2O production = 23.1 nmol g_{DW}^{-1} h^{-1}.
[e]Ratios of the duplicates and the mean of the control microcosms (%).
[f]N_2O production = 54.9 nmol g_{DW}^{-1} h^{-1}.
[g]N_2O production = 16.7 nmol g_{DW}^{-1} h^{-1}.
[h]N_2O production = 22.6 nmol g_{DW}^{-1} h^{-1}.

Table 4 Analyses of *in silico*-translated amino-acid sequences of *narG*, *nirK*, *nirS* and *nosZ* derived from peat soil

Gene marker	Unturbated						Cryoturbated						β-Diversity	
	No. of sequences	Library coverage (%)[a]	No. of OTUs observed	No. of OTUs estimated[b]	H^c	E^d	No. of sequences	Library coverage (%)[a]	No. of OTUs observed	No. of OTUs estimated[b]	H^c	E^d	S_S^e	BC_S^f
narG forward	1 825	99.8	16	17 (16–27)	1.28 (1.23–1.34)	0.46 (0.44–0.48)	2526	99.8	14	25 (17–78)	1.36 (1.33–1.40)	0.50 (0.49–0.52)	0.84	0.67
narG reverse	2 047	99.9	9	10 (9–23)	0.89 (0.85–0.94)	0.41 (0.39–0.43)	3806	99.9	8	9 (8–22)	0.81 (0.77–0.84)	0.39 (0.37–0.40)	0.93	0.70
nirK	12 187	100	19	20 (19–24)	1.17 (1.15–1.19)	0.40 (0.39–0.40)	10 219	100	10	13 (10–33)	0.13 (0.12–0.15)	0.06 (0.05–0.06)	0.48	0.03
nirS	2 942	99.9	14	16 (14–30)	1.78 (1.75–1.80)	0.67 (0.66–0.68)	285	98.6	6	9 (6–30)	0.69 (0.59–0.79)	0.39 (0.33–0.44)	0.30	0.12
nosZ forward	2 097	100	7	7 (7)	0.23 (0.19–0.27)	0.12 (0.10–0.14)	3709	100	9	9 (9)	0.77 (0.73–0.80)	0.35 (0.33–0.36)	0.59	0.72
nosZ reverse	1 919	99.9	6	6 (6)	0.23 (0.19–0.27)	0.13 (0.11–0.15)	3664	99.9	11	14 (12–22)	0.75 (0.72–0.79)	0.31 (0.30–0.33)	0.67	0.68

Abbreviation: OTU, operational taxonomic unit.
[a]Percentage library coverage $C=(1-n_s\ n_t^{-1})\times 100$ (n_s = OTUs that occur only once, n_t = total number of sequences).
[b]Chao1 richness estimate with upper and lower 95% confidence intervals given in parentheses.
[c]Shannon–Weaver diversity index with upper and lower 95% confidence intervals given in parentheses.
[d]Species evenness with upper and lower 95% confidence intervals given in parentheses.
[e]Sørensen similarity index.
[f]Bray–Curtis similarity index.

trends to those from forward reads (Figure 3, Supplementary Figures S5 and 6). Thus, the information presented below refers to forward reads only. In total, *narG* sequences were assigned to 18 species-level OTUs (Figure 3). OTU 1 dominated *narG* in both soils. In all, 16 of the 18 OTUs including OTU 1 were only distantly related to *narG* of cultured organisms or environmental sequences (that is, sequence dissimilarities of OTU representatives were 20–35%), indicating phylogenetic new *narG* in cryoturbated and unturbated peat soils (Figure 3). Overall, 95% and 76% of *narG* from cryoturbated and unturbated peat soils, respectively, affiliated with *Actinobacterial narG*. OTUs 2 and 3 were more abundant in cryoturbated than in unturbated peat soil amplicon libraries (Figure 4a). OTU 4 was exclusively detected in unturbated peat soil amplicon libraries and accounted for 19% of *narG* (Figure 3). Confidence intervals of Shannon–Weaver diversity indices and species evenness values of *narG* from cryoturbated and unturbated peat soils overlapped. Sørensen and Bray–Curtis indices for the β-diversity of *narG* were high (that is, 0.84 and 0.67, respectively; Table 4) indicating a high proportion of shared OTUs among both soils. UniFrac analysis of *narG* phylogenetic trees likewise indicated that the *narG* communities were similar ($P \approx 1$).

In total, *nirK* was assigned to 22 species-level OTUs (Figure 5). Less OTUs were detected in cryoturbated than in unturbated peat soil (Table 4). *nirK* of cryoturbated peat soil was dominated by OTU 1 with a relative abundance of 97% (Figures 4 and 5). OTUs 2 and 3 both had a relative abundance of ∼44% and dominated *nirK* in unturbated soil amplicon libraries (Figures 4 and 5). Overall, 99% and 94% of *nirK* from cryoturbated and unturbated peat soils, respectively, affiliated with *Alphaproteobacterial nirK* (Figures 4 and 5). Major OTUs of both soils were related to environmental *nirK* from upland soil. In all, 10 of the 22 *nirK* OTUs were only distantly related (that is, sequence dissimilarities of OTU representatives were 15–23%) to *nirK* of cultured organisms or environmental sequences, indicating phylogenetic new *nirK*.

In total, 19 species-level OTUs of *nirS* occurred (Figure 6). Less OTUs occurred in cryoturbated than in unturbated peat soil (Figure 6, Table 4). *nirS* of cryoturbated peat was dominated by OTU 6, whereas OTUs 1 and 2 dominated *nirS* in unturbated soil (Figures 4 and 6). Overall, 99% and 12% of *nirS* from cryoturbated and unturbated peat soils, respectively, affiliated with *Alphaproteobacterial nirS*. Overall, 1% and 82% of *nirS* from cryoturbated and unturbated peat soils, respectively, affiliated with putative *Betaproteobacterial nirS*. Many OTUs from both soils were related to environmental *nirS* from wetlands or marine sediments, and distantly related to pure cultures (Figure 6). In all, 8 of the 19 *nirS* OTUs were only distantly related (that is, sequence dissimilarities of OTU representatives

were 15–25%) to *nirS* of cultured organisms or environmental sequences, indicating phylogenetically new *nirS*.

Diversity measures of *nirK* and *nirS* were consistently lower in cryoturbated than in unturbated peat soil (Table 4, Supplementary Figure S5). The 95%

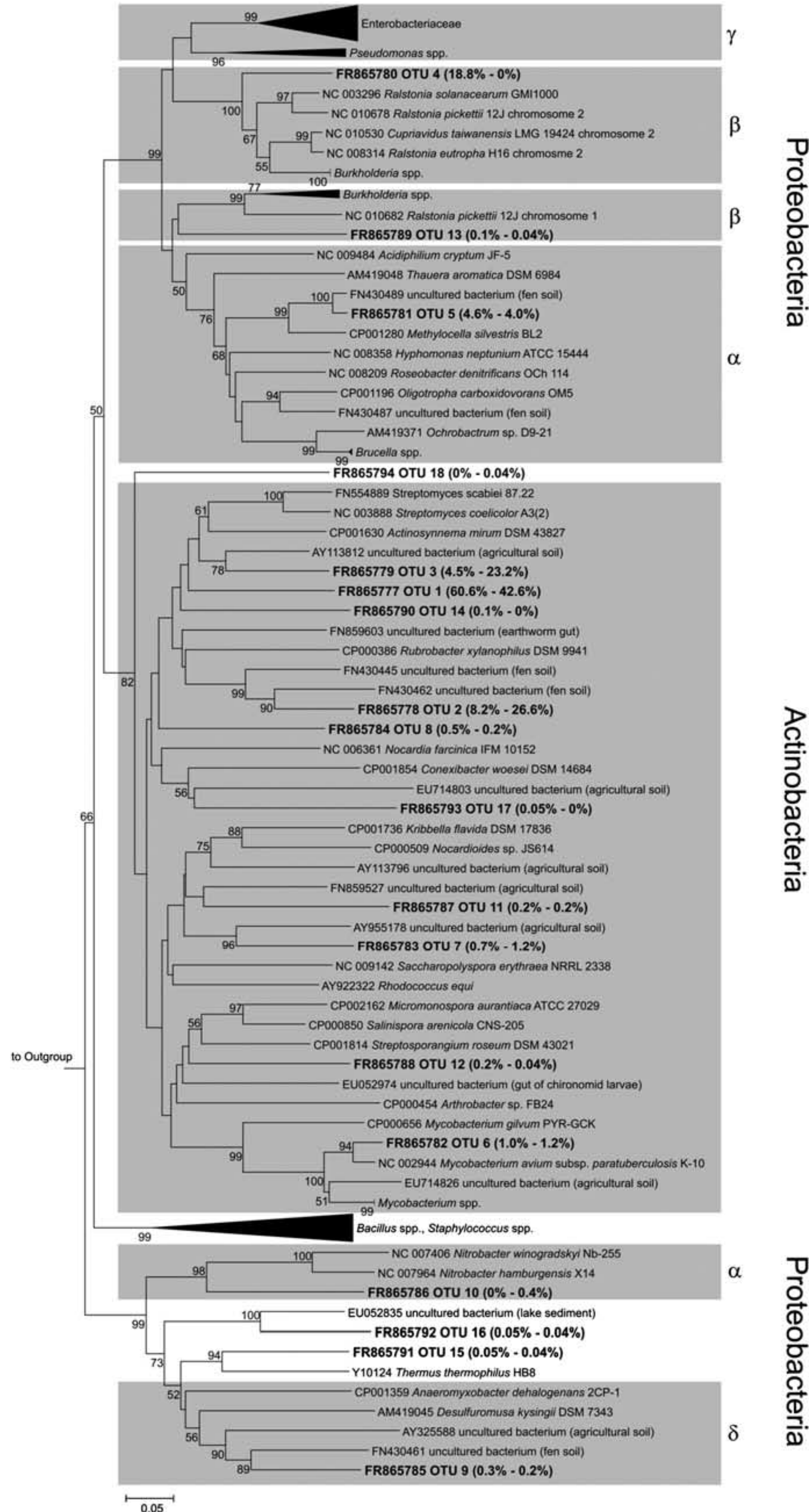


Table 5 Abundance of denitrification-associated genes in peat soil

Gene marker	*Unturbated*		*Cryoturbated*	
	Copy no. per 16S rRNA gene (%)[a,b]	*Copy no. per ng DNA*[a]	*Copy no. per 16S rRNA gene (%)*[a,c]	*Copy no. per ng DNA*[a]
narG	$(3.8 \pm 1.3) \times 10^{-2}$	$(6.5 \pm 2.5) \times 10^{2}$	$(7.6 \pm 2.8) \times 10^{0}$	$(6.5 \pm 2.0) \times 10^{4}$
nirK	$(7.7 \pm 1.6) \times 10^{-3}$	$(3.5 \pm 1.1) \times 10^{1}$	$(5.2 \pm 1.6) \times 10^{-4}$	$(5.1 \pm 2.1) \times 10^{0}$
nirS	$(8.8 \pm 1.3) \times 10^{-1}$	$(7.2 \pm 0.9) \times 10^{3}$	$(3.4 \pm 0.8) \times 10^{-1}$	$(4.6 \pm 1.0) \times 10^{3}$
nosZ	$(1.0 \pm 0.6) \times 10^{-4}$	$(2.7 \pm 1.2) \times 10^{0}$	$(1.7 \pm 0.4) \times 10^{-3}$	$(1.2 \pm 0.2) \times 10^{1}$

[a]Mean of 3 (sites) × 6 (technical) replicates ± s.e. (see the 'Materials and methods' section).
[b]16S rRNA gene copy numbers were $(1.9 \pm 0.2) \times 10^{6}$ per ng DNA.
[c]16S rRNA gene copy numbers were $(8.0 \pm 1.7) \times 10^{5}$ per ng DNA.

confidence intervals of Shannon–Weaver indices and evenness values did not overlap indicating that the detected diversity of *nirK*- and *nirS*-type denitrifier communities was lower in cryoturbated than in unturbated peat soil (Table 4). A similar trend was observed in the rarefaction curves generated from *nirK* and *nirS* obtained from both soils, even though 95% confidence intervals overlapped in the case of *nirK* (Supplementary Figure S5). The Sørensen and Bray–Curtis indices for β-diversity of *nirK* and *nirS* were low (0.48 and 0.03 for *nirK* and 0.30 and 0.12 for *nirS*, respectively), suggesting differences in the community composition in cryoturbated and unturbated peat soils. UniFrac analysis confirmed significant differences in *nirK* and *nirS* community compositions of cryoturbated and unturbated peat soils ($P<0.002$ and $P<0.002$, respectively).

In total, *nosZ* forward reads were assigned to 11 species-level OTUs (Figure 7). OTU 1 dominated *nosZ* of cryoturbated and unturbated peat soils (Figures 4 and 7). Most of the *nosZ* from both soils were affiliated with *Alphaproteobacterial nosZ*. In all, 7 of the 11 *nosZ* OTUs from cryoturbated and unturbated peat soils were only distantly related (that is, sequence dissimilarities of OTU representatives were 16–22%) to *nosZ* of cultured organisms, and 2 were likewise distantly related to *nosZ* from environmental sequences, indicating hitherto uncultured acid-tolerant denitrifiers capable of N_2O reduction in both soils. *nosZ* sequences clustered with *nosZ* of wetland and upland soils

Figure 3 Phylogenetic tree of representative *narG* sequences (forward reads) retrieved from unturbated and cryoturbated peat soils. The tree is based on *in silico*-translated amino-acid sequences. One representative sequence per OTU is shown. Codes preceeding sequence names represent sequence accession numbers in public databases. Values in parentheses represent relative abundances of sequences from unturbated (left) and cryoturbated (right) peat soils. In total, 1825 and 2526 sequences from forward reads were obtained from unturbated and cryoturbated peat soils, respectively. Gray boxes indicate branches where the majority of sequences are derived from reference strains of a certain phylogenetic class. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches. Bootstrap values below 50% are not shown. The outgroup was *narG* of *Haloarcula marismortui* ATCC 43049 (NC 006396).

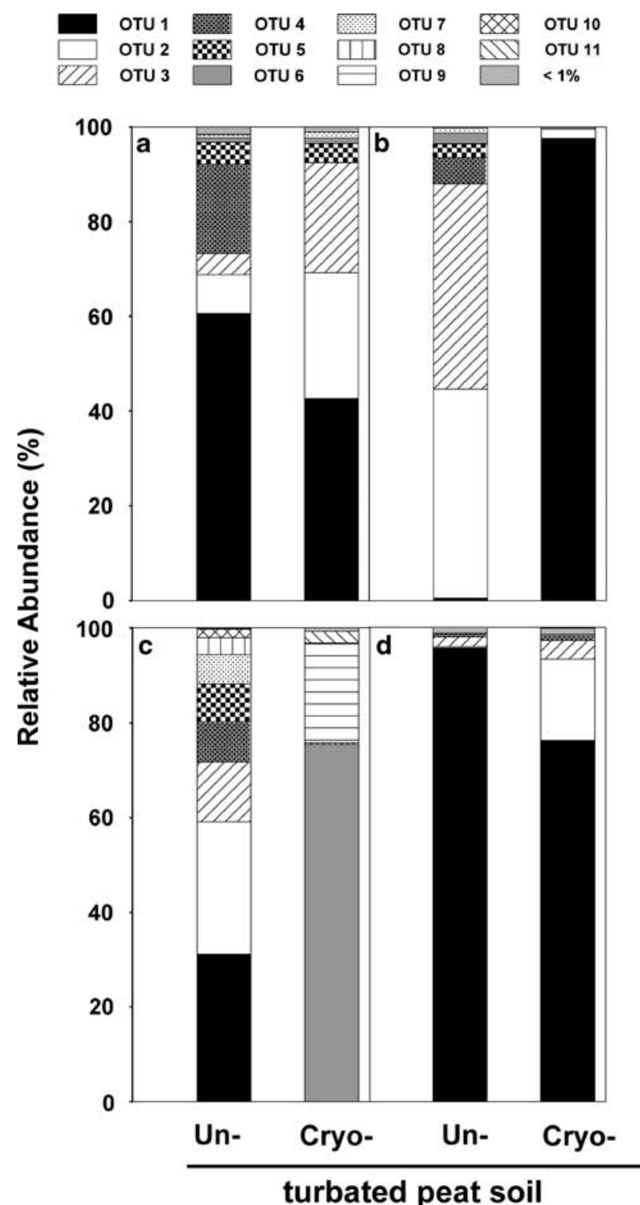
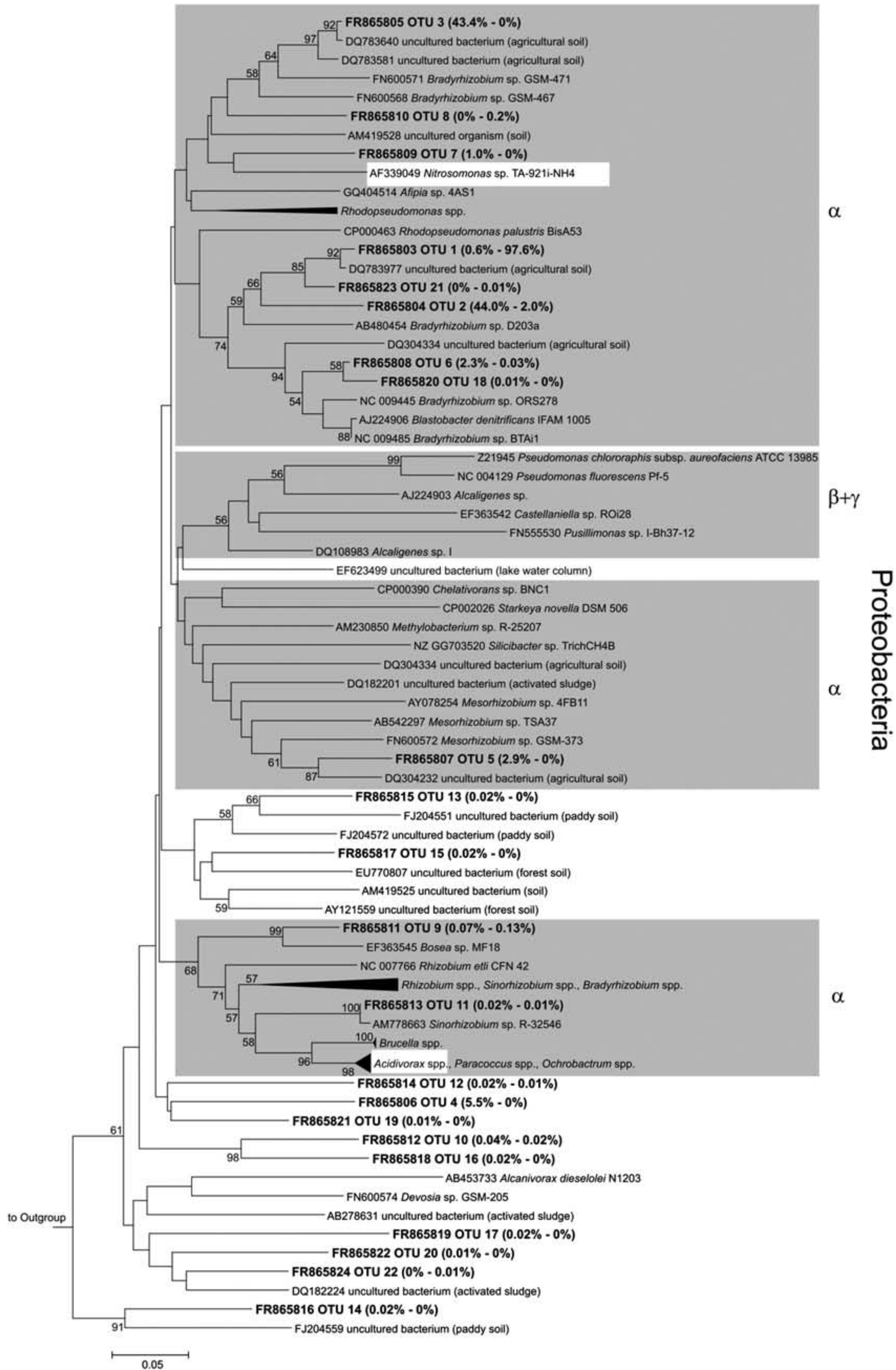


Figure 4 Relative abundances of (**a**) *narG*- (forward reads), (**b**) *nirK*-, (**c**) *nirS*- and (**d**) *nosZ*- (forward reads) derived OTUs retrieved from unturbated and cryoturbated peat soils. Sequences were assigned to OTUs using sequence similarity thresholds of 67% (*narG*), 83% (*nirK*), 82% (*nirS*) and 80% (*nosZ*). All OTUs that had relative abundances below 1% in both soils were grouped.

(Figure 7). *nosZ* reverse reads yielded similar results (Table 4, Supplementary Figure S7). Diversity measures of *nosZ* were higher in cryoturbated than in unturbated peat soil (Table 4, Supplementary Figure S5). Confidence intervals of Shannon–Weaver diversity indices and species evenness values did



not overlap. Similar trends were indicated by the rarefaction curves of *nosZ* forward and reverse sequences, even though 95% confidence intervals overlapped (Supplementary Figure S5). β-Diversity as indicated by Sørensen and Bray–Curtis diversity indices of *nosZ* tended to be lower than those of *narG* but higher than for *nirK* and *nirS* (Table 4). However, UniFrac analysis of *nosZ* phylogenetic trees did not reveal significant differences in *nosZ* communities ($P \approx 1$). Such analyses might suggest a marginally higher detected diversity of putative denitrifiers capable of N_2O reduction in cryoturbated than in unturbated peat soil.

Quantification of narG*,* nirK*,* nirS *and* nosZ *relative to 16S rRNA genes*

Copy numbers of all genes determined in this study were corrected by inhibition factors that were experimentally determined for every DNA extract and gene analyzed to overcome the effect of PCR-interfering substances that contaminate most environmental DNA (see the 'Materials and methods' section). Copy numbers of *narG* approximated 7×10^4 per ng DNA in cryoturbated peat soil, and accounted for 8% of 16S rRNA gene copy numbers, indicating that a substantial portion of bacteria in cryoturbated peat soil was capable of dissimilatory nitrate reduction (Table 5). *narG* copy numbers in unturbated peat soil were significantly lower (~ 100 times, $P = 0.02$) than those in cryoturbated peat soil (Table 5). The data are in agreement with the high and low capacities of cryoturbated and unturbated peat soils, respectively, to sustain nitrate-dependent denitirification (Figure 2).

Copy numbers of *nirK* were 5 and 35 per ng DNA in cryoturbated and unturbated peat soils, suggesting a minor role of *nirK*-type denitrifiers and marginally significant differences in the abundance of *nirK*-type denitrifiers ($P = 0.066$; Table 5). The same tendency was reflected in *nirK*/16S rRNA gene copy number ratios. Copy numbers of *nirS* were in the same range (that is, $5–7 \times 10^3$ per ng DNA, $P = 0.650$) for both soils and accounted for up to 1% of 16S rRNA gene copy numbers (Table 5). *nirS* $nirK^{-1}$ copy number ratios approximated 1000 and 100 for cryoturbated and unturbated peat, respectively, and differed significantly ($P = 0.02$). *nirS* $narG^{-1}$ copy number ratios approximated 0.05 and 15 for cryoturbated and unturbated peat, respectively, and differed significantly ($P = 0.05$).

Copy numbers of *nosZ* approximated 10^1 per ng DNA in cryoturbated peat soil, and accounted for 0.002% of 16S rRNA and 0.6% of *nirS* gene copy numbers (Table 5). Detected *nosZ* copy numbers per ng DNA detected in cryoturbated peat soil were five times higher than in unturbated peat soil, although such differences were not significant ($P = 0.247$; Table 5). In unturbated peat soil, *nosZ* 16S $rRNA^{-1}$ and *nosZ* $nirS^{-1}$ gene copy number ratios were 0.0001% and 0.02%, respectively. *nosZ* $narG^{-1}$ copy number ratios approximated 0.0002 and 0.003 for cryoturbated and unturbated peat, respectively. Such diferrences were only marginally significant ($P = 0.08$).

Discussion

Denitrification as major source of N_2O in cryoturbated peat

Cryoturbated acidic peat circles are 'hotspots' of N_2O emission in the arctic permafrost region, which was previously regarded as an insignificant source of N_2O (Denman *et al*., 2007; Repo *et al*., 2009; Marushchak *et al*., 2011). Denitrification, dissimilatory nitrate reduction to ammonium, nitrification and chemodenitrification are potential sources of N_2O in soils (Smith, 1983; Conrad, 1996; Bremner, 1997; van Cleemput, 1998; Kresovic *et al*., 2009). Although chemodenitrification of nitrite might occur under anoxic conditions at low pH, major products are NO and NO_2 rather than N_2O, and biotic denitrification is much quicker (van Cleemput, 1998; Kappelmeyer *et al*., 2003; Kresovic *et al*., 2009). Nitrification is suggested to be the main source of N_2O in well-aerated soils with a water-filled pore space of $<60\%$ (Conrad, 1996; Pihlatie *et al*., 2004). However, a water-filled pore space of 70–80% in cryoturbated peat circles, nitrate concentrations in the mM range, correlation of high water contents with high N_2O emission and a C-to-N-ratio of ~ 25 suggest denitrification rather than nitrification as the primary source of N_2O (Pihlatie *et al*., 2004; Repo *et al*., 2009; Marushchak *et al*., 2011). Indeed, anoxic microcosms at *in situ* pH with cryoturbated peat soil showed an immediate production of N_2O from endogenous nitrate, and 75% of the initial nitrate-N was recovered in N_2O, indicating denitrification rather than dissimilatory nitrate reduction of non-denitrifiers (DNR) (Figure 1a). Organisms catalyzing DNR produce N_2O by an unspecific reaction of nitrate reductase with accu-

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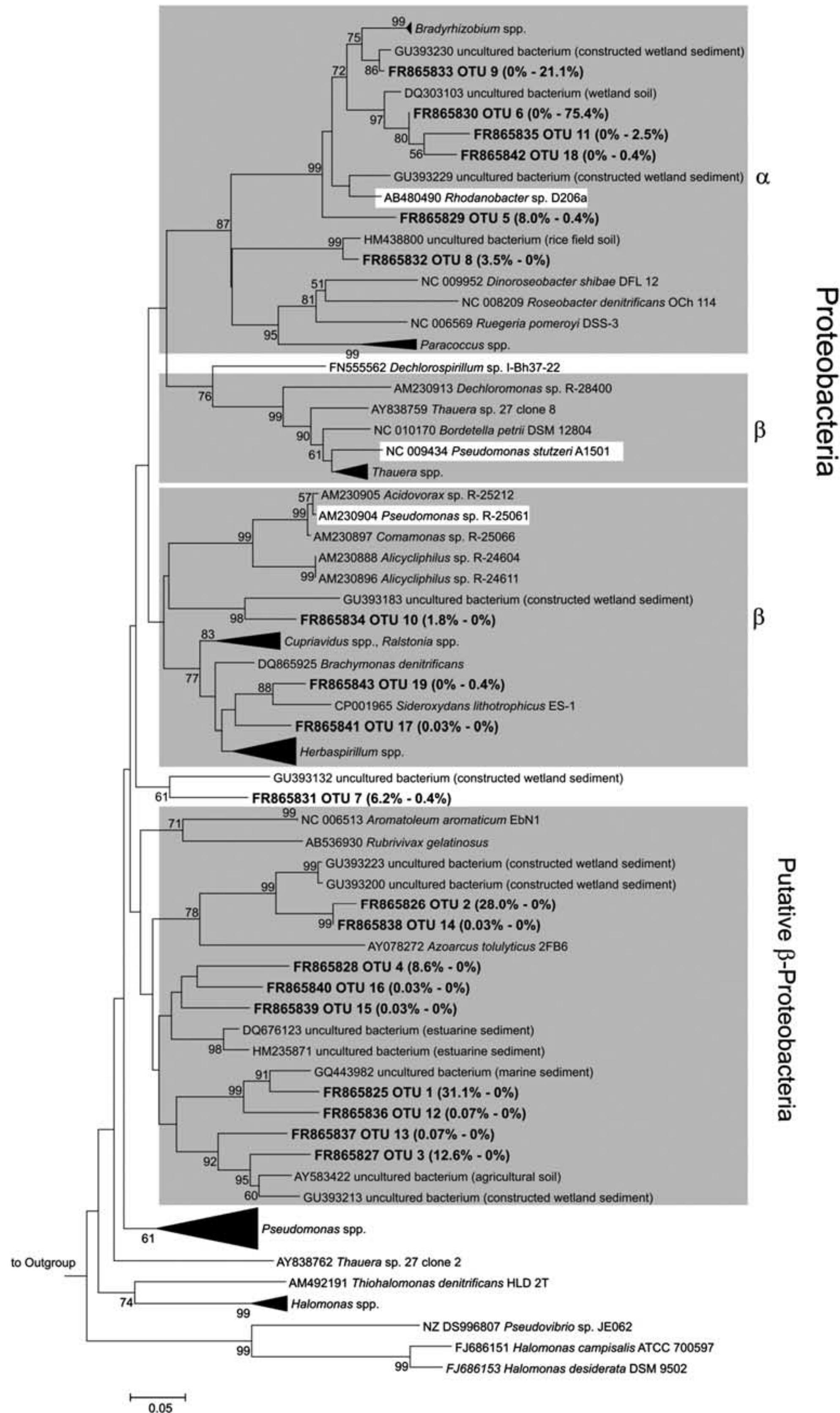
Figure 5 Phylogenetic tree of representative *nirK* sequences retrieved from unturbated and cryoturbated peat soils. The tree is based on *in silico*-translated amino-acid sequences. One representative sequence per OTU is shown. Values in parentheses represent relative abundances of sequences from unturbated (left) and cryoturbated (right) peat soils. Codes preceeding sequence names represent sequence accession numbers in public databases. In total, 12 187 and 10 219 sequences were obtained from unturbated and cryoturbated peat soils, respectively. Gray boxes indicate branches where the majority of sequences are derived from reference strains of a certain phylogenetic class; white boxes indicate minority sequences from genera not affiliated with the indicated class. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches. Bootstrap values below 50% are not shown. The outgroup was *nirK* of *Nitrosomonas* sp. C-56 (AF339044).

mulated nitrite (Smith 1983; Tiedje, 1988). Nitrite is virtually absent in cryoturbated peat (Repo *et al*., 2009), indicating that DNR is negligible as a direct source of N_2O. Nitrate, nitrite and acetylene stimulated net N_2O production under anoxic conditions (Figures 1b and 2). Extrapolation of N_2O production



from soil endogenous nitrate in microcosms to the field level largely exceed N_2O emissions measured *in situ*. Thus, current findings demonstrate that cryoturbated peat soil denitrifiers (1) are prone to react to anoxia, (2) are active under acidic conditions and (3) have the potential to account for the *in situ* N_2O emissions of cryoturbated peat circles (Repo *et al*., 2009; Marushchak *et al*., 2011).

Contrasting denitrifiers in cryoturbated and unturbated peat soils

Denitrification potentials, affinities for electron acceptors as indicated by K_M, v_{max}, nitrate tolerance and the potential to consume N_2O were higher in cryoturbated than in unturbated peat soil (Figures 1 and 2, Supplementary Figure S2, Table 2). Such data provided ecophysiological evidence that denitrifier communitities of cryoturbated and unturbated peat soils were dissimilar.

Pyrosequencing and quantitative kinetic real-time PCR of denitrification-associated genes substantiated the previous conclusion (Figure 4, Tables 4 and 5). *narG* copy numbers of cryoturbated peat soil were higher than or in the same range as in agricultural soils or glacier forelands (Deiglmayr *et al*., 2006; Kandeler *et al*., 2006; Bru *et al*., 2007), and significantly higher than in unturbated peat soil (Table 5). Such findings are in agreement with the high *in situ* concentrations of nitrate in cryoturbated peat soil (Repo *et al*., 2009), and the inability of unturbated peat soil to cope with high nitrate concentrations (Figure 2).

nirS and *nirK* diversity, *nirS* $nirK^{-1}$ copy number ratios and dominant OTUs in cryoturbated differed from those in unturbated peat soil (Tables 4 and 5, Figures 4–6). *nirS* diversity is higher than *nirK* diversity in some aquifers, marsh and costal sediments, suggesting that (semi-)aquatic systems sustain diverse *nirS*-type denitrifiers (Braker *et al*., 2000; Prieme *et al*., 2002; Santoro *et al*., 2006). Such findings are in agreement with the high detected *nirS* diversity in acidic peat soils (Table 4). Copy numbers of *nirS* outnumbered *nirK* by 2 to 3 orders of magnitude in both acidic peat soils (Table 5), indicating that *nirS*- rather than *nirK*-type denitrifiers were associated with denitrification in acidic peat soils. Indeed, *nirS* abundance in spruce forest soil was positively correlated with decreasing pH from 6.1 to 3.7, whereas *nirK* abundance was negatively correlated (Barta *et al*., 2010). Such data suggest that low pH and high moisture contents might favor *nirS*-type rather than *nirK*-type denitrifiers in acidic peat soils and highlight differences in detected nitrite reductase gene containing denitrifier communities of cryoturbated and unturbated peat soils.

In both soils, the proportion of detected denitrifiers that possess a N_2O reductase was rather low, as suggested by the low *nosZ* $nirS^{-1}$ ratios (Table 5). The relative abundance of N_2O reductases in the bacterial community is reflected in the ratio of N_2O to total N gases (Philippot *et al*., 2009), and an increased percentage of denitrifiers lacking N_2O reductase can increase the relative amount of emitted N_2O (Philippot *et al*., 2011). Indeed, N_2O to total N-gas ratios approximated 100% for both soils, when 500 μM of nitrate or nitrite was supplied (Supplementary Figure S2). Diversity measures of detected *nosZ* consistently suggested that denitrifiers capable of N_2O reduction (that is, harboring the *nosZ* gene) were more diverse in cryoturbated than in unturbated peat soil (Table 4 and 5, Supplementary Figure S5). Condsidering the contrasting response of cryoturbated and unturbated peat soil denitrifiers to various concentrations of nitrate and nitrite in terms of their N_2O to total N gas production (Supplementary Figure S2), and the consistent (although sometimes marginal) differences in diversity measures of *nosZ*, the data indicate that denitrifers capable of N_2O reduction likewise differed between both soils.

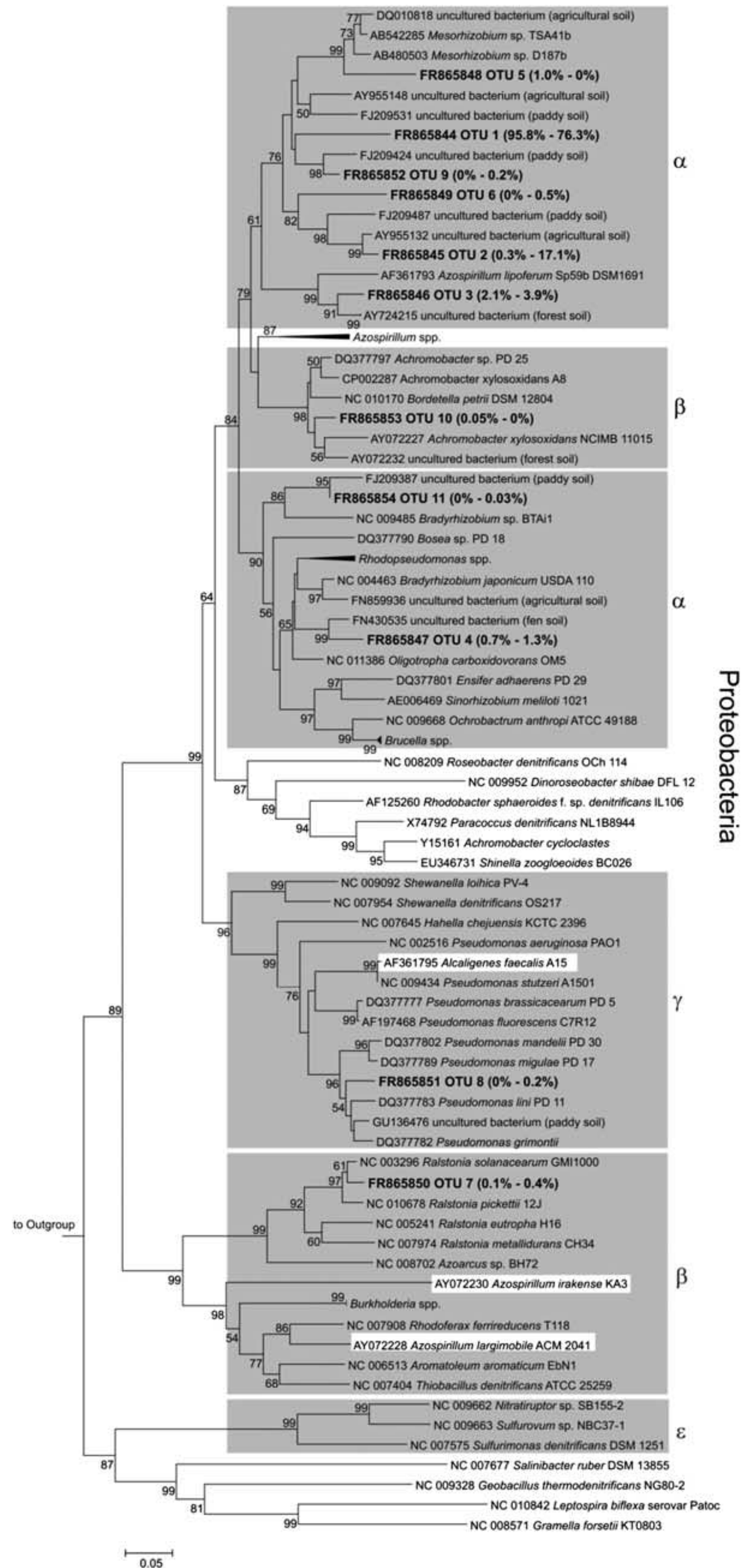
Regulation of net N_2O production by peat denitrifiers

Stimulation of denitrifiers in cryoturbated and unturbated peat soils by nitrite was more pronounced than by nitrate (Figures 1 and 2), suggesting that denitrifiers lacking nitrate reductases might contribute to N_2O production and/or nitrate reduction is rate limiting (Vangnai and Klein, 1974; Mahne and Tiedje, 1995; Zumft, 1997).

Denitrifiers in cryoturbated peat thrive at a low pH of 4 (Figures 1 and 2, Supplementary Figure S2). Denitrification occurs at acidic soil pH in other systems as well, although denitrification capacities of neutral soils are often higher (Parkin *et al*., 1985). However, denitrification capacities of cryoturbated peat soil were much higher than those of many more neutral habitats, indicating an acid-tolerant denitrifier community in cryoturbated peat that can cope remarkably well with low pH (Cuhel *et al*., 2010). Apparent K_M values for both nitrate and nitrite

Figure 6 Phylogenetic tree of representative *nirS* sequences retrieved from unturbated and cryoturbated peat soils. The tree is based on *in silico*-translated amino-acid sequences. One representative sequence per OTU is shown. Values in parentheses represent relative abundances of sequences from unturbated (left) and cryoturbated (right) peat soils. Codes preceeding sequence names represent sequence accession numbers in public databases. In total, 2942 and 285 sequences were obtained from unturbated and cryoturbated peat soils, respectively. Gray boxes indicate branches where the majority of sequences are derived from reference strains of a certain phylogenetic class; white boxes indicate minority sequences from genera not affiliated with the indicated class. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches. Bootstrap values below 50% are not shown. The outgroup was *nirS* of *Rhodothermus marinus* DSM 4252 (CP001807).

at pH 4 were $<10\,\mu M$ for cryoturbated peat soil denitrifiers (Table 2), indicating a high affinity of the denitrifiers for both substrates. Apparent K_M values for both nitrate and nitrite were in the same range or lower than in other more neutral soil types or pure cultures (Betlach and Tiedje, 1981; Strong



and Fillery, 2002; Palmer *et al*., 2010), supporting the conclusion that peat denitrifiers cope well with low pH.

Unturbated peat has the same acidic pH as cryoturbated peat soil, but a dissimilar denitrifier community, and does not emit N_2O *in situ* (Repo *et al*., 2009, Marushchak *et al*., 2011). Although soil pH has a significant impact on denitrifiers in temperate soils (Bru *et al*., 2011), data suggest that the low nitrate content of the vegetated unturbated peat soil and the dissimilar denitrifier communities rather than soil pH might account for the contrasting N_2O emission patterns of cryoturbated and unturbated peat soils (Figure 1; Repo *et al*, 2009, Marushchak *et al*., 2011). The contrasting denitrifier communites of such soils reacted differently to nitrate and nitrite supplementations (Figures 1b and 2, and Supplementary Figure S2), lending further support to the hypothesis that denitrifier community composition impacts regulation and thus prediction of N_2O fluxes (Holtan-Hartwig *et al*., 2000; Philippot *et al*., 2009, 2011; Ma *et al*., 2011).

Cryoturbated peat soil consumed N_2O that was initially produced from internal-N sources (Figure 1a), indicating the capability of peat soil denitrifiers for complete denitrification to N_2 under acidic conditions, which is in agreement with capabilities of a previously analyzed acidic fen denitrifier community and the genetic potential for complete denitrification detected in acidic Antarctic permafrost-affected wetland soils (Yergeau *et al*., 2007; Yergeau and Kowalchuk, 2008; Palmer *et al*., 2010). Ratios of N_2O to total N gases were below 40% at low nitrate and nitrite concentrations and ~100% at 500 μM (Supplementary Figure S2). Increasing concentrations of nitrate and nitrite were correlated with an increase in the ratio of N_2O to total N gases, a phenomenon that has been observed in various soils (Blackmer and Bremner, 1978; Gaskell *et al*., 1981; Palmer *et al*., 2010). Low pH and low electron donor availability favor increased ratios of N_2O to total N gases when nitrate is not limiting (Blackmer and Bremner, 1978; Schalk-Otte *et al*., 2000; Simek and Cooper, 2002; van den Heuvel *et al*., 2010). Indeed, denitrifiers of cryoturbated peat were saturated with less than half of the nitrate concentrations occurring *in situ*, suggesting that electron donor availabiltity might limit denitrification (Figure 2). *In situ* nitrate concentrations exceed 1 mM and might be explained by constant replenishment of carbon and nitrogen due to mixing in the cryoturbated soil and by the absence of plants as competitors for nitrate (Bockheim, 2007; Repo *et al*., 2009; Kuhry *et al*., 2010). Thus, cryoturbation favors denitrifiers and N_2O as the main end product of denitrification in cryoturbated peat soil.

New acid-tolerant peat denitrifers

Most of the *narG* OTUs retrieved from acidic peat soils contained hitherto unknown sequences, and the major ones clustered with *Actinobacterial narG* (for example, OTUs 1 and 3; Figures 3 and 4). Interestingly, detected agricultural soil *narG* communities are likewise dominated by *Actinobacteria*-related *narG*, indicating a wide distribution of *Actinobacterial* nitrate reducers (Philippot *et al*., 2002).

Many OTUs of *nirK* and *nirS* contained new sequences indicative of new and uncultured denitrifiers (Figures 5 and 6). Major OTUs affiliated with *Alphaproteobacterial* sequences and were substantially more abundant in cryoturbated peat *nirS* amplicon libraries than in those from unturbated peat (Figures 4 and 6). *nirS*-based phylogenies are more congruent with the 16S rRNA-based phylogenies of their hosts than *nirK*-based phylogenies; thus, the data suggest that uncultured acid-tolerant denitrifiers of the *Alphaproteobacteria* occur in cryoturbated peat soil (Heylen *et al*., 2006). Certain *nirK* harboring acid-tolerant *Rhodanobacter* strains of the *Gammaproteobacteria* that are known to be capable of complete denitrification to N_2 at pH 4 were not detected (van den Heuvel *et al*., 2010). However, the primers used for the amplification of *nirK* from the peat soils do not target *nirK* of *Rhodanobacter* sp. (Green *et al*., 2010). Thus, it is still unclear whether *Rhodanobacter*-like denitrifiers occur in acidic peat soils. *nosZ* OTUs were also indicative of new and uncultured denitrifiers capable of N_2O reduction (Figure 7). Thus, the collective analysis of denitrification gene-associated data suggests that the permafrost-affected, acidic tundra peat soil harbors diverse, new and acid-tolerant, uncultured denitrifiers.

Conclusions and limitations

Microbial communities including denitrifiers in permafrost-affected habitats are rather stable under repeated freeze-thaw cycles and rapidly resume activity upon the onset of soil thawing (Yergeau

Figure 7 Phylogenetic tree of representative *nosZ* sequences retrieved from unturbated and cryoturbated peat soils. The tree is based on *in silico*-translated amino-acid sequences. One representative sequence per OTU is shown. Values in parentheses represent relative abundances of sequences from unturbated (left) and cryoturbated (right) peat soils. Codes preceeding sequence names represent sequence accession numbers in public databases. In total, 2097 and 3709 sequences from forward reads were obtained from unturbated and cryoturbated peat soils, respectively. Gray boxes indicate branches where the majority of sequences are derived from reference strains of a certain phylogenetic class; white boxes indicate minority sequences from genera not affiliated with the indicated class. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches. Bootstrap values below 50% are not shown. The outgroup was *nosZ* of *Haloarcula marismortui* ATCC 43049 (NC 006396).

and Kowalchuk, 2008; Männistö *et al*., 2009; Sawicka *et al*., 2010). Increasing thaw-depth and frequencies of freeze-thaw cycles may increase the availability of organic carbon and nitrogen stored in permafrost-affected soils, finally fueling denitrification-associated N_2O emissions (Mørkved *et al*., 2006; Sharma *et al*., 2006; Kuhry *et al*., 2010). This highlights the potential susceptibility of such systems to global change. Ecophysiological and molecular data collected in this study indicate pronounced differences and a high diversity of denitrifier communities in cryoturbated and unturbated peat soils. However, the molecular data are largely dependent on the choice of primers. Although the primer systems used in this study are well evaluated and widely applied for estimating denitrifier diversity, and four denitrification-associated genes were analyzed in parallel to maximize the detectability of denitrifiers, it is known that not all denitrifers are detectable by the primer systems used (Throbäck *et al*., 2004; Enwall *et al*., 2010; Green *et al*., 2010; Palmer *et al*., 2010). Considering the rather high threshold distances used in this study for calling OTUs, the diversity analyses of cryoturbated and unturbated peat soil denitrifiers might be regarded as a minimal estimate of the 'real' denitrifier diversity.

Within these limitations, this study nonetheless provides evidence that (1) the exceptionally high N_2O emissions from cryoturbated peat circles are associated with a specific diverse, and acid-tolerant denitrifier community, (2) contrasting denitrifier community compositions are associated with high and low N_2O emission patterns in acidic permafrost-affected peat soil and (3) such soils represent a hitherto overlooked reservoir of new microbial diversity associated with N_2O production. Such new and uncultured diversity might coincide with new ecophysiological traits, necessitating future in-depth studies addressing denitrifiers in permafrost-affected peat soils with respect to global warming.

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