Denitrification Activity of a Remarkably Diverse Fen Denitrifier Community in Finnish Lapland Is N-Oxide Limited

Katharina Palmer*, Marcus A. Horn*

Department of Ecological Microbiology, University of Bayreuth, Bayreuth, Germany

* Current address: Water Resources and Environmental Engineering Research Group, University of Oulu, Oulu, Finland
* marcus.horn@uni-bayreuth.de

Abstract

Peatlands cover more than 30% of the Finnish land area and impact N₂O fluxes. Denitrifiers release N₂O as an intermediate or end product. In situ N₂O emissions of a near pH neutral pristine fen soil in Finnish Lapland were marginal during gas chamber measurements. However, nitrate and ammonium fertilization significantly stimulated in situ N₂O emissions. Stimulation with nitrate was stronger than with ammonium. N₂O was produced and subsequently consumed in gas chambers. In unsupplemented anoxic microcosms, fen soil produced N₂O only when acetylene was added to block nitrous oxide reductase, suggesting complete denitrification. Nitrate and nitrite stimulated denitrification in fen soil, and maximal reaction velocities (v_max) of nitrate or nitrite dependent denitrification where 18 and 52 nmol N₂O h⁻¹ gDW⁻¹, respectively. N₂O was below 30% of total produced N gases in fen soil when concentrations of nitrate and nitrite were <500 μM. v_max for N₂O consumption was up to 36 nmol N₂O h⁻¹ gDW⁻¹. Denitrifier diversity was assessed by analyses of narG, nirK/nirS, and nosZ (encoding nitrate-, nitrite-, and nitrous oxide reductases, respectively) by barcoded amplicon pyrosequencing. Analyses of ~14,000 quality filtered sequences indicated up to 25 species-level operational taxonomic units (OTUs), and up to 359 OTUs at 97% sequence similarity, suggesting diverse denitrifiers. Phylogenetic analyses revealed clusters distantly related to publicly available sequences, suggesting hitherto unknown denitrifiers. Representatives of species-level OTUs were affiliated with sequences of unknown soil bacteria and Actinobacterial, Alpha-, Beta-, Gamma-, and Delta-Proteobacterial sequences. Comparison of the 4 gene markers at 97% similarity indicated a higher diversity of narG than for the other gene markers based on Shannon indices and observed number of OTUs. The collective data indicate (i) a high denitrification and N₂O consumption potential, and (ii) a highly diverse, nitrate limited denitrifier community associated with potential N₂O fluxes in a pH-neutral fen soil.
Introduction

Northern peatlands are important players in the global carbon and nitrogen cycles, and store more than 30% of soil carbon and nitrogen even though they cover only about 3% of the terrestrial surface [1]. Greenhouse gases such as methane (CH4) and nitrous oxide (N2O) are produced in and released from northern peatlands soils [2]. High latitude peatlands have been intensively studied with respect to their capacity to emit CH4 due to the large amount of stored carbon in peat soils (e.g., [3–5]). N2O has a high global warming potential (approximately 300 times higher than CO2), is a major ozone-depleting substance, and 6% of the greenhouse effect is attributed to N2O [6–8]. Stored organic N in certain peatlands fuels N2O release via coupling of ammonification, ammonia oxidation, and denitrification [9]. Thus, potential N2O emissions from northern peatlands are of major interest. Northern peatlands are very diverse ecosystems, including many types of pristine and managed soils. Many studies investigating N2O emissions from peatlands have focused on N2O emissions from managed peatlands, and only recently N2O fluxes from pristine northern peat soils have been investigated [10–15]. Pristine northern fens include significant net sources of N2O even though emission rates are generally low [10, 13]. Negative N2O fluxes suggest that peatlands can act as temporary sinks for N2O [2, 14, 16, 17]. Understanding of the mechanisms and regulation of N2O fluxes in such systems is still incomplete.

N2O in soils is generally produced during nitrification, denitrification, or chemical processes [18, 19]. Denitrification is considered to be the main source of N2O in water-saturated soils including peatlands [19, 14]. During denitrification, nitrate or nitrite are sequentially reduced via nitric oxide (NO) and N2O to dinitrogen (N2) [20]. The reductions are catalyzed by a set of oxidoreductases, namely nitrate reductases (encoded by narG and napA), nitrite reductases (encoded by nirK and nirS), NO reductases (encoded by norBC), and N2O reductases (encoded by nosZ) [20]. N2O or N2 can be released into the atmosphere. The ratio of N2O to N2 is determined by in situ parameters such as pH, temperature, as well as nitrate/nitrite and electron donor availability [21].

High latitude peatlands are likely to be strongly affected by increasing temperatures due to climate change [3, 4, 9, 22]. Global warming might reduce the water table in northern peatlands and influence the amount of N2O released from the soil [9]. A constantly lowered water table increases N2O fluxes from nutrient rich peat, whereas fluxes from nutrient-poor peat remain largely unaffected [12]. Dissimilar denitrifier communities are related with dissimilar N2O fluxes [15]. Detailed knowledge about the microbial catalysts involved in N2O turnover in northern peatlands is scarce. Thus, the aim of this study was to assess denitrification in a pH neutral pristine fen. The main objectives were to (i) assess in situ N2O emissions of a pH-neutral fen soil, (ii) determine depth-related N2O production and consumption capacities of fen soil, and (iii) link differences in the denitrifier community composition to physiological differences of the denitrification in the peat soil over two depths.

Material and Methods

Sampling site and soil parameters

Puukkosuo fen is located in northeastern Finland (66°22’38”N, 29°18’28”E) at an elevation of 220 m above sea level. The mean annual air temperature is (-0.43±0.09)°C, and mean annual precipitation approximates (772±12) mm (average of years 1966 to 2011, measured at Oulanka research station). The fen is meso-eutrophic and water saturated. Vegetation consists mainly of mosses (Sphagnum spp.) and grasses (e.g., Carex spp.). Four replicate soil cores from layers 0 to 20 cm and 20 cm to 40 cm were taken on July 28th 2010. Soil temperatures on the day of
sampling were 17.2°C in surface soil and 15.1°C in deeper soil layers (below 15 cm). Samples were transported on ice to the laboratory and stored at 4°C for microcosm analyses or at -80°C for nucleic acid extractions. Microcosm experiments were conducted within 2 weeks after sampling. Nitrate, nitrite and ammonium concentrations, soil pH, soil moisture content, total carbon (TC), dissolved organic carbon (DOC) and total nitrogen (TN) were determined from pooled soil samples as described previously [23]. Permission to access and sample Puukkosuo fen was granted by Metsähallitus (www.metsa.fi) on 12th of July 2010.

Assessment of in situ gas emissions

In situ gas emissions of unfertilized soil and soil supplemented with either nitrate or ammonium were determined in closed poly(methyl methacrylate) (PMMA) chambers. Chambers were placed onto the soil surface and surrounded by metal collars, which had been inserted into the soil for a few centimeters to ensure that the chambers were gas tight. The transition between the plexiglas chamber and the metal ring was sealed with a rubber band to avoid exchange of gases from the chamber with the surrounding air. Before the installation of the gas chambers, 2l of fen pore water with 20 mM of added nitrate or ammonium was applied homogeniously onto the soil surface in 4 replicate treatments each and unsupplemented controls received pure fen pore water. Gas samples (5 ml per sampling timepoint) were taken from gas outlets and injected into gas tight evacuated containers (Exetainer, Labco Limited, High Wycombe, UK) at the start of the experiment, after 0.5, 1 and 3 hours.

Assessment of denitrification potentials in soil microcosms

Denitrification potentials of pH-neutral fen soil (0 to 20 cm and 20 to 40 cm) were assessed in unsupplemented and nitrate-, nitrite- or N2O-supplemented anoxic microcosms as described earlier [14, 15, 23]. Supplemental nitrate and nitrite ranged from 0 to 1000 μM, while supplemental N2O ranged from 0 to 4 μM. Acetylene blockage was used to distinguish between total N2O production and total denitrification as described earlier [15, 24]. Incubations were conducted at 20°C in the dark. N2O production rates and apparent kinetic parameters [Michaelis-Menten constants (K_M) and maximum reaction velocities (v_max)] were determined as described [14]. Michaelis-Menten regressions obtained for different incubation conditions were compared using the “extra sum of squares” principle to test for significant differences between the regressions [25]. Obtained values for K_M and v_max were compared by t-tests.

Molecular characterisation of fen denitrifier communities

Nucleic acids were extracted from homogenized pooled fen soil of both soil layers as previously described using a bead-beating protocol [15, 26]. DNA yields were 4 to 12 μg DNA per gram (fresh weight) of soil. A260/A230 values approximating 0.94 to 1.56 indicated DNA with moderate to low humic acid content. The structural genes narG, nirK, nirS, and nosZ were amplified using the primer pairs narG1960f (TAY GTS GGS CAR GAR AA)/narG2650r (TTY TCR TAC CTR CBR GC) [27], F1aCu (ATC ATG GTS GTS CGG CG)/R3Cu (GCC TCG ATC AGR TGG TGG TT) [28], cd3aF (GTS AAC GTS AAG GAR ACS GG)/R3cd (GAS TTC GGR TGS GTC TGG A) [28], and nosZF (CGC TGT TCI TCG ACA GYC AG)/nosZR (ATG TGC AKI GCR TGG CAG AA) [29], respectively, and subjected to barcoded pyrosequencing as previously described [15, 23]. Barcodes used to identify sequences after pyrosequencing were ACTGCG and AGTATG for 0 to 20 cm and 20 to 40 cm fen soil, respectively. Pyrosequencing was performed at the Göttingen Genomics Laboratory using the Roche GS-FLX 454/Titanium technology as previously described [15, 23]. Pyrosequencing and PCR errors of the obtained reads were corrected using the AmpliconNoise pipeline [30] and sequences were clustered at
species-level (i.e., for narG, nirK, nirS, and nosZ, respectively), and 97% threshold similarities using Qiime as previously described [23, 31]. Species-level threshold similarities were determined from pairwise comparisons of 16S rRNA gene similarities and structural gene similarities of cultured denitrifiers [32]. Such OTUs indicate a minimal estimate of species-level diversity, i.e., is likely to underestimate “real” species-level diversity. Phylogenetic trees with cluster representatives were constructed in MEGA 5.0 [33]. Alpha- and beta-diversity measures were calculated in Qiime from rarified OTU tables as described [23, 34] to allow statistical comparison of the structural gene diversity from both soil layers. Rarified OTU tables were generated in Qiime by randomly subsampling original OTU tables 100 times at depth of 1000, 1500, 2500, and 500 sequences for narG, nirK, nirS, and nosZ, respectively. OTU representative sequences of narG, nirK, nirS, and nosZ were deposited at EMBL under accession numbers HE995549 to HE995577. Complete sequence data sets were deposited deposited in the European Nucleotide Archive (ENA) under the study accession number ERP008864.

Quantitative kinetic real-time PCRs (qPCRs) were performed in 6 technical replicates as described [15]. Obtained gene copy numbers were corrected for inhibition with inhibition factors ranging from 0.5–0.6, 0.3–0.4, 0.5–1.0, 0.9–1.0, and 0.9–1.0 for narG, nirK, nirS, nosZ, and 16S rRNA genes, respectively [35]. Normal distribution of the data was verified by Kolmogorov-Smirnov as well as Shapiro-Wilk tests. Copy numbers of narG, nirK, nirS, and nosZ in 0 to 20 cm and 20 to 40 cm soil were statistically evaluated using Student’s t-test (based on the 6 replicates for each gene).

Results

Soil parameters

Soil moisture content of Puukkosuo fen soil was 90% in both soil layers (Table 1). Soil pH in water was 6.8 and 6.9 in 0 to 20 cm and 20 to 40 cm fen soil, respectively. Nitrate was below the detection limit of 5.8 μg gDW⁻¹ (Table 1). Values for carbon and nitrogen contents appeared to be marginally higher in 20 to 40 cm than in 0 to 20 cm fen soil, but C/N ratios and DOC concentrations were similar in both soil layers (Table 1).

In situ gas emissions of fen soil

During gas chamber measurements, only minor amounts of N₂O accumulated in gas chambers placed on unsupplemented fen soil on average (Fig 1). Increases of about 10 ppb in N₂O mixing ratio were observed in two of the four replicate gas chambers, while decreases in N₂O mixing ratio were observed in the other two replicate gas chambers (-1 to -17 ppb decrease in mixing}

| Soil layer (cm) | pH | Moisture content (%) | NO₃⁻ (μM) | NO₂⁻ (μg gDW⁻¹) | NO₃⁻ (μM) | NO₂⁻ (μg gDW⁻¹) | NH₄⁺ (μM) | NH₄⁺ (μg gDW⁻¹) | Total C (g kgDW⁻¹) | DOC (mg l⁻¹) | Total N (g kgDW⁻¹) | C/N |
|----------------|----|----------------------|-----------|-----------------|-----------|-----------------|-----------|-----------------|-------------------|-------------|-------------------|-----|
| 0 to 20        | 6.8| 90                   | 14.4      | 7.7             | < 48.5    | < 19.2          | 77.2      | 11.9            | 434               | 63.2        | 29                | 15  |
| 20 to 40       | 6.9| 90                   | < 10.7    | < 5.8           | < 48.2    | < 19.2          | 37.7      | 5.9             | 492               | 65.1        | 35                | 14  |
| Pore water     | n.a.|                     | < 2.5     | n.a.            | < 10.9    | n.a.            | < 1.4     | n.a.            | n.a.              | 8.8         | n.a.              | n.a.|

1. Total carbon
2. Dissolved organic carbon (per l porewater)
3. Total nitrogen
4. Carbon to nitrogen ratio
5. Not applicable

doi:10.1371/journal.pone.0123123.t001
Nitrate-addition initially lead to accumulation of N2O in the gas chambers. However, this accumulation of N2O was restricted to the first 30 minutes after nitrate-addition, and initially accumulated N2O was subsequently consumed after 30 minutes (Fig 1). Ammonium likewise led to accumulation of N2O in the gas chambers, however this initial accumulation of N2O was slower than after nitrate-addition (Fig 1). Moreover, initially accumulated N2O was subsequently consumed after the first hour.

Denitrification potentials in fen soil microcosms

In anoxic microcosms, unsupplemented fen soil from both soil layers produced only minor amounts of N2O in the absence of acetylene, and initially produced N2O was subsequently consumed (Fig 2). However, N2O production was significantly higher in anoxic microcosms when N2O-reductase was blocked by acetylene (Fig 2). N2O mixing ratios increased from 0.04 ± 0.004 to about 35 ± 3.5 ppm within the first 94 hours in acetylene-amended microcosms with 0 to 20 cm fen soil, and the concentration of N2O plateaued out after the first 94 hours.
indicating that endogenous nitrate had been consumed. The increase in $N_2O$ mixing ratio was significantly lower in microcosms with 20 to 40 cm fen soil than in those with 0 to 20 cm fen soil (Fig 2). $N_2O$ mixing ratios increased from $0.03 \pm 0.001$ to $1.4 \pm 0.5$ ppm within the first 94 hours in acetylene-amended microcosms with 20 to 40 cm fen soil. In 20 to 40 cm fen soil microcosms without acetylene, mixing ratios increased only to $91 \pm 21$ bbp $N_2O$ within the first 94 hours. The initially accumulated $N_2O$ was subsequently consumed within the next 74 hours. Both soil layers displayed the capability to consume subatmospheric concentrations of $N_2O$ in microcosms without acetylene. 0 to 20 cm fen soil reduced $N_2O$ from 290 ppb to 55 ppb, while 20 to 40 cm fen soil reduced $N_2O$ from 91 ppb to 39 ppb (Fig 2).

Supplemental nitrate and nitrite stimulated the production of $N_2O$ without apparent delay in microcosms with fen soil from both soil layers (Fig 3 A and S1 Fig), while $N_2O$ consumption was stimulated in $N_2O$ supplemented microcosms (Fig 3 B). Stimulation of $N_2O$ production with nitrate was smaller than with nitrite, and $N_2O$ production in nitrate-supplemented microcosms was less than 25% of that in nitrite-supplemented microcosms (Fig 3 A and S1 Fig). In microcosms with fen soil from 0 to 20 cm, $N_2O$ production in acetylene-amended microcosms was in a similar magnitude for all supplemented nitrate concentrations $\geq 100 \mu M$. $N_2O$
production in microcosms with fen soil from 20 to 40 cm was highest when 50 μM nitrate were supplied, and decreased with increasing nitrate concentrations, indicating that denitrifiers in fen soil were saturated at low nitrate concentrations, and were inhibited by higher nitrate concentrations (Fig 3 A). In nitrite-supplemented microcosms, N2O production rates increased with increasing nitrite concentrations in both soil layers (Fig 3 A). N2O consumption was likewise stimulated by increasing N2O concentrations (Fig 3 B). N2O production and consumption capacities were higher in 0 to 20 cm fen soil than in 20 to 40 cm fen soil.

The ratio of N2O to (N2 + N2O) was below 30% and 40% for all supplied nitrate concentrations in microcosms with 0 to 20 cm and 20 to 40 cm fen soil, respectively (S2 Fig), indicating that more than half of the N2O produced from nitrate was further reduced to N2 in fen soil. The ratio of N2O to (N2 + N2O) was below 30% in microcosms with fen soil from 0 to 20 cm when nitrite concentrations were 100 μM or smaller and increased to about 75% for higher nitrite concentrations. In microcosms with 20 to 40 cm fen soil, the ratio of N2O to (N2 + N2O) was between 50% and 100% for all supplied nitrite concentrations (S2 Fig), indicating that N2O...
was a major product of denitrification in that soil layer when nitrite was provided as electron acceptor.

Initial nitrite-dependent N₂O production rates of fen soil microcosms amended with acetylene followed apparent Michaelis-Menten kinetics, as did nitrate-dependent N₂O production rates of fen soil microcosms from 0 to 20 cm depth and N₂O-dependent N₂O consumption rates in both layers (Fig 3). The Michaelis–Menten kinetics differed significantly between the different treatments and soil layers (p ≤ 0.03 for all comparisons). Apparent maximal reaction velocities (v<sub>max</sub>) were highest for nitrite-dependent N₂O production, followed by N₂O-dependent N₂O consumption rates. v<sub>max</sub> was lowest for nitrate-dependent N₂O production (Table 2). v<sub>max</sub> values for nitrate and nitrite dependent N₂O production, as well as N₂O-dependent N₂O consumption were significantly higher in 0–20 cm than 20–40 cm fen soil (p < 0.001, and p < 0.001, as well as p = 0.02, respectively). Apparent Michaelis-Menten constants K<sub>M</sub> were about 60 to 140 times lower for N₂O consumption than for nitrite dependent N₂O production in 0 to 20 cm fen soil (p = 0.003), indicating a high affinity of fen denitrifiers for N₂O (Table 2).

### Table 2. Kinetic parameters of denitrification in Puukkosuo fen soil.

| Soil layer (cm) | Nitrate amended | Nitrite amended | N₂O amended |
|-----------------|-----------------|-----------------|--------------|
|                 | KM (μM) | v<sub>max</sub> (nmol h<sup>-1</sup> g<sub>DW</sub>⁻¹) | KM (μM) | v<sub>max</sub> (nmol h<sup>-1</sup> g<sub>DW</sub>⁻¹) | KM (μM) | v<sub>max</sub> (nmol h<sup>-1</sup> g<sub>DW</sub>⁻¹) |
| 0 to 20         | 28.7 ± 16.8 | 18.3 ± 2.6    | 61.8 ± 14.2 | 52.3 ± 3.3 | 0.43 ± 0.12 | - 36.0 ± 3.5 |
| 20 to 40        | n.a.     | n.a.          | 18.3 ± 21.0 | 13.3 ± 3.3 | 0.30 ± 0.09 | - 21.4 ± 2.0 |

<sup>1</sup> Kinetic parameters (calculated from Fig 3) ± standard error.

<sup>2</sup> Not applicable.

doi:10.1371/journal.pone.0123123.t002

Phylogenetic analysis of denitrifiers in high latitude peatlands

Approximately 14 000 denoised quality-filtered sequences of the structural gene markers narG, nirK, nirS, and nosZ were utilized in total for further analyses. Forward and reverse reads for nirK and nirS showed a sufficiently long overlap (amplicon lengths of approximately 470 and 410 bp, respectively) to allow combined assessment of forward and reverse reads per gene for further analyses. Only forward reads of narG and nosZ were analyzed, as the overlap of forward and reverse reads was not sufficient to allow a combined analysis of forward and reverse reads (amplicon lengths approximately 670 and 700 bp for narG and nosZ, respectively), and previous studies indicate that results obtained from forward and reverse reads of narG and nosZ are similar [15, 23]. More than 99% of sequences generated from amplicons of a certain gene specific (i.e., narG, nirK, nirS, nosZ) primer set were specific amplicons of the target gene. All library coverages were greater than 99% at species-level DNA sequence dissimilarities of 33%, 17%, 18%, and 20% for narG, nirK, nirS, and nosZ, respectively, and varied from 80% to 97% at 3% sequence dissimilarity (Table 3), indicating that the number of sequences generated was sufficient.

narG sequences were assigned to 7 species-level OTUs in total. 7 and 4 OTUs were detected in 0 to 20 cm and 20 to 40 cm of fen soil, respectively (Table 3). narG community composition was similar in both sampled soil layers (Fig 4 A). Three OTUs had a relative abundance greater than 1%. Of those OTUs, OTU 1 dominated narG in fen soil (about 60% in both soil layers). About 40% of narG belonged to OTUs 2 and 3 (Fig 4 A). OTU 2 was more abundant in 0 to 20 cm than...
in 20 to 40 cm fen soil (relative abundances of 33% and 9%, respectively), whereas OTU 3 was more abundant in 20 to 40 cm fen soil (23% vs. 6% in 0 to 20 cm fen soil; Fig 4 A). Most of the OTUs were only distantly related to *nirG* of cultured organisms or environmental sequences (i.e., sequence dissimilarities of OTU representatives were 10–23%) (Table 4 and S3 Fig). Sequences of OTUs 1, 2, and 3 affiliated with *nirG* of Alphaproteobacteria, Actinobacteria, and Deinococci, respectively, more specifically they were related to *nirG* of uncultured bacteria and to those of *Oligotrophi carboxidovorans*, *Salinispora arenicola*, and *Marinithermus hydrothermalis*, respectively (Table 4 and S3 Fig). Observed *nirG* diversity was higher at 97% threshold similarity than at species-level threshold similarity (Table 3). At 97% threshold similarity, 359 and 230 OTUs were detected in 0 to 20 cm and 20 to 40 cm fen soil, respectively (Table 3). Shannon diversity, species evenness indices, and the observed number of OTUs calculated from rarified OTU tables indicated significantly higher diversity in 0–20 cm than 20–40 cm fen soil at 97% and species-level threshold similarity (Table 3). Beta-diversity measures indicated greater differences in community composition at 97% than at 67% threshold similarity (Table 3).

*nirK* were assigned to 24 species-level OTUs in total. 23 and 17 OTUs were detected in fen soil from 0 to 20 cm and from 20 to 40 cm, respectively (Table 3). Community composition differed significantly between the soil layers (Fig 4 B). OTU 2 dominated *nirK* in fen soil from 0 to 20 cm (about 60%), while OTU 1 dominated *nirK* in fen soil from 20 to 40 cm, respectively (about 70%; Fig 4 B). Similarities of OTU representative sequences to *nirK* of cultured organisms ranged from 75–100% (Table 4). Most OTUs were related to Alphaproteobacterial *nirK*. OTUs 1, 2, and 3 were related to *nirK* of *Brucella canis*, *Rhizobium etli*, and *Castellaniella sp.*, respectively (Table 4 and S4 Fig). Further OTUs were related to *nirK* of *Bosea sp.*, *Afipia sp.*, or uncultured bacteria (Table 4 and S4 Fig). *nirS* were assigned to 25 species-level OTUs in total. 22 and 23 OTUs were detected in fen soil from 0 to 20 cm and from 20 to 40 cm, respectively (Table 3). Differences in community composition of *nirS* from the soil layers were more pronounced than those of *nirK* (Fig 4 B and 4 C). *nirS* of fen soil was dominated by OTUs affiliated to Betaproteobacterial *nirS*. However, about 26% of detected *nirS* from 20 to 40 cm affiliated with Alphaproteobacterial *nirS* (S5 Fig). *nirS* of OTU representatives were only distantly related to *nirS* of cultured organisms (i.e., similarities ranged from 74–84%, Table 4). Many OTUs of both soil layers were related to *nirS* of uncultured wetland or marine sediment bacteria, and distantly related to *nirS* of e.g., *Thiobacillus denitrificans*, *Dechloromonas sp.*, and *Arthrobacter sp.* (Table 4 and S5 Fig). Diversity estimates calculated from rarified OTU tables of *nirK* based on species-level threshold similarities differed significantly between 0–20 and 20–40 cm fen soil (Table 3). Chao1 richness estimates of *nirS* did not differ significantly at species-level similarity thresholds, amounting to about 24 in both soil layers, while Shannon diversity as well as species evenness were significantly higher in the lower soil layer (Table 3). On the contrary, Shannon diversity, species Evenness, and Chao1 richness estimates of *nirK* and *nirS* calculated from rarified OTU tables based on 97% threshold similarity were consistently higher in 0 to 20 cm than 20 to 40 cm fen soil (Table 3).

*nosZ* forward reads were assigned to 10 species-level OTUs in total. 8 OTUs were detected in each soil layer (Table 3). OTU 1 dominated *nosZ* of fen soil from both soil layers (Fig 4 D). Essentially all *nosZ* from both soil layers affiliated with Alphaproteobacterial *nosZ* (S6 Fig). Most *nosZ* sequences from fen soil were distantly related to *nosZ* of cultured organisms with sequence dissimilarities ranging from 11–27% (Table 4), indicating hitherto uncultured denitrifiers capable of N₂O reduction in fen soil. *nosZ* sequences clustered with *nosZ* of wetland and upland soils, as well as *Achromobacter sp.*, *Herbaspirillum sp.*, and *Ralstonia sp.* (Table 4 and S6 Fig). Shannon diversity and species evenness calculated from rarified OTU tables at species-level threshold similarity were significantly higher in 0 to 20 cm than in 20 to 40 cm soil, while there was no significant difference in Chao1 richness estimates (Table 3).
Table 3. Diversity measures of amplicon pyrosequencing libraries of fen soil from rarified and non-rarified OTU tables of \(\text{narG}, \text{nirK}, \text{nirS}\) and \(\text{nosZ}\).

| Gene marker | Threshold similarity (%) | Soil depth (cm) | Original non-rarified OTU tables | \(\alpha\)-Diversity (based on rarified OTU tables) | \(\beta\)-Diversity (based on rarified OTU tables) |
|-------------|--------------------------|----------------|----------------------------------|-----------------------------------|-----------------------------------|
|             | No. of sequences | Library coverage (%) | No. of OTUs observed | No. of OTUs estimated | \(H^4\) | \(E^5\) | \(S_s^6\) | \(BC_s^7\) | \(D_{UU}^8\) | \(D_{WU}^9\) |
| \(\text{narG}\) | 67 | 0 to 20 | 1 141 | 99.7 | 7 | 9 ± 0.1 (A) | 1.28 ± 0.001 (A) | 0.46 ± 0.002 (A) | 0.31 ± 0.25 | 0.40 ± 0.20 |
|              | 20 to 40 | 1 697 | 99.9 | 4 | 4 ± 0.1 (B) | 1.20 ± 0.002 (B) | 0.65 ± 0.007 | 0.01 | 0.001 | 0.006 | 0.001 |
|              | 97 | 0 to 20 | 1 141 | 79.8 | 359 | 814 ± 5 (A) | 7.03 ± 0.003 (A) | 0.84 ± 0.001 (A) | 0.78 ± 0.85 | 0.71 ± 0.56 |
|              | 20 to 40 | 1 697 | 93.7 | 230 | 312 ± 3 (B) | 5.35 ± 0.006 (B) | 0.72 ± 0.001 (B) | 0.001 | 0.001 | 0.001 | 0.001 |
| \(\text{nirK}\) | 63 | 0 to 20 | 1 814 | 99.7 | 23 | 26 ± 0.3 (A) | 1.53 ± 0.002 (A) | 0.84 ± 0.001 (A) | 0.22 ± 0.64 | 0.41 ± 0.30 |
|              | 20 to 40 | 1 876 | 99.8 | 17 | 19 ± 0.4 (B) | 1.41 ± 0.002 (B) | 0.35 ± 0.001 (B) | 0.003 | 0.001 | 0.004 | 0.001 |
| \(\text{nirS}\) | 82 | 0 to 20 | 3 146 | 99.9 | 22 | 24 ± 0.3 | 2.14 ± 0.002 (A) | 0.49 ± 0.001 (A) | 0.13 ± 0.78 | 0.19 ± 0.32 |
|              | 20 to 40 | 3 382 | 99.9 | 23 | 24 ± 0.2 | 2.78 ± 0.001 (B) | 0.62 ± 0.001 (B) | 0.003 | 0.001 | 0.005 | 0.001 |
| \(\text{nosZ}\) | 80 | 0 to 20 | 572 | 100.0 | 8 | 8 ± 0.0 | 1.78 ± 0.003 (A) | 0.60 ± 0.001 (A) | 0.25 ± 0.30 | 0.30 ± 0.18 |
|              | 20 to 40 | 530 | 99.8 | 8 | 8 ± 0.0 | 0.79 ± 0.002 (B) | 0.26 ± 0.001 (B) | 0.001 | 0.001 | 0.001 | 0.001 |

Original OTU tables were rarified 100 times at sequence depths of 1000, 1500, 2500, and 500 for \(\text{narG}, \text{nirK}, \text{nirS}\), and \(\text{nosZ}\), respectively. Different letters in parentheses indicate that \(\alpha\)-diversity measures of 0 to 20 and 20 to 40 cm depth fen soil differed significantly (Student’s T-test, \(p < 0.001\)).

1 Percent library coverage \(C = (1 - ns/nt) \times 100\) (\(ns\) = OTUs that occur only once, \(nt\) = total number of sequences).

2 Number of OTUs observed in non-rarified OTU tables ± standard error.

3 Chao1 richness estimate of rarified OTUs ± standard error.

4 Shannon diversity index of rarified OTUs ± standard error.

5 Species Evenness of rarified OTUs ± standard error.

6 Sørensen similarity index of rarified OTUs ± standard error.

7 Bray Curtis similarity index of rarified OTUs ± standard error.

8 Unweighted Unifrac distance of rarified OTUs ± standard error.

9 Weighted Unifrac distance of rarified OTUs ± standard error.

doi:10.1371/journal.pone.0123123.t003
Fig 4. Relative abundances of denitrification associated genes in amplicon libraries of pH-neutral fen soil. OTUs of narG forward reads (A), nirK (B), nirS (C), and nosZ forward reads (D) were derived at species-level thresholds of 33%, 17%, 18%, and 20%, respectively.

doi:10.1371/journal.pone.0123123.g004
97% threshold similarity, all diversity estimates calculated from rarified OTU tables were significantly higher in the upper soil layer (Table 3). The difference in threshold similarity most strongly affected on the number of observed and estimated OTUs, which were similar at species-level threshold similarity (around 8 in both soil layers), but were about 3 times higher in 0 to 20 cm soil at 97% similarity threshold (Table 3). Beta-diversity was higher at 97% than at species-level threshold similarity (Table 3).

Quantification of narG, nirK, nirS, and nosZ relative to 16S rRNA genes
Copy numbers of all genes investigated in this study were corrected by inhibition factors that were experimentally determined for every DNA extract and gene analyzed (see Material and Methods). 16S rRNA gene copy numbers of 0–20 and 20–40 cm fen soil were $(5.3 \pm 0.3) \times 10^5$ and $(8.6 \pm 0.3) \times 10^5$ per ng DNA. Copy numbers of narG accounted for 7 and 3% of bacterial 16S rRNA gene copy numbers in 0 to 20 cm and 20 to 40 cm fen soil, respectively (Fig 5). Copy numbers of nirK, nirS, and nosZ were lower than narG copy numbers (Fig 5). Copy numbers of nirS were app. 100x and 10x higher than copy numbers of nirK and nosZ, respectively, in both soil layers (Fig 5). Copy numbers of narG and nosZ were 3 x higher ($P < 0.01$), and those of nirK were slightly lower in 0 to 20 cm than 20 to 40 cm fen soil ($P = 0.1$). Ratios of nosZ to narG were similar in both soil layers. Those of nosZ to nirK and nirS were 30 and 3 x higher, respectively, in 0 to 20 cm than 20 to 40 cm fen soil (Fig 5).

Discussion
pH neutral fen soil as N₂O sink
Peatlands are important ecosystems in the northern hemisphere and cover more than 30% of the Finnish land surface [36]. The potential of those peatlands to produce or consume greenhouse gases is of great interest, especially in respect to climate warming which is predicted to have a strong impact on peatlands [37]. N₂O emissions from natural wetlands are highly variable, and many water-saturated soils are also sinks for N₂O [2, 16, 17]. Many studies demonstrate that undrained, pH-neutral and acidic fens are sources of molecular nitrogen, and act as sinks for N₂O depending on environmental conditions [10, 14, 16, 38–40]. N₂O accumulation in gas chamber experiments from Puukkosuo fen were also variable, ranging from 10 ppb to -17 ppb at the time of soil sampling. Fen soil in situ consumed initially produced N₂O during nitrate or ammonium fertilization experiments (Fig 1). Previous studies show that mainly complete denitrification to N₂ occurs in pristine pH-neutral fens at in situ nitrate concentrations [10, 40]. Thus, the absence of in situ N₂O emission from Puukkosuo fen soil is likely due to complete denitrification to N₂ as the major end product (Fig 1). Even though the amount of stored nitrogen in the soil is high, low concentrations of available nitrate are observed in a northern boreal fen, where denitrification is thus N-limited [10]. Nitrate concentrations in Puukkosuo fen soil were likewise low, nitrite was not detected (Table 1), and nitrate as well as nitrite stimulated denitrification (Fig 3), indicating nitrate- and nitrite-limitation of fen denitrifiers. Microcosms and in situ fertilization with nitrate resulting in temporary in situ emission of N₂O with subsequent consumption indicated ongoing complete denitrification (Fig 1).

Peatland soils are temporarily or permanently water-logged, and oxygen generally penetrates only the uppermost centimeters, leading to oxygen-limitation in lower soil layers. In the absence of oxygen and nitrate, nitrous oxide is a potent sink for electrons released during the oxidation of organic carbon compounds, as the reduction of N₂O by H₂ is even more exergonic than O₂ reduction by H₂ (N₂O half-cell potential of $E_0$ (pH 7.0) = 1.35 V; $\Delta G_0^\circ = -339.5$ kJ mol⁻¹; reviewed in [41]). In situ relevant concentrations of dissolved organic carbon (app. 5 μM glucose equivalents; e.g., [42]) and atmospheric concentrations of
Table 4. OTU representatives retrieved from pH-neutral fen soil.

| Gene marker | OTU (accession No.) | Closest relative (accession No.) | Similarity (%) | Closest cultured relative (accession No.) | Similarity (%) | Relative abundance of OTUs in amplicon libraries (%) |
|-------------|---------------------|----------------------------------|----------------|-------------------------------------------|---------------|-----------------------------------------------|
|             |                     |                                  |                |                                           |               | 0 to 20 cm  Below 20 cm                        |
| nfrG        | 1 (HE616587)        | Oligotropha carboxidovorans OM5 (CP001196) | 90             | Oligotropha carboxidovorans OM5 (CP001196) | 90            | 59.7  67.2                                    |
|             | 2 (HE616588)        | Salinispora arenicola CNS-205 (CP000850) | 77             | Salinispora arenicola CNS-205 (CP000850) | 77            | 33.4  9.0                                     |
|             | 3 (HE616589)        | uncultured bacterium (FJ566669)     | 88             | Marinithermus hydrothermales DSM 14884 (CP002630) | 75            | 6.4  23.7                                    |
|             | 4 (HE616590)        |                      |                |                                           |               |                                               |
|             | 5 (HE616591)        |                      |                |                                           |               |                                               |
|             | 6 (HE616592)        |                      |                |                                           |               |                                               |
|             | 7 (HE616593)        |                      |                |                                           |               |                                               |
|             | 8 (HE616594)        |                      |                |                                           |               |                                               |
|             | 9 (HE616595)        |                      |                |                                           |               |                                               |
|             | 10 (HE616596)       |                      |                |                                           |               |                                               |
|             | 11 (HE616597)       |                      |                |                                           |               |                                               |
|             | 12 (HE616598)       |                      |                |                                           |               |                                               |
|             | 13 (HE616599)       |                      |                |                                           |               |                                               |
|             | 14 (HE616600)       |                      |                |                                           |               |                                               |
|             | 15 (HE616601)       |                      |                |                                           |               |                                               |
|             | 16 (HE616602)       |                      |                |                                           |               |                                               |
|             | 17 (HE616603)       |                      |                |                                           |               |                                               |
|             | 18 (HE616604)       |                      |                |                                           |               |                                               |
|             | 19 (HE616605)       |                      |                |                                           |               |                                               |
|             | 20 (HE616606)       |                      |                |                                           |               |                                               |
|             | 21 (HE616607)       |                      |                |                                           |               |                                               |
|             | 22 (HE616608)       |                      |                |                                           |               |                                               |

(Continued)
| Gene marker | OTU (accession No.) | Closest relative (accession No.) | Similarity (%) ¹ | Closest cultured relative (accession No.) | Similarity (%) ¹ | Relative abundance of OTUs in amplicon libraries (%) |
|-------------|---------------------|---------------------------------|-----------------|------------------------------------------|-----------------|--------------------------------------------------|
|             |                     |                                 |                 |                                          |                 | 0 to 20 cm  Below 20 cm                           |
| nosZ        | 1 (HE616616)        | uncultured bacterium (DQ010777) | 99               | Bosea sp. PD 24 (DQ377796)              | 89              | 60.7 88.3                                       |
|             | 2 (HE616617)        | Achromobacter sp. PD 27 (DQ377799) | 85              | Achromobacter sp. PD 27 (DQ377799)     | 85              | 13.6 5.3                                       |
|             | 3 (HE616618)        | Azospirillum largimobile ACM 2041 (AY072228) | 88              | Azospirillum largimobile ACM 2041 (AY072228) | 88              | 13.1 0.6                                       |
|             | 4 (HE616619)        | uncultured bacterium (FN859926)  | 95               | Herbaspirillum sp. TSA29 (AB542280)     | 75              | 7.3 1.5                                        |
|             | 5 (HE616620)        | uncultured bacterium (FN859707)  | 98               | Ralstonia solanacearum GMI1000 (AL646053) | 73              | 3.3 1.5                                        |
|             | 6 (HE616621)        | uncultured bacterium (DQ324384)  | 90               | Ralstonia eutropha H16 (NC 005241)      | 73              | 0.5 1.3                                        |
|             | 7 (HE616622)        | uncultured bacterium (FN430515)  | 99               | Rhodobacter sphaeroides f. sp. denitrificans IL106 (AF125260) | 78              | 0.0 1.3                                        |

¹ Determined after alignment in MEGA 5.0.

doi:10.1371/journal.pone.0123123.t004
N₂O result in -360 kJ mol⁻¹ N₂O (http://cms.uni-konstanz.de/schink/dg-calculator/), allowing for high energy conservation of organisms capable of N₂O reduction. Lower below-surface N₂O concentrations than at atmospheric equilibrium are observed in fens and suggest ongoing N₂O consumption [10, 16, 38]. Indeed, apparent KM values of Puukkosuo fen soil were approximately 60 times lower for N₂O than for nitrate (Table 2), indicating a higher affinity of fen denitrifiers for N₂O than for nitrate. The assumed absence of oxygen, the observed nitrate-limitation and high N₂O affinity indicate a strong in situ sink potential of Puukkosuo fen for N₂O.

Diverse denitrifier communities are associated with denitrification activities in pH-neutral fen soil

Unsupplemented fen soil from both sampled soil layers produced N₂O in acetylene-amended microcosms, demonstrating the denitrification potential of the fen soil. However, nearly no N₂O was produced in the absence of acetylene and initially produced N₂O was subsequently consumed (Fig 2). Nitrate- and oxygen-limitation might select for denitrifiers capable of complete denitrification, and hitherto unknown denitrifiers as well as N₂O reducers might occur in Puukkosuo fen soil. Indeed, nosZ copy numbers in 0 to 20 cm fen soil were of a similar magnitude as nitrite reductase copy numbers (Fig 5), and newly-discovered nirK/S and nosZ (Table 4) indicate that a high percentage of uncharacterized denitrifiers in that soil layer possessed a complete denitrification pathway.

Supplemental nitrate and nitrite resulted in immediate N₂O production in fen soil after internal nitrate and nitrite were consumed. Stimulation was greater with nitrite than with nitrate.
in both soil layers (Fig 3 A). This reflects the fact that all denitrifiers “sensu stricto” use nitrite as electron acceptor, while many cultured denitrifiers lack the ability to use nitrate as electron acceptor [20, 43]. Stimulation was also greater in the top soil layer (Fig 3 A), reflecting a greater denitrification potential of the top soil. In other wetland and also agricultural soils, denitrification potentials are also highest in the top soil layers (e.g., [14, 44]). In 20 to 40 cm fen soil, N₂O production and total denitrification decreased with increasing nitrate concentrations, indicating substrate inhibition of denitrification at high nitrate concentrations. This finding is in contrast to denitrification potentials reported for deeper horizons of agricultural soils, suggesting that the fen denitrifier community of 20–40 cm depth is well adapted to low nitrate concentrations (e.g., [44]). Nitrate and nitrite reduction compete for electrons at high nitrate concentrations, and nitrate reduction is favored over the rest of the denitrification pathway, causing eventually accumulation of nitrite when electron donors are limiting [45].

The ratio of N₂O to (N₂+N₂O) was lower in nitrate- and nitrite-amended microcosms with 0 to 20 cm fen soil than in 20 to 40 cm fen soil when nitrate or nitrite were supplied (S2 Fig), and consumption of supplied N₂O was about 2-fold higher in 0 to 20 cm than in 20 to 40 cm fen soil (Fig 3 B). Indeed, the ratio of nitrite to nitrous oxide reductases was higher in 20 to 40 cm fen soil than in 0 to 20 cm fen soil (Fig 5), indicating an increased amount of denitrifiers lacking nitrous oxide reductase in the lower soil layer. The ratio of nitrite reductase genes to N₂O reductase genes is highly variable in soils, and often nitrite reductase copy numbers largely exceed N₂O reductase copy numbers [15, 23, 46]. However, non-denitrifying NO₃ consumers were recently shown to be quantitatively important in certain soils [47, 48]. Relative abundances of both atypical and typical nosZ assigned to non-denitrifiers and denitrifiers, respectively, are variable in soil metagenomes. Hence, further analyses including both groups are demanded for better understanding of N₂O reducers in fens [49]. Nevertheless, N₂O produced in lower layers of fen soil can diffuse upwards and be further reduced to N₂ in upper soil layers, and thus emission of N₂O into the atmosphere can be reduced [14, 16, 38]. It is thus hypothesized that also in Puukkosuo fen soil lower soil layers are N₂O sinks while upper soil layers are N₂O sinks.

The analysis of denitrification-specific gene markers indicated a higher diversity of these genes in 0 to 20 cm than in 20 to 40 cm fen soil (Table 3). Detected narG and nosZ were more similar in 0 to 20 cm and 20 to 40 cm fen soil than nirK and nirS (Fig 4), indicating that nitrite reductases show a higher variability in fen soil than nitrate and N₂O reductases. Nitrite reductase community composition is highly variable in other types of peatland soils, including permafrost affected systems, while variations in nitrate and N₂O reductase community composition are much less pronounced [15, 23]. Indeed, the distribution of nitrite reductases is more heavily impacted by changes in environmental conditions than those of nitrate or N₂O reductases [50–52]. Nitrite reductase genes from fen soil were affiliated with Proteobacterial nirK/S (Table 4 and S4 Fig and S5 Fig). For nirS, sequences related to Rhodanobacter/Bradyrhizobium were detected (S5 Fig). Such sequences are also detected in other peatland soils such as permafrost affected tundra and palsa peat soils [15, 23]. Proteobacteria-affiliated sequences of narG and nosZ (Table 4 and S3 Fig and S6 Fig) further support that Proteobacteria play an important role for denitrification in this pH-neutral fen soil. Denitrification-associated genes related to Proteobacteria are also found in acidic fen soils or permafrost-affected peatlands [14, 15, 23], indicating that Proteobacteria represent general peatland denitrifiers. Sequences of narG were also affiliated with Actinobacterial narG (10–30%; S3 Fig). Actinobacteria are common in soils, include many genera capable of nitrate reduction, and are in general considered to be more tolerant to extreme environmental conditions such as low pH or low temperature [53–55]. Actinobacteria and Actinobacteria-affiliated gene markers are frequently detected in a variety of peatlands including acidic fen soils, permafrost-affected tundra and palsa peat soils.
However, in those more extreme environments, *Actinobacteria* often dominate the *narG* communities, indicating that *Actinobacteria* are further important players involved in nitrate reduction and potentially denitrification in pH-neutral fen soil [14, 15, 23]. The nitrate reducer community in pH-neutral fen soil also contained a substantial portion of *Deinococci*-affiliated *narG* (Fig 4A and S3 Fig.), which are not detected in the above mentioned more extreme habitats such as acidic fens, frost-affected tundra and palsa peat soils [14, 23]. Soil pH is a driver of the general microbial community structure [56]. Denitrifier diversity in pH-neutral fen soil is high when compared to more acidic pristine peatland soils [14, 15, 23], suggesting that soil pH likewise plays an important role in shaping denitrifier communities.

Denitrifier diversity and quantity is routinely underestimated due to choice of primer sets, e.g., gram-positive denitrifiers escaped detection in many studies [46, 50, 57]. Soil metagenomes might represent an alternative strategy to obtain a more complete picture of denitrifier diversity in soils. However, the low abundance of denitrification associated genes on denitrifier genomes (i.e., app. 1%; most of the genes on denitrifier genomes are associated with other functions than denitrification like anabolism, motility, etc.) in combination with a low number of denitrifiers compared to total number of prokaryotes in soil (app. 1%) limits their detection by metagenomics [49, 58, 59]. However, metagenomes are extremely useful for the design of denitrification gene specific primers. Although amplicon based approaches combined with next generation sequencing depend on the choice of primers, such approaches currently provide a cost-effective way for the detection of a large denitrifier diversity.

The collective data indicate that (i) a core nitrate reducer/denitrifier community might be common to all kinds of (northern) peatlands, (ii) some nitrate reducers/denitrifiers are unique in pH-neutral fen soil, possible due to the lack of environmental stress that might be induced by acidic pH, (iii) denitrifier communities are from upper and lower layers are dissimilar as indicated by apparent Michaelis-Menten kinetics and structural gene marker analyses, and (iv) pH-neutral fens are a strong potential sink for atmospheric N₂O.

**Supporting Information**

**S1 Fig. Effect of supplemental nitrate (1) and nitrite (2) on N₂O production and consumption in microcosms with fen soil.** Squares and circles represent fen soil from 0 to 20 cm and 20 to 40 cm depth, respectively. Microcosms with and without acetylene are represented by closed and open symbols, respectively. Supplied concentrations of nitrate or nitrite were 0 μM (A), 10 μM (B), 20 μM (C), 50 μM (D), 100 μM (E), 500 μM (F), and 1000 μM (G). Mean values and standard errors of three replicate microcosms are shown.

(TIF)

**S2 Fig. Effect of supplemental nitrate (black) or nitrite (white) on the ratio of N₂O to (N₂+N₂O) in anoxic microcosms with fen soil from 0 to 20 cm (A) and 20 to 40 cm (B) depth.** Mean values and standard errors of three replicates are shown.

(TIF)

**S3 Fig. Phylogenetic tree of *narG* OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil.** The trees was calculated based in translated amino acid sequences of *narG* forward reads. OTUs were grouped at a species-level threshold dissimilarity of 33%. Numbers preceding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parentheses show the relative abundances of each OTU in 0 to 20 cm (left) and 20 to 40 cm (right) fen soil. Grey boxes indicate reference sequences belonging to the same phylogenetic group. The percentage of replicate trees that produced the observed clustering of taxa in the bootstrap test (10 000 replications) are shown next to the
branches. Bootstrap supports below 50% are not displayed. *narG* of *Haloarcula marismortui* ATCC 43049 was used as outgroup to root the tree.

(TIF)

**S4 Fig. Phylogenetic tree of nirK OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil.** The tree was calculated based on translated amino acid sequences. OTUs were grouped at a species-level threshold dissimilarity of 17%. Numbers preceding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parentheses show the relative abundances of each OTU in 0 to 20 cm (left) and 20 to 40 cm (right) fen soil. Grey boxes indicate reference sequences belonging to the same phylogenetic group. The percentages of replicate trees that produced the observed clustering of taxa in the bootstrap test (10,000 replications) are shown next to the branches. Bootstrap supports below 50% are not displayed. *nirK* of *Nitrosomonas* sp. C-56 was used as outgroup to root the tree.

(TIF)

**S5 Fig. Phylogenetic tree of nirS OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil.** The tree was calculated based on translated amino acid sequences. OTUs were grouped at a species-level threshold dissimilarity of 18%. Numbers preceding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parentheses show the relative abundances of each OTU in 0 to 20 cm (left) and 20 to 40 cm (right) fen soil. Grey boxes indicate reference sequences belonging to the same phylogenetic group, white boxes indicate single taxa not belonging to the major phylogenetic group. The percentages of replicate trees that produced the observed clustering of taxa in the bootstrap test (10,000 replications) are shown next to the branches. Bootstrap supports below 50% are not displayed. *nirS* of *Rhodothermus marinus* DSM 4252 was used as outgroup to root the tree.

(TIF)

**S6 Fig. Phylogenetic tree of nosZ OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil.** The tree was calculated based on translated amino acid sequences of *nosZ* forward reads. OTUs were grouped at a species-level threshold dissimilarity of 20%. Numbers preceding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parentheses show the relative abundances of each OTU in 0 to 20 cm (left) and 20 to 40 cm (right) fen soil. Grey boxes indicate reference sequences belonging to the same phylogenetic group, white boxes indicate single taxa not belonging to the major phylogenetic group. The percentages of replicate trees that produced the observed clustering of taxa in the bootstrap test (10,000 replications) are shown next to the branches. Bootstrap supports below 50% are not displayed. *nosZ* of *Haloarcula marismortui* ATCC 43049 was used as outgroup to root the tree.

(TIF)

**Acknowledgments**

We are thankful to Jyrki Manninen for organisational help, the team at Oulanka research station for excellent on-site support, Christian Hofmann for assistance with gas measurements, the Central Analytics Department of BayCEER for analyses of nitrate, nitrite, ammonium, TC, TN, and TOC, Rolf Daniel and Andrea Thürmer for pyrosequencing, and Steffen Kolb, Markus Nebel, Sebastian Wild, Justin Kuczynski and Christopher Quince for help with sequence analyses.
Author Contributions
Conceived and designed the experiments: KP MAH. Performed the experiments: KP. Analyzed the data: KP MAH. Contributed reagents/materials/analysis tools: MAH. Wrote the paper: KP MAH.

References
1. Gorham E. Northern peatlands—role in the carbon-cycle and probable responses to climatic warming. Ecol Appl 1991; 1: 182–195.
2. Takakai F, Desyatkin AR, Lopez CML, Fedorov AN, Desyatkin RV, Hatano R. CH₄ and N₂O emissions from a forest-as alas ecosystem in the permafrost taiga forest region, eastern Siberia, Russia. J Geophys Res—Biogeio 2008; 113: G02002.
3. Christensen TR, Ekberg A, Strom L, Mastepanov M, Panikov N, Oquist M, et al. Factors controlling large scale variations in methane emissions from wetlands. Geophys Res Lett 2003; 30: 1414.
4. Roulet N, Moore T, Bubier J, Lalfluer P. Northern fens—methane flux and climatic change. Tellus B 1992; 44: 100–105.
5. Whalen SC. Biogeochemistry of methane exchange between natural wetlands and the atmosphere. Environ Eng Sci 2005; 22: 73–94.
6. Forster P, Ramaswamy V, Artaxo P, Berntsen T, Betts R, Fahey D, et al. Changes in atmospheric constituents and in radiative forcing. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, et al., editors. Climate Change 2007: The physical science basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge: Cambridge University Press; 2007. pp. 129–234.
7. Schulze ED, Luyssaert S, Ciais P, Freibauer A, Janssens IA, Soussana JF, et al. Importance of methane and nitrous oxide for Europe’s terrestrial greenhouse-gas balance. Nature Geosci 2009; 2: 842–850.
8. Ravishankara AR, Daniel JS, Portmann RW. Nitrous oxide (N₂O): The dominant ozone-depleting substance emitted in the 21st century. Science 2009; 326: 123–125. doi: 10.1126/science.1176985 PMID: 19713491
9. Smith KA. The potential for feedback effects induced by global warming on emissions of nitrous oxide by soils. Glob Change Biol 1997; 3: 327–338.
10. Lohila A, Aurela M, Hatakka J, Pihlajamäki M, Minkkinen K, Penttilä T, et al. Responses of N₂O fluxes to temperature, water table and N deposition in a northern boreal fen. Eur J Soil Sci 2010; 61: 651–661.
11. Maljanen M, Sigurdsson BD, Guomundsson J, Oskarsson H, Huttunen JT, Martikainen PJ. Greenhouse-gas balances of managed peatlands in the nordic countries—present knowledge and gaps. Biogeosciences 2010; 7: 2711–2738.
12. Martikainen PJ, Nykänen H, Crill P, Silvola J. Effect of a lowered water-table on nitrous-oxide fluxes from northern peatlands. Nature 1993; 366: 51–53.
13. Marushchak M, Pitkämäki A, Koponen H, Biasi C, Seppälä M, Martikainen P. Hot-spots for nitrous oxide emissions found in different types of permafrost peatlands. Glob Change Biol 2011; 17: 2601–2614.
14. Palmer K, Drake HL, Horn MA. Association of novel and highly diverse acid-tolerant denitrifiers with N₂O fluxes of an acidic fen. Appl Environ Microb 2010; 76: 1125–1134. doi: 10.1128/AEM.02256-09 PMID: 20023077
15. Palmer K, Biasi C, Horn MA. Contrasting denitrifier communities relate to contrasting N₂O emission patterns from acidic peat soils in arctic tundra. ISME J 2012; 6: 1058–1077. doi: 10.1038/ismej.2011.172 PMID: 22134649
16. Goldberg SD, Knorr KH, Gebauer G. N₂O concentration and isotope signature along profiles provide deeper insight into the fate of N₂O in soils. Isot Environ Healt S 2008; 44: 377–391. doi: 10.1080/10256010802507433 PMID: 19061068
17. Kolb S, Horn MA. Microbial CH₄ and N₂O consumption in acidic wetlands. Front Microbiol 2012; 3: 78. doi: 10.3389/fmicb.2012.00078 PMID: 22403579
18. Bremner JM. Sources of nitrous oxide in soils. Nutr Cycl Agroecosys 1997; 49: 7–16.
19. Conrad R, Soil microorganisms as controllers of atmospheric trace gases (H₂, CO, CH₄, OCS, N₂O, and NO). Microbiol Rev 1996; 60: 609–640. PMID: 8987358
20. Zumft WG. Cell biology and molecular basis of denitrification. Microbiol Mol Biol R 1997; 61: 533–615. PMID: 9409151
21. van Cleemput O. Subsoils: Chemo- and biological denitrification, N₂O and N₂ emissions. Nutr Cycl Agroecosyst 1998; 52: 187–194.
22. Moore TR, Roulet NT, Waddington JM. Uncertainty in predicting the effect of climatic change on the carbon cycling of Canadian peatlands. Clim Change 1998; 40: 229–245.
23. Palmer K, Horn MA. Actinobacterial nitrate reducers and Proteobacterial denitrifiers are abundant in N₂O-metabolizing palsa peat. Appl Environ Microb 2012; 78: 5584–5596. doi:10.1128/AEM.00810-12 PMID: 22660709
24. Yoshinari T, Knowles R. Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem Bioph Res Co 1976; 69: 705–710.
25. Ratkowsky D. Nonlinear regression modeling: a unified and practical approach. 1st ed. New York: Marcel Dekker Inc.; 1983.
26. Persoh D, Theuerl S, Buscot F, Rambold G. Towards a universally adaptable method for quantitative extraction of high-purity nucleic acids from soil. J Microbiol Meth 2008; 72: 6121–6128. doi:10.1016/j.jmim.2008.02.016 PMID: 18234587
27. Philippot L, Piutti S, Martin-Laurent F, Hallet S, Germon JC. Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Appl Environ Microb 2002; 68: 5974–5982. PMID: 12450836
28. Throback IN, Enwall K, Jarvis A, Hallin S. Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiol Ecol 2004; 49: 401–417. doi: 10.1111/j.1574-6941.2004.tb01974.x PMID: 15234336
29. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. Qiime allows analysis of high-throughput community sequencing data. Nat Methods 2009; 11: 95–95. doi:10.1038/nmeth.f.303 PMID: 20383131
30. Hughes JB, Hellmann JJ, Ricketts TH, Bohannan BJM. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 2001; 67: 4399–4406. PMID: 11571135
31. Zaprasis A, Liu YJ, Liu SJ, Drake HL, Horn MA. Abundance of novel and diverse tfdA-like genes, encoding putative phenoxyalkanoic acid herbicide-degrading dioxygenases, in soil. Appl Environ Microbiol 2010; 76: 119–128. doi:10.1128/AEM.01727-09 PMID: 19880651
32. Eurola S, Hicks S, Kaakinen E. Key to Finnish mire types. In: Moore PD, editor. European mires. London: Academic Press Inc; 1984. pp. 11–118.
33. Lemke P, Ren J, Alley R, Allison I, Carrasco J, Flato G, et al. Observations: Changes in snow, ice and frozen ground. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, et al., editors. Climate Change 2007: The physical science basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge: Cambridge University Press; 2007. pp. 337–283.
34. Blücher-Mathiesen G, Hoffmann C. Denitrification as a sink for dissolved nitrous oxide in a freshwater riparian fen. J Environ Qual 1999; 28: 257–262.
35. Chapuis-Lardy L, Wrage N, Metay A, Chotte JL, Bernoux M. Soils, a sink for N₂O? A review. Glob Change Biol 2007; 13: 1–17.
36. Roobroeck D, Butterbach-Bahl K, Bruegge mann N, Boeckx P. Dinitrogen and nitrous oxide exchanges from an undrained monolith fen: shortterm responses following nitrate addition. Eur J Soil Sci 2010; 61: 662–670.
37. Zumft WG, Kroneck PMH. Respiratory transformation of nitrous oxide (N₂O) to dinitrogen by Bacteria and Archaea. Adv Microb Physiol 2007; 52: 107–227. PMID: 17027372
38. Kirchman DL, Meon B, Ducklow HW, Carlson CA, Hansell DA, Steward GF. Glucose fluxes and concentrations of dissolved combined neutral sugars (polysaccharides) in the Ross Sea and Polar Front Zone, Antarctica. Deep Sea Res. Part II Top Stud Oceanogr 2001; 48: 4179–4197.
43. Mahne I, Tiedje JM. Criteria and methodology for identifying respiratory denitrifiers. Appl Environ Microbiol 1995; 61: 1110–1115. PMID: 16534960

44. Jahangir MMR, Khalil MI, Johnston P, Cardenas LM, Hatch DJ, Butler M, et al. Denitrification potential in subsoils: A mechanism to reduce nitrate leaching to groundwater. Agric Ecosyst Environ 2012; 147: 13–23.

45. Almeida JS, Reis MAM, Carrondo MJT. Competition between nitrate and nitrite reduction in denitrification by Pseudomonas fluorescens. Biotechnol Bioeng 1995; 46: 476–484. PMID: 18623340

46. Henry S, Bru D, Stres B, Hallet S, Philippot L. Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. Appl Environ Microbiol 2006; 72: 5181–5189. PMID: 16885263

47. Sanford RA, Wagner DD, Wu Q, Chee-Sanford JC, Thomas SH, Cruz-Garcia C, et al. Unexpected non-denitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc Nat Acad Sci USA 2012; 109: 19709–19714. doi: 10.1073/pnas.1211238109 PMID: 23150571

48. Jones CM, Graf DRH, Bru D, Philippot L, Hallin S. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISMEJ 2013; 7: 417–426. doi: 10.1038/ismej.2012.125 PMID: 23151640

49. Orellana LH, Rodríguez-R LM, Higgins S, Chee-Sanford JC, Sanford RA, Ritalahti KM, et al. Detecting nitrous oxide reductase (nosZ) genes in soil metagenomes: Method development and implications for the nitrogen cycle. mBio 2014; 5: e01193–14. doi: 10.1128/mBio.01193-14 PMID: 24895307

50. Braker G, Conrad R. Diversity, structure, and size of N₂O-producing microbial communities in soils: what matters for their functioning? Adv Appl Microbiol 2011; 75: 33–70. doi: 10.1016/B978-0-12-387046-9.00002-5 PMID: 21807245

51. Bru D, Ramette A, Saby NPA, Dequiet S, Ranjard L, Jolivet C, et al. Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. ISME J 2011; 5: 532–542. doi: 10.1038/ismej.2010.130 PMID: 20703315

52. Cuhel J, Simek M, Laughlin RJ, Bru D, Cheneby D, Watson CJ, et al. Insights into the effect of soil pH on N₂O and N₂ emissions and denitrifier community size and activity. Appl Environ Microbiol 2010; 76: 1870–1878. doi: 10.1128/AEM.02484-09 PMID: 20118356

53. Männistö MK, Häggblom MM. Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. Syst Appl Microbiol 2006; 29: 229–243. PMID: 16564959

54. Zenova GM, Manucharova NA, Zvyagintsev DG. Extremophilic and extremotolerant actinomycetes in different soil types. Eurasian Soil Sci 2011; 44: 417–436.

55. Whitman WB, Goodfellow M, Kämpfer P, Busse HJ, Trujillo ME, Suzuki KI, et al. Bergey's manual of systematic bacteriology: Volume 5—The Actinobacteria. 2 nd ed. New York: Springer; 2012.

56. Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. Proc Nat Acad Sci USA 2006; 103: 626–631. PMID: 16407148

57. Jones CM, Welsh A, Throback IN, Dorsch P, Bakken LR, Hallin S. Phenotypic and genotypic heterogeneity among closely related soil-borne N₂O- and N₂-producing Bacillus isolates harboring the nosZ gene. FEMS Microbiol Ecol 2011; 76: 541–552. doi: 10.1111/j.1574-6941.2011.01071.x PMID: 21348884

58. Demanèche S, Philippot L, David MM, Navarro E, Vogel TM, Simonet P. Characterization of denitrification gene clusters of soil bacteria via a metagenomic approach. Appl Environ Microbiol 2009; 75: 534–537. doi: 10.1128/AEM.01706-08 PMID: 19011059

59. Singh BK, Campbell CD, Sorenson SJ, Zhou J. Soil genomics. Nat Rev Micro 2009; 7: 756–756.