Greenhouse gas metabolizing prokaryotes in peatlands

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We know what we are, but not what we may be.

William Shakespeare - Hamlet

I shall be telling this with a sigh

Somewhere ages and ages hence:

Two roads diverged in a wood, and I -

I took the one less traveled by,

And that has made all the difference.

Robert Frost - The road not taken

A truly happy person is one who can enjoy the scenery while on a detour.

Author Unknown

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BES	Bromoethanesulfonate
CH_4	methane
CO_2	carbon dioxide
DW	dry weight
H^+	proton
H_2	molecular hydrogen
H_2O	water
N_2	molecular nitrogen
NAG	N-Acetylglucosamine
NO_3^-	nitrate
NO_2^-	nitrite
NO	nitric oxide
N_2O	nitrous oxide
O_2	molecular oxygen
OTU	Operational Taxonomic Unit
PC	Cryoturbated permafrost peat (Peat Circles)
ppb	parts per billion
ppm	parts per million
PS	Fen Puukkosuo
PT	Peat plateau permafrost tundra
SB	Fen Schlöppnerbrunnen
SV	Skalluvaara palsa peat
v/v	volume per volume
$W \cdot m^{-2}$	Watt per square meter

Summary

Peatlands in the northern hemisphere store substantial amounts of carbon and nitrogen, and are predicted to react sensitively to global warming. Most peatlands are sources of the greenhouse gas methane (CH_4), which is produced by methanogens in peatland soil that are trophically linked to fermenters. The greenhouse gas nitrous oxide (N_2O) can be produced or consumed by denitrifiers in peatland soils. Source and sink strengths for CH_4 and N_2O show great variability between different peatland types. Thus, the main objective of the study was to study microbial processes involved in greenhouse gas fluxes from peatlands, namely fermentation and methanogenesis as important contributors to CH_4 fluxes, and denitrification as a contributor to N_2O fluxes. Five model peatlands with contrasting features were assessed (two fens (pH 7 and pH 5), peat plateau tundra (pH 4), palsa peat (pH 4.5), and cryoturbated peat circles (pH 4)). Those contrasting peatlands differed in *in situ* CH_4 and N_2O emissions as well as in e.g., nitrate content, mean annual temperature and/or pH.

Processes involved in CH_4 production were assessed in pH-neutral fen soil as a model system. The soil produced CH_4 *in situ* as well as in unsupplemented anoxic microcosms with fen soil. Supplemental N-Acetylglucosamine (NAG) stimulated formation of fermentation products as well as CH_4 and lead to increased accumulation of fermentation products (mainly acetate, H_2/CO_2, as well as minor amounts of ethanol, formate and propionate) when methanogenesis was inhibited by Bromoethanesulfonate (BES), indicating that methanogens use fermentation products as substrates, being thus trophically linked to fermenters. Supplemental H_2/CO_2 and formate greatly stimulated methanogenesis, while acetate and methanol stimulated methanogenesis to a much lesser extent, indicating that hydrogenotrophic methanogenesis is an important CH_4 producing process in pH-neutral fen soil. A

high diversity of bacterial families was detected in fen soil by barcoded amplicon pyrosequencing of bacterial 16S rRNA genes, including *Firmicutes*, *Alpha-* and *Deltaproteobacteria* as well as many novel families. Within the detected families, genera known for syntrophic interactions with methanogens (e.g., *Clostridium*, *Syntrophobacter*) were found, indicating that those genera might be important providers for methanogenic substrates. Diversity of methanogens was lower than bacterial diversity, as only *Methanomicrobiales* and *Methanocellales* (methanogens of both orders are hydrogenotrophs) were detected by analysis of the structural gene marker *mcrA* (encoding the methyl-coenzyme M reductase), likewise indicating that hydrogenotrophic methanogens are dominating in pH-neutral fen soil. Both process data and molecular data suggest that (i) hydrogenotrophic methanogenesis is the main process of CH_4 formation in pH-neutral fen soil, and (ii) a high diversity of bacterial families occur in pH-neutral fen soil that are likely involved in diverse fermentations, thus providing substrates for fen methanogens.

Denitrification is considered to be the main source of N_2O in waterlogged soils such as peatland soils, as high water saturation promotes anoxia in the soil. As N_2O is an intermediate in the denitrification process, denitrifiers can be producers as well as consumers of N_2O. Despite their important role in the global N_2O budget, factors controlling denitrification in pristine peatlands and the associated diversity of the denitrifier community are virtually unknown. Thus, denitrification and N_2O consumption potentials as well as denitrifier community composition were assessed in all five northern peatlands. *In situ* N_2O emissions range from < 0.01 mg $N_2O \cdot m^{-2} \cdot d^{-1}$ in pH-neutral fen soil to approximately 10 mg $N_2O \cdot m^{-2} \cdot d^{-1}$ in cryoturbated peat circle soil, thus cryoturbated peat soil is an important N_2O source, emitting in the same range as tropical or agricultural soils. *In situ* N_2O emissions were positively and negatively correlated with soil nitrate and ammonia contents, respectively. All

soils produced and consumed N_2O in anoxic microcosms without apparent delay. N_2O production capacities and apparent affinities (v_{max}/K_M) for nitrate were likewise positively correlated with soil nitrate content. N_2O production capacities were especially high in acidic, permafrost-affected cryoturbated peat soil, and co-occurred with high *in situ* N_2O emissions and high nitrate contents. On the other hand, N_2O consumption capacities were highest in pH-neutral fen soil, and co-occurred with low *in situ* emissions and low nitrate content. Phylogenetic analyses of the nitrate reduction- and denitrification-associated genes *narG*, *nirK/nirS*, and *nosZ* (encoding nitrate, nitrite, nitrite, and N_2O reductases, respectively) indicated that the diversity of the denitifier community was highest in pH-neutral fen soil, and that diversity was positively correlated with pH. Detected nitrate reductase genes affiliated mainly with *Betaproteobacterial* and *Actinobacterial narG*. *Betaproteobacterial narG* dominated in pH-neutral fen soil, while *Actinobacterial narG* were predominant in all more acidic soils, indicating that *Actinobacterial* nitrate reducers might have a higher tolerance to acidity. The number and the identity of observed operational taxonomic units (OTUs) of *nirK*, *nirS*, and *nosZ* in pH-neutral fen soil was clearly distinct from those of the more acidic soils, and indicated *Alpha-*, *Beta-*, and *Gammaproteobacterial* denitrifiers in all peatlands. These results were confirmed by canonical correspondence analysis (CCA) of relative OTU abundances in amplicon libraries, suggesting that the denitrifier community of the pH neutral fen was unique among the analyzed peatlands. Permafrost-affected soils mainly clustered together in the CCA plots based on all analyzed genes. pH was the most important factor determining the community composition of nitrate reducers (as indicated by *narG*) and denitrifiers (as indicated by all analyzed genes). Significant influences of soil carbon content (on *narG*, *nirK*), precipitation (on *narG*), or temperature (on *nosZ*) were also detected. The ratio of *narG/nosZ* copy numbers was positively correlated

with N_2O emissions, as was the occurrence of certain OTUs of *nirK* and *nirS*. The collective data indicate that (i) denitrification is an ongoing processes in different types of pristine peatland soils, (ii) source and sink function of peatland denitrifiers for N_2O are influenced by soil nitrate content as well as denitrifier community composition, and (iii) nitrate reducer and denitrifier community composition are affected by pH, temperature, precipitation, and soil carbon content.

Global warming is predicted to increase the frequency of extreme weather events, causing periods of prolonged drought or excess rainfall. Those events affect the water table level in peatlands and might thus affect microbial communities involved in the production of CH_4 and N_2O. Thus, the influence of short-term water table manipulations including application of artificial drought conditions or excessive flooding was assessed in the acidic fen as a model system. Fermentative, methanogenic and denitrifying potentials that were assessed in anoxic microcosm studies with fen soil taken at different timepoints of the water table manipulation revealed that the potential activity of methanogens and denitrifiers was affected by changing water tables (i.e., methanogenic activity based on instantaneous CH_4 production potentials was lowered by drought and increased by flooding, denitrifying activity was increased by flooding), whereas the potential activity of fermenters was largely unaffected. Changes in the copy numbers of *mcrA*, *narG*, and *nosZ* detected by quantitative PCR were rather small when compared to the observed changes in potential activity, indicating that the community size of methanogens, nitrate reducers, and N_2O reducers, respectively, is rather unaffected by short-term water table manipulations. Community composition of methanogens, nitrate reducers and denitrifiers (as assessed by TRFLP-fingerprinting of *mcrA*, *narG*, and *nosZ*, respectively) was similar at all sampled timepoints of the manipulation experiments, indicating that the microbial community composition is not affected by enhanced water table fluctuations.

The collective data indicate a stable microbial community in fen soil that is able to adapt its activity to the changing conditions quite rapidly.

The collective observations underline the importance of peatland ecosystems for greenhouse gas fluxes. CH_4 is produced in a model peatland, and the studied peatlands can be sources as well as sinks for N_2O, and thus might contribute significantly to the global N_2O budget. Changing water tables affected the potential activity of methanogens and denitrifiers, even though the community composition of methanogens and denitrifiers were quite stable. Thus, greenhouse gas metabolizing microorganisms in peatland ecosystems are prone to react sensitively to global change, which might in turn affect the source and sink strengths of peatland ecosystems for CH_4 and N_2O and as a consequence change their contribution to the global budget of those greenhouse gases.

Zusammenfassung

Moorgebiete der nördlichen Hemisphäre speichern beträchtliche Mengen Kohlenstoff und Stickstoff, und es wird vorhergesagt, dass sie empfindlich auf die globale Erwärmung reagieren. Die meisten Moorgebiete sind Quellen des Treibhausgases Methan (CH_4), welches von Methanogenen im Moorboden in trophischer Interaktion mit Gärern gebildet wird. Das Treibhausgas Distickstoffmonoxid (N_2O) kann im Moorboden von Denitrifikanten produziert und verbraucht werden. Quellen- und Senkenfunktionen für CH_4 und N_2O variieren stark zwischen verschiedenen Moortypen. Das Hauptziel der Arbeit waren daher die Untersuchung der mikrobiellen Prozesse, welche an Treibhausgasflüssen aus Moorgebieten beteiligt sind, und zwar Gärungen und Methanogenese als wichtige mitwirkende Prozesse an CH_4-Flüssen und Denitrifikation als mitwirkender Prozess an N_2O-Flüssen. Fünf Beispiel-Moore mit unterschiedlichen Eigenschaften (zwei Niedermoore (pH 7 und pH 5), Hochlandtundra (pH 4), ein Palsamoor (pH 4,5) und cryoturbierte Torfkreise (pH 4). Diese unterschiedlichen Moorgebiete differierten in ihren *in situ* CH_4- und N_2O-Emissionen sowie z.B. im Nitratgehalt, der Jahresdurchschnittstemperatur und/oder im pH.

Prozesse, die an der CH_4-Bildung beteiligt sind, wurden am Beispiel des pH-neutralen Niedermoores untersucht. Das Niedermoor produzierte CH_4 *in situ* sowie in unsupplementierten anoxischen Mikrokosmeninkubationen mit Niedermoorboden. Supplementierung mit N-Acetylglucosamin (NAG) stimulierte die Bildung von Gärungsprodukten sowie CH_4 und resultierte in der verstärkten Akkumulation von Gärungsprodukten (hauptsächlich Acetat, H_2/CO_2, sowie geringe Mengen Ethanol, Formiat und Propionat) als die Methanogenese durch Bromoethansulfonat (BES) inhibiert wurde, was darauf hindeutet, dass Methanogene Gärungsprodukte als Substrate verwenden und somit trophisch mit den Gärern verknüpft sind. Supplemen-

tierung mit H_2/CO_2 und Formiat stimulierte die Methanogenese stark, während die Stimulierung der Methanogenese mit Acetat und Methanol deutlich geringer war. Dies deutet darauf hin, dass hydrogenotrophe Methanogenese ein wichtiger CH_4-bildender Prozess im pH-neutralen Niedermoorboden ist. Mittels kodierter Amplicon-Pyrosequenzierung von bakteriellen 16S rRNA Genen wurde eine große Diversität bakterieller Familien im Niedermoorboden detektiert, dazu gehörten Familien der *Firmicutes*, *Alpha-* und *Deltaproteobacteria* sowie viele neue Familien. Innerhalb der detektierten Familien wurden Gattungen, welche für syntrophe Interaktionen mit hydrogenotrophen Methanogenen bekannt sind (z.B. *Clostridium*, *Syntrophobacter*), gefunden. Dies weist darauf hin, dass diese Gattungen wichtige Lieferanten für methanogene Substrate darstellen können. Die Diversität der Methanogenen war niedriger als die der Bakterien, da nur *Methanomicrobiales* und *Methanocellales* (Methanogene beider Ordnungen sind Hydrogenotrophe) durch Analyse des strukturellen Genmarkers *mcrA* (kodierend für die Methyl-Coenzym M Reduktase) detektiert wurden. Auch dies deutet auf eine Dominanz der hydrogenotrophen Methanogenen im pH-neutralen Niedermoor hin. Sowohl Prozess- als auch molekulare Daten deuten darauf hin, dass (i) hydrogenotrophe Methanogenese der Hauptprozess der CH_4-Bildung im pH-neutralen Niedermoor ist, und (ii) eine hohe Diversität bakterieller Familien im pH-neutralen Niedermoor vorkommt, welche wahrscheinlich an diversen Gärungsprozessen beteiligt sind und somit Substrate für die Methanogenen bereitstellen.

Denitrifikation wird als Hauptquelle von N_2O in staunassen Böden wie z.B. Moorböden angesehen, da hohe Wassersättigung Anoxia im Boden fördert. Da N_2O ein Intermediat im Denitrifikationsprozess ist, können Denitrifikanten sowohl N_2O-Produzenten als auch -Konsumenten sein. Obwohl sie eine wichtige Rolle für den globalen N_2O-Haushalt darstellen, sind Faktoren, welche die Denitrifikation in un-

berührten Moorgebieten kontrollieren, sowie die Diversität der assoziierten Denitrifikantengemeinschaft weitgehend unbekannt. Daher wurden Denitrifikations- und N_2O-Verbrauchspotentiale sowie die Zusammmensetzung der Denitrifikantengemeinschaft in allen fünf nördlichen Moorgebieten untersucht. *In situ* N_2O-Emissionen bewegen sich im Bereich von $< 0,01$ mg $N_2O \cdot m^{-2} \cdot d^{-1}$ in pH-neutralem Niedermoorboden bis zu ungefähr 10 mg $N_2O \cdot m^{-2} \cdot d^{-1}$ in cryoturbiertem Torfkreisboden, daher stellt cryoturbierter Moorboden eine wichtige N_2O-Quelle dar und emittiert in der selben Größenordnung wie tropische Böden oder Ackerböden. *In situ* N_2O-Emissionen waren positiv bzw. negativ mit dem Nitrat- bzw. Ammoniumgehalt des Bodens korreliert. Alle Böden produzierten und verbrauchten N_2O in anoxischen Mikrokosmen-Inkubationen ohne erkennbare Verzögerung. N_2O-Produktionskapazitäten und apparente Affinitäten (v_{max}/K_M) für Nitrat waren ebenfalls positiv mit dem Nitratgehalt des Bodens korreliert. N_2O-Produktionskapazitäten waren in sauren, Permafrost-beeinflussten cryoturbierten Torfkreisböden besonders hoch und traten gemeinsam mit hohen *in situ* N_2O-Emissionen und hohen Nitratgehalten auf. N_2O-Verbrauchskapazitäten waren dagegen am höchsten in pH-neutralem Niedermoorboden und traten zusammen mit niedrigen *in situ* Emissionen und niedrigem Nitratgehalt auf. Phylogenetische Analysen der Nitratreduktions- und Denitrifikations-assoziierten Genmarker *narG*, *nirK/nirS* und *nosZ* (kodierend für Nitrat-, Nitrit-, Nitrit- und N_2O-Reduktasen) deuteten darauf hin, dass die Diversität der Denitrifikantengemeinschaft in pH-neutralem Niedermoorboden am höchsten war und dass die Diversität positiv mit dem pH korreliert war. Detektierte Nitrat-Reduktasegene waren haupstächlich mit *narG* der *Betaproteobacteria* und *Actinobacteria* verwandt. *Betaproteobacteria*-verwandte *narG* dominierten in pH-neutralem Niedermoorboden, währen *Actinobacteria*-verwandte *narG* in allen saureren Böden dominierten, was darauf hindeutet, dass *Actinobacteria*-verwandte Nitratre-

duzierer eine höhere Säuretoleranz aufweisen könnten. Die Zahl und Identität der beobachteten operativen taxonomischen Einheiten (operational taxonomic units, OTUs) of *nirK*, *nirS* und *nosZ* unterschieden sich klar von denen der saureren Böden, und deuteten auf *Alpha-*, *Beta-* und *Gammaproteobacteria*-verwandte Denitrifikanten in allen Moorgebieten hin. Diese Ergebnisse wurden durch kanonische Korrespondenzanalysen (canonical correspondence analysis, CCA) der relativen OTU-Häufigkeiten in den Amplicon-Bibliotheken bestätigt, was darauf hindeutet, dass die Denitrifikantengemeinschaft des pH-neutralen Niedermoores unter den untersuchten Moorgebieten einzigartig war. Permafrost-beeinflusste Böden fielen in den CCA-Ordinationen aller analysierten Genmarker meist zusammen. pH war der wichtigste die Gemeinschaftszusammmensetzung der Nitratreduzierer (abgeleitet von *narG*) und Denitrifikanten (abgeleitet von den anderen Genmarkern) bestimmende Faktor. Signifikante Einflüsse des Kohlenstoffgehaltes des Bodens (auf *narG*, *nirK*) der Niederschlagsmenge (auf *narG*) oder der Temperatur (auf *nosZ*) wurden ebenfalls detektiert. Das Verhältnis der *narG/nosZ* Kopienzahlen und das Auftreten gewisser OTUs von *nirK* und *nirS* waren positiv mit den N_2O-Emissionen korreliert. Die gesammelten Daten weisen darauf hin, dass (i) Denitrifikation in verschiedenen Arten unberührter Moorgebiete abläuft, (ii) N_2O Quellen- und Senkenfunktionen der Moordenitrifikanten von dem Nitratgehalt des Bodens sowie der Gemeinschaftzusammensetzung der Denitrifikanten beeinflusst werden und (iii) die Gemeinschaftszusammensetzung der Nitratreduzierer und Denitrifikanten von pH, Temperatur, Niederschlagsmenge und Kohlenstoffgehalt des Bodens beeinflusst wird.

Aufgrund der globalen Erwärmung werden erhöhte Häufigkeiten von Extremwetter-Ereignissen erwartet, welche längere Dürreperioden oder verstärkte Niederschläge bewirken. Diese Ereignisse wirken sich auf den Wasserstand in Moorgebieten aus und könnten daher die mikrobiellen Gemeinschaften, welche an der Bildung von CH_4

und N_2O beteiligt sind, beeinflussen. Daher wurde der Einfluss von kurzzeitigen Manipulationen des Wasserstands, welche künstliche Dürre und verstärkte Überflutung beinhalteten, am Beispiel des sauren Niedermoores untersucht. Gärungs-, CH_4-Bildungs- und Denitrifikationspotentiale, welche in anoxischen Mikrokosmenstudien mit zu verschiedenen Zeitpunkten der Manipulationsexperimente genommenen Niedermoorbodenproben untersucht wurden, zeigten, dass die potentielle Aktivität der Methanogenen und Denitrifikanten von durch die Änderung des Wasserstands beeinflusst wurden (d.h. methanogene Aktivität basierend auf spontanen CH_4-Bildungspotentialen wurde durch die Dürre erniedrigt und durch die Überflutung erhöht, denitrifizierende Aktivität wurde durch die Überflutung erhöht), wohingegen die potentielle Aktivität der Gärer kaum beeinflusst wurde. Änderungen in der Kopienzahl von *mcrA*, *narG* und *nosZ*, welche mittels quantitativer PCR bestimmt wurden, waren eher gering im Vergleich zu den beobachteten Änderungen der potentiellen Aktivitäten, was darauf hindeutet, dass die Größe der Methanogenen-, Nitratreduzierer- und N_2O-Reduzierer-Gemeinschaft kaum durch die kurzzeitigen Manipulationen des Wasserstands beeinflusst wird. Die Gemeinschaftszusammensetzung der Methanogenen, Nitrat- und N_2O-Reduzierer (untersucht mittels TRFLP-Fingerprinting von *mcrA*, *narG* bzw. *nosZ*) war zu allen beprobten Zeitpunkten der Manipulationsexperimente ähnlich, was darauf hindeutet, dass die mikrobielle Gemeinschaftzusammensetzung nicht durch verstärkte Wasserstandsschwankungen beeinflusst wird. Die gesammelten Daten weisen auf eine stabile mikrobielle Gemeinschaft im Niedermoorboden hin, welche ihre Aktivität relativ schnell an sich ändernde Bedingungen anpassen kann.

Die gesammelten Beobachtungen unterstreichen die Bedeutung von Moor-Ökosystemen für Treibhausgasflüsse. CH_4 wird in einem Modell-Moorgebiet gebildet, und die untersuchten Moorgebiete können Quellen sowie Senken für N_2O sein und somit

signifikant zum globalen N_2O-Haushalt beitragen. Sich ändernde Wasserstände beeinflussten die potentielle Aktivität der Methanogenen und Denitrifikanten, obwohl die Gemeinschaftszusammensetzung der Methanogenen und Denitrifikanten relativ stabil war. Treibhausgas-metabolisierende Mikroorganismen sind daher geneigt empfindlich auf globale Änderungen zu reagieren, was wiederum die CH_4 und N_2O Quellen- und Senkenstärken der Moor-Ökosysteme beeinflussen könnte und als Konsequenz daraus ihren Beitrag zum globalen Treibhausgas-Haushalt.

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1 General introduction

1.1 Greenhouse gases and their impact on earth's climate

Greenhouse gases are known to cause the greenhouse gas effect [88]. Short-waved incoming solar radiation can penetrate the atmosphere and reach the earth's surface. At the surface, part of the incoming radiation is reflected, while another part is absorbed, leading to heating of the earth's surface. Due to its surface temperature the earth emits radiation in the infrared-range, which is absorbed by greenhouse gases and water vapour in the atmosphere. The absorbed energy causes global warming [88]. The greenhouse gas effect is in part natural, and in part anthropogenic, as human activities have caused an increase in greenhouse gas emissions [88]. Naturally occuring levels of greenhouse gases in the atmosphere elevate the earth's surface temperature from $-19°C$ (without atmosphere and greenhouse gas effect) to $14°C$ [88]. Water vapour has the greatest contribution to the natural greenhouse gas effect, causing about 60% of the warming [66]. Greenhouse gases include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), halocarbons e.g., hydrofluorocarbons (HFCs), chlorofluorocarbons (CFCs), perfluorocarbons (PFCs), or hydrofluorochlorocarbons (HCFCs), as well as sulfur hexafluoride (SF_6) and ozone (O_3) [36]. The effect of an individual greenhouse gas on the earth's climate is expressed by its radiative forcing. The radiative forcing of a greenhouse gas (in $W \cdot m^{-2}$) indicates the extend to which it affects the energy balance of the system. Gases with a radiative forcing > 0 cause heating of the system, while gases with a radiative forcing < 0 cause cooling of the system [36]. In total, radiative forcing has increased by 2.63 $W \cdot m^{-2}$ since 1750; this increase is mainly caused by rising atmospheric concentrations of CO_2 (increase in radiative forcing: 1.66 $W \cdot m^{-2}$), CH_4 (increase in radiative forcing: 0.48 $W \cdot m^{-2}$), and N_2O (increase in radiative forcing:

0.16 $W \cdot m^{-2}$) [36].

In respect of radiative forcing, CO_2 is the most important greenhouse gas. In 2005, the CO_2 concentration in the atmosphere was 379 ppm (i.e., 100 ppm higher than in 1750; Figure 1) [36]. From 1960 to 2005 the increase of the atmospheric CO_2 concentration was linear, approximating 1.4 ppm per year. 75% of this increase is due to anthropogenic causes.

Respiration of organisms on land releases 119.6 Gt CO_2 per year, while 70.6 Gt CO_2 per year are released from the ocean; those processes are the major natural sources of CO_2. Fixation of CO_2 by plants and microorganisms on land (120 Gt CO_2 per year) and in the oceans (70 Gt CO_2 per year) constitute the major CO_2 sinks [28]. The use of fossil fuels causes a release of an additional 7 Gt CO_2 per year into the atmosphere, deforestation accounts for an additional 1-2 Gt CO_2 per year [28]. Further anthropogenic sources of CO_2 include the production of cement and changes in land use [28].

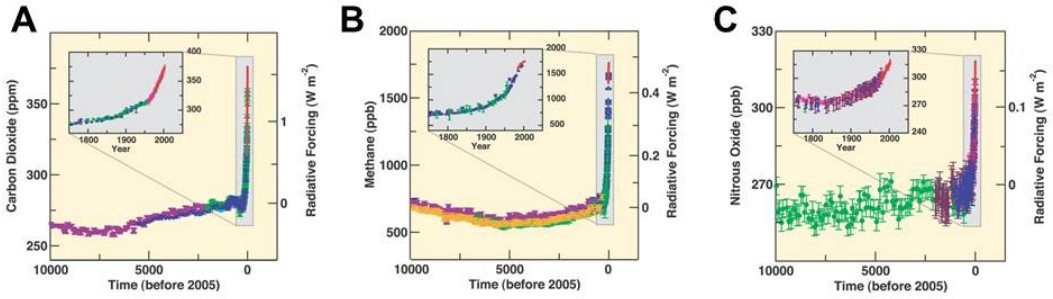

Figure 1: Changes in the concentrations of the greenhouse gases carbon dioxide (CO_2, A), methane (CH_4, B), and nitrous oxide (N_2O, C) and their radiative forcing with time. Insets show an enlargement for the time period from 1750 to 2005. Red lines represent concentrations measured in atmospheric samples, while colored symbols represent concentrations based on ice-core measurements (different colors represent different studies [57])(based on [2].)

CH_4 is the greenhouse gas with the second-largest radiative forcing after CO_2

[36]. The CH_4 concentration in the atmosphere increased from 715 ± 4 ppb to 1774 ± 1.8 ppb from 1750 to 2005 (Figure 1. The rise of the atmospheric CH_4 concentration has slowed down since 1980 [36]. The contribution of the increased CH_4 concentration to the radiative forcing is 0.48 $W \cdot m^{-2}$. From 2000 to 2004, mean yearly CH_4 emissions approximated 582 $Tg \cdot y^{-1}$. 70% of observed CH_4 emissions are of biogenic origin, 30% of abiogenic origen [28]. Biogenic CH_4 production occurs mainly in wetlands and in the digestive systems of ruminants or termites, while CH_4 is produced abiogenically via fossile fuel and biomass burning, as well as gas leakage from ocean floors and vulcanoes [28]. The mean atmospheric lifetime of CH_4 is 8.4 years [28]. The major CH_4 sink is oxidation by OH-radicals in the troposhere (511 $Tg \cdot y^{-1}$). However also biological CH_4 oxidation in soils (30 $Tg \cdot y^{-1}$) and loss in the stratosphere (40 $Tg \cdot y^{-1}$) contribute to the total CH_4 sinks [28].

Due to its long atmospheric lifetime of approximately 120 years and a strong warming potential (310 times the warming potential of CO_2 on a 100 year time frame), N_2O is another important greenhouse gas [36]. Moreover, N_2O is considered to be the major ozone depleting substance on earth [135]. The atmospheric concentration of N_2O has been rising from 270 ppb in 1750 to 319 ppb in 2005 (Figure 1) [36]. 17.7 Tg N $\cdot y^{-1}$ are released as N_2O into the atmosphere, 6.7 $Tg \cdot y^{-1}$ are of anthropogenic origin, while 11.0 Tg $\cdot y^{-1}$ are emitted from natural sources [28]. Even though the exact terrestrial sources and sinks of N_2O remain uncertain [23], soils are considered to be the major source (approximately 70%) [109]. Tropical soils are the single most important source of N_2O and release approximately 1.34 Tg N per year [180], whereas N_2O emissions from peatland soils in the northern hemisphere were generally considered to be insignificant [20, 138, 161]. However, more recent studies showed that especially permafrost peatland systems in the northern hemisphere can be significant but highly variable sources of N_2O [32, 100, 138].

1.2 Peatland ecosystems as important sources of greenhouse gases

1.2.1 Peatland formation and peatland types

Northern wetlands store substantial amounts of carbon and nitrogen and are thus important players in the carbon and nitrogen cycles [133, 167]. Wetland soils are either temporarily or permanently waterlogged which provides anoxic conditions in most parts of the soil [23, 181]. Peatlands are caracterized by the accumulation of peat which forms when assimilation exceeds decomposition under oxygen limited conditions [22, 153]. Peatlands develop when organic material accumulates in a lake or pond (Figure 2). The initial state of a peatland is a fen peat which is rich in inorganic nutrients (minerotrophic) as it receives nutrient from groundwater, surface runoff and rainwater. Fens are generally pH-neutral to slightly acidic habitats and are vegetated by grasses and mosses [173]. As peat accumulation continues, the peat surfaces is raised above the groundwater table and a bog peat is formed (Figure 2). Bogs receive all nutrients from rainwater and are thus usually nutrient limited. The main vegetation are *Sphagnum* mosses and the pH is generally more acidic than in fen soils [176, 181].

1.2.2 Permafrost peatlands

Permafrost-affected soils in the northern hemisphere cover about 16% of the global soil surface, and store substantial amounts of carbon and nitrogen [133, 167]. Permafrost-affected peatlands in the continuous and discontinuous permafrost zone of the northern hemisphere include palsa peats and cryoturbated peat circles [138, 150, 179]. Palsas are elevations of peat soil above the ground level due to uplifting of peat layers by a frozen ice lense and are widely distributed in the circumarctic regions (i.e., Canada, USA, Finland, Sweden, Iceland, Russia) [150, 194]. Palsa

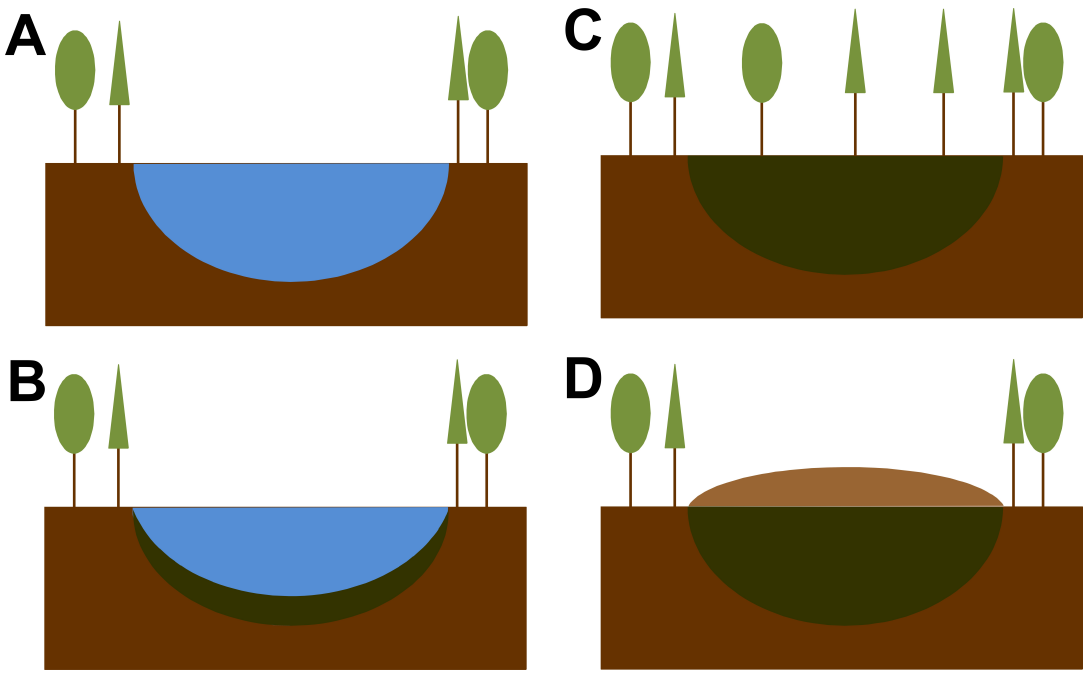

Figure 2: Peatland development. Accumulation of organic matter in a pond (A, B), fen (C), raised bog peat (D); based on [176]. Brown=mineral substratum, blue=water, dark green=fen peat, light brown=bog peat, light green=trees. Vegetation cover changes with time due to changes in ground properties.

development is affected by various environmental factors including depth of snow cover, ground water table depth, vegetation cover and degree of wind erosion [150]. Palsa development includes many freeze-thaw cycles leading to accumulation of ice in the active layer and subsequent upheaval of the peat soil [150]. However, as palsas mature the ice lenses will eventually melt and the palsa collapses, forming a thermokarst lake [150]. On the other hand, cryoturbated peat circles are bare surface peat areas in arctic tundra in which vegetation has been removed due to mixing by frost action [138, 177]. Vegetation cover is absent from approximately 12% of the arctic soil surfaces, including cryoturbated peat circles [177]. Cryoturbation occurs mainly in soils with poor soil drainage and frequent freeze-thaw cycles, leading to formation of patterned ground [11, 128, 179]. Cryoturbated soils contain high amounts of incompletely degraded soil organic matter [85]. Organic carbon stored in permafrost soil is redistributed to the active layer (i.e., the layer that thaws in

summer months) by cryoturbation, and cryoturbation can increase the amount of organic carbon stored in permafrost soil [11, 63].

Wetlands are the most important single CH_4 source, as their emissions account for 25% of the observed global CH_4 emissions [182]. In northern peatlands, about 20% of the assimilated CO_2 are released into the atmosphere as CH_4 [139]. However, total CH_4 production in wetland soils is even higher, as about 20 to 40% of the produced CH_4 is oxidized on root surfaces or in more oxic surface layers [182]. N_2O emission from northern peatlands have been studied to a much lesser extent than CH_4 emissions, and most studies have focused on N_2O emissions from managed peatlands [93, 98, 99]. Pristine northern peatlands can be net sources of N_2O, even though their emission rates are generally low [93, 161]. On the other hand, northern peatlands can act as permanent or temporary sinks for N_2O [16, 43, 117, 100, 161]. Permafrost-affected peatlands like certain palsa peats and especially cryoturbated peat circles are large point sources of N_2O with emission rates comparable to those of tropical and agricultural soils [100, 138]. N_2O emissions from palsa peat soils are highly variable [100]. The ability of permafrost-affected peatlands to emit N_2O is increased by a low degree of plant cover (resulting in reduced competition for N-sources), high nitrate content, low C:N ratios and high gross mineralization activities [20, 100, 138].

Peatland ecosystems in the northern hemisphere and especially permafrost-affected tundra ecosystems are predicted to be severely affected by global warming, as warming occurs at faster than average rates in those systems [4, 38, 150, 174]. Increasing temperatures are likely to lower the water table in northern peatlands and thus increase the amount of CO_2, CH_4 and N_2O released from peatland soils [4, 99, 100]. Moreover, permafrost thawing may cause high emissions of CO_2, CH_4 and N_2O from stored carbon and nitrogen [32, 147].

1.3 Processes involved in greenhouse gas production in anoxic peatland soils

Peatland soils are oxygen limited due to the high degree of water-saturation, and organic matter is thus degraded mainly anaerobically to CO_2 and CH_4 (Figure 3, Figure 4). Complex structural polymers like cellulose, chitin or lignin are first degraded to monomers (e.g., glucose, xylose, N-acetyl-gucosamine, or aromatic compounds) which are further degraded by fermentation (producing alcohols, short chain fatty acids, CO_2, and H_2) [30, 82, 192]. Monomers as well as fermentation products are electron donors in respiratory processes that use alternative electron acceptors like nitrate, manganese, iron, sulfate or CO_2 (Table 2) [30, 192]. Primary fermentation products such as alcohols and short chain fatty acids are utilized by syntrophic secondary fermenters which produce acetate, CO_2 and H_2 [30, 82]. Primary and secondary fermentation products are finally converted to CH_4 by methanogens [30, 82].

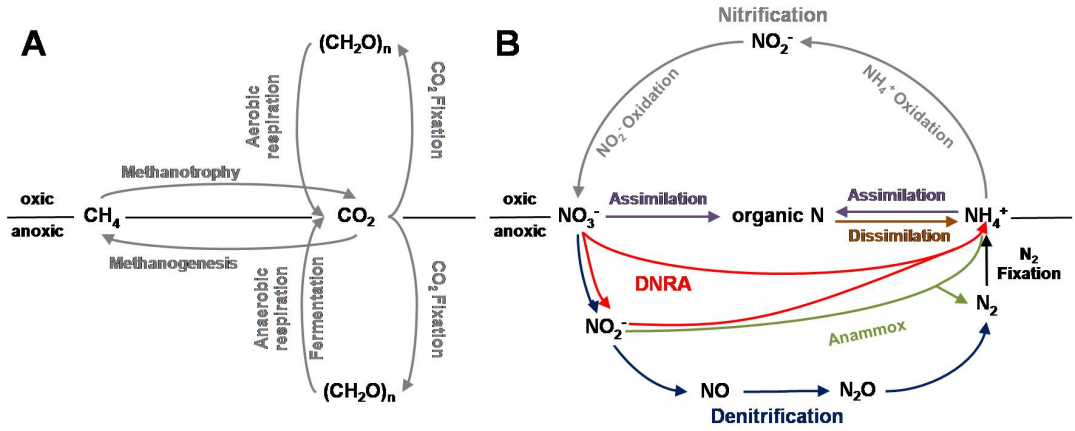

Figure 3: Schematic overview of the main processes in the biological carbon (A) and nitrogen (B) cylcles. DNRA=dissimilatory nitrate reduction to ammonium, Anammox=anaerobic ammonium oxidation (based on [96, 59]).

Methanogenesis competes with other anaerobic processes under anoxic conditions [30]. The reduction of alternative electron acceptors like nitrate or iron is thermodynamically more favorable than methanogenesis, and methanogens are thus often out-

competed (Table 2) [92, 94]. However, concentrations of alternative electron acceptors in wetlands are spacially and temporally variable and often low [1, 13, 125, 149], and methanogenesis can although occur simultaneously with those processes, given a sufficently high concentration of H_2 [23].

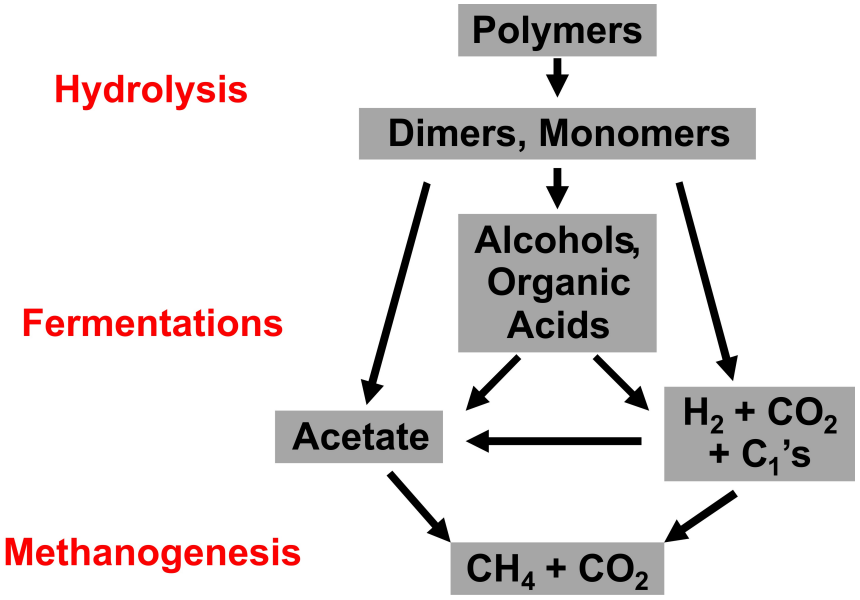

Figure 4: Schematic overview of anaerobic processes in wetland soils that lead to the degradation of organic material and the production of CH_4. C_1=one-carbon organic compounds (excluding CH_4) such as formate, methanol, and methylamine. Based on [30].

Table 2: Redoxpotentials of different half-cell reactions under standard conditions (E_0'), based on [89, 96, 168].

Half-cell reaction	n_{e^-} [1]	E_0' (V) [2]	Process
O_2/H_2O	2	0.82	Aerobic respiration
$NO_3^-/0.5\ N_2$	5	0.74	Denitrification
NO_3^-/NO_2^-	2	0.43	Nitrate reduction
Mn_4^+/Mn_2^+	2	0.39	Mn(IV) reduction
Fe^{3+}/Fe^{2+}	1	0.20	Fe(III) reduction
SO_4^{2-}/S^{2-}	8	-0.22	Sulfate reduction
CO_2/CH_4	8	-0.24	Methanogenesis
CO_2/Acetate	8	-0.29	Acetogenesis

[1] Number of transfered electrons
[2] Redoxpotentials under standard conditions (Temperature: 25 °C, Pressure: 101.3 kPA, pH 7.0).

1.3.1 Fermentation

In fermentative processes, electrons obtained by oxidation of a substrate are transfered to a partly oxidized intermediate [168]. Substrates include carbohydrates, amino acids, alcohols, and carbonic acids [168]. Energy is conserved in form of ATP via substrate-level phosphorylation by kinase-reactions from activated compounds such as acetyl-coenzyme A (Figure 5). Those activated compounds are formed in previous steps via lyase- or dehydrogenase-reactions [168] (Figure 5).

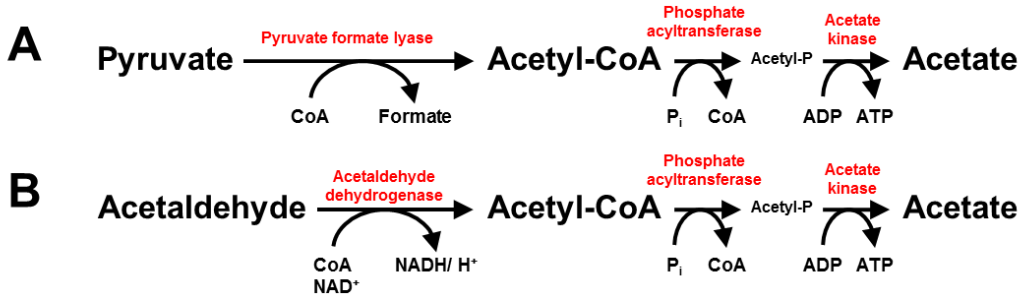

Figure 5: Examples for ATP generation by substrate-level phosphorylation (SLP). Acetate kinase forms ATP from acetyl-CoA after an initial lyase- (A) or dehydrogenase-reaction (B). Based on [168].

Table 3: Examples of primary fermentations, based on [96, 168].

Fermentation type	Reaction [1]	ΔG_0' (kJ/mol) [2]	Example organisms
Alcoholic fermentation	Hexoses → 2 EtOH + 2 CO_2	-218	*Saccharomyces* sp.
Lactic acid fermentation	Hexoses → 2 Lactat + 2 H^+ (homofermentative)	-198	*Lactobacillus* sp., *Enterococcus* sp.
	Hexoses → Lactate + EtOH + 2 H^+ + CO_2 (heterofermentative)	-208	*Leuconostoc* sp., *Lactobacillus* sp.
Butyric acid fermentation	Hexoses → Butyrate + Acetate + H_2 + 2 CO_2	-247	*Clostridium* sp.
Mixed acid fermentation	Hexoses → EtOH + 2,3-Butanediol + Succinate + Lactate + Acetate + Formate + H_2 + CO_2	-200 to -260	*Escherichia* sp., *Enterobacter* sp.

[1] Equations are not stoiciometrically balanced.

[2] Gibbs free energy under standard conditions (Temperature: 25 °C, Pressure: 101.3 kPA, pH 7.0; given for glucose utilization).

Primary fermentation products include ethanol, acetate, H_2 and CO_2 (Table 3). Alcohols (e.g. ethanol, propanol), organic acids (e.g. acetate, succinate, butyrate) or aromatic compounds (e.g. benzoate) are subjected to secondary fermentations by syntrophic organisms (Table 4) [145]. As those secondary fermentations are endergonic under standard conditions, syntrophic partners (e.g. methanogens) are

required to remove products such as H_2 or CO_2 and thus drive the reaction [96, 145, 168]. The ability to conserve energy via fermention is widespread in bacteria, *Archaea*, and *Eukarya*, and organisms can be facultative or obligate fermenters [168].

Table 4: Examples of secondary fermentations (syntrophic processes coupled to methanogenesis), based on [96, 145].

Substrate	Reaction [1]	ΔG_0' (kJ) [2]	Example organisms [3]
Butyrate	$2\ CH_3CH_2CH_2COO^- + 2\ H^+ + 2H_2O \rightarrow 5\ CH_4 + 3\ CO_2$	-177	*Syntrophomonas* sp.
Propionate	$4\ CH_3CH_2COO^- + 4\ H^+ + 2\ H_2O \rightarrow 7\ CH_4 + 5\ CO_2$	-249	*Syntrophobacter* sp.
Ethanol	$2\ CH_3CH_2OH + CO_2 \rightarrow 2\ CH_3COO^- + 2\ H^+ + CH_4$	-112	*Desulfovibrio vulgaris*
Acetate	$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$	-36	*Clostridium* sp.

[1] Chemical equation of the combined syntrophic reaction including secondary fermentation and methanogenesis.

[2] Gibbs free energy under standard conditions (Temperature: 25 °C, Pressure: 101.3 kPA, pH 7.0).

[3] Examples of secondary fermenters involved in this type of syntrophic reaction.

1.3.2 Methanogenesis

Methanogenesis is an anaerobic respiration process catalyzed by strictly anaerobic obligately methanogenic *Archaea* [6, 92]. Methanogens belong to the Euryarchaeota [184] and form 6 orders (*Methanobacteriales*, *Methanocellales*, *Methanococcales*, *Methanomicrobiales*, *Methanopyrales*, *Methanosarcinales*) including 32 genera [51, 144, 169].

Methanogens have a limited substrate range and produce CH_4 hydrogenotrophically, acetoclastically or methylotrophically [51, 92] (Figure 6, Table 5). Hydrogenotrophic methanogens reduce CO_2 with H_2 to form CH_4 [6, 92]. Many hydrogenotrophic methanogens also utilize formate, while some can utilize secondary alcohols like 2-propanol or 2-butanol, ethanol, or CO [92]. Hydrogenotrophic methanogenesis is widespread and occurs in all methanogenic orders [6]. Acetoclastic methanogens belong to the order *Methanosarcinales* (genera *Methanosarcina* and *Methanosaeta*) and utilize acetate by oxidizing the carboxyl-group to CO_2 and reducing the methyl-group to CH_4 [92]. While *Methanosarcina* produce CH_4 hydrogenotrophically, methylotrophically and acetoclastically, *Methanosaeta* are strictly acetoclastic [51, 92]. Methylotrophic methanogens utilize methylated compounds such as methanol, methylamines, and methylated sulfides and occur only within the *Methanosarcinales* (with exception of *Methanosaeta*) and within the genus *Methanosphaera* (*Methanobacteriales*) [6, 92]. The methyl-coenzyme M reductases Mcr and Mrt (catalytical subunits encoded by *mcrA* or *mrtA*) are central enzymes involved in all types of methanogenic pathways (Figure 6; [34]), and *mcrA* is frequently used as a structural genemarker to assess the community composition of methanogens in environmental samples [47, 55, 101, 191].

1.3.2.1 Hydrogenotrophic methanogenesis

In hydrogenotrophic methanogenesis, CO_2 is reduced with H_2 or formate as a pri-

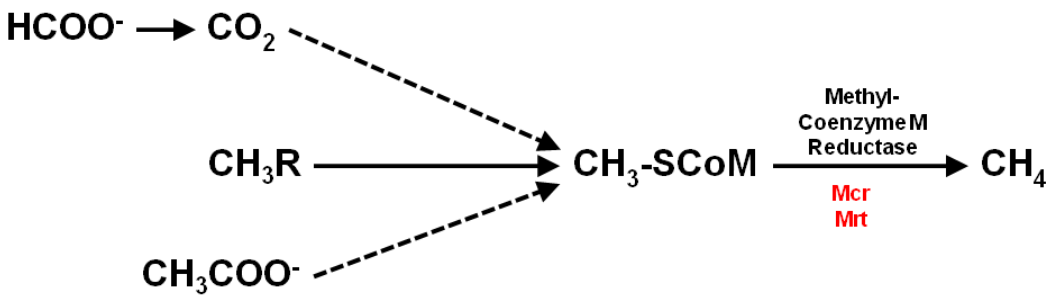

Figure 6: Schematic overview of the three methanogenic pathways. Methyl-Coenzyme M reductase functions as a key enzyme in all three methanogenic pathways. Further enzymes, cofactors and compounds are not shown. Dashed lines indicate that more than one step is needed for the conversion. R=e.g., -SH, -OH, or NH_2. Mcr=Methyl coenzyme-M reductase I; Mrt=Methyl coenzyme-M reductase II. Based on [34].

Table 5: Types of methanogenesis.

Type	Reaction	ΔG_0' (kJ) [1]	Organisms [2]
Hydrogenotrophic	$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$	-35	Most methanogens
Acetoclastic	$CH_3COOH \rightarrow CH_4 + CO_2$	-33	*Methanosarcina*, *Methanosaeta*
Methylotrophic	$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + H_2O$	-105	*Methanosarcina* and others

[1] Gibbs free energy under standard conditions (Temperature: 25 °C, Pressure: 101.3 kPA, pH 7.0).

[2] Examples of secondary fermenters involved in this type of syntrophic reaction. [92]

mary electron donor to CH_4 [92]. When formate is used as an electron donor, 4 molecule of formate are oxidized to CO_2 and the obtained reduction equivalents are used to reduced 1 molecule of CO_2 [92]. In a first step, CO_2 binds to methanofuran (MF) and is reduced by ferredoxin to a formyl-group, the ferrodoxin in turn is reduced by H_2 (Figure 7; [169]). The formyl-group is transfered to tetrahydromethanopterin (H_4MPT), dehydrated to a methenyl-group and subsequently reduced to methylene-H_4MPT and methyl-H_4MPT by reduced F_{420} [92, 169]. The methyl-group is then transfered to coenzyme-M (CoM) and reduced to CH_4 in a final step by the methy CoM reductase [92, 169].

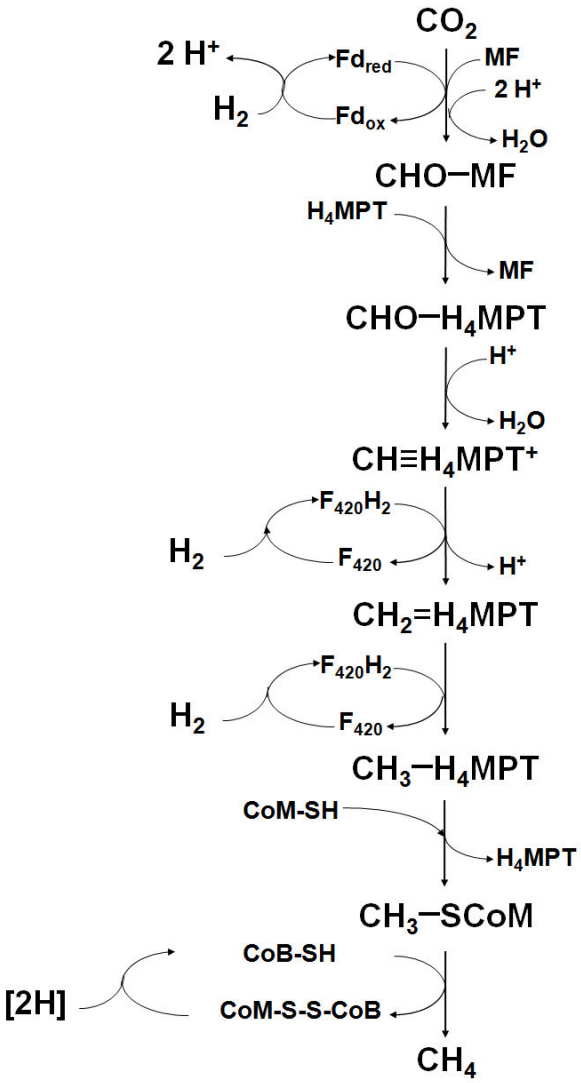

Figure 7: Reactions involved in hydrogenotrophic methanogenesis. F_{420}=coenzyme F_{420}; Fd=ferredoxin; MF=methanofuran; H_4MPT=tetrahydromethanopterin; HS-CoB=coenzyme B; HS-CoM=coenzyme M; based on [92, 169]

1.3.2.2 Acetoclastic methanogenesis

In acetoclastic methanogenesis, acetate is split, the carboxyl-group is oxidized to CO_2, while the methyl-group is reduced to CH_4 [92]. Acetate is first activated with ATP and transfered to coenzyme A by acetate kinase-phospotransacetylase, forming acetyl-CoA. Acetyl-CoA is cleaved to methyl-H_4MPT and CO-CoA by the CO

dehydrogenase/acetyl-CoA synthase system. CO-CoA is further oxidized to CO_2, electrons are first transfered to oxidized ferredoxin, then to H_2 in a hydrogenase reaction. The methyl-group is transfered to CoM and reduced to CH_4 as in hydrogenotrophic methanogenesis [92].

1.3.2.3 Methylotrophic methanogenesis

In methylotrophic methanogenesis, the methyl-groups of methylated compounds like methanol are transfered to CoM to form methyl-CoM. Methyl-CoM is reduced to CH_4 by the methyl CoM reductase. The reduction equivalents for this reduction are obtained by oxidation of additional methyl-groups to CO_2 via a reversed hydrogenotrophic pathway (i.e. via methyl-H_4MPT, methylene-H_4MPT, methenyl-H_4MPT and formyl-MFR) [92].

1.3.2.4 Factors influencing methanogenesis in soils

Methanogenesis is affected by pH, temperature, groundwater level, the amout of available organic carbon, and the amount of available alternative electron acceptors [23, 148].

Most cultured methanogens show optimal growth at near-neutral pH, and methanogenesis is generally inhibited at ph <5 [148]. However, acid-tolerant methanogenic strains have been isolated from peatlands [183]. Indeed, methanogenesis is active in many acidic wetland systems [15, 14, 54, 81]. CH_4 production rates of methanogenic communities from acidic wetlands are higher at acidic than at neutral pH, indicating an adaptation of the methanogens to *in situ* pH [14, 81]

Most wetland methanogenic communities are mesophilic and show optimum CH_4 production capacities at 20-35°C [14, 81, 102, 103]. Increased soil temperatures result in increased CH_4 production rates [23]. However, methanogenesis in wetland soils occurs also at lower temperatures and is still comparably high at 4°C [81, 102,

103] .Moreover, the contribution of the methanogenic precursors differs according to temperature. At lower temperatures, CH_4 is mainly derived from acetate [23]. However, this is likely attributed to a change in the fermentation pathways that occur prior to methanogenesis than to the methanogenic processes themselves, as e.g. the production of H_2 is reduced at low temperatures [23]. On the other hand, an increased contribution of hydrogenotrophic methanogenesis at low temperatures is observed in acidic wetland soils, and is attributed to the occurence of further sinks for acetate or H_2 in those soils [54, 82].

The groundwater level affect the degree of soil aeration and determines the position of the oxic/anoxic interface [72]. A lower water table results in higher soil aeration and a concomitant rise of the redox potential [70]. Oxygen (O_2) inhibits methanogenesis, and increased soil aeration leads to a decrease in CH_4 emissions from soil as O_2 inactivates the methyl-coenzyme M reductase [67]. Even though methanogens are detected in more oxic systems such as desert soils, CH_4 production in these systems is negligible [64, 127, 186]. Decreased CH_4 production in more oxic systems is attributed to a combination of O_2 inhibition and desiccation [35]. Methanogens can survive or even grow under oxic conditions, however, the exact mechanisms is not yet resolved, since there are no known resting stages of methanogens [35]. However, methanogens show enzymatic protection against oxidative stress, e.g. by the enzymes superoxide dismutase and catalase [69, 162].

1.3.3 Denitrification

Denitrification is one of the major processes in the nitrogen cycle (Figure 3; [59]). Nitrogen oxides are used as alternative electron acceptors under anoxic conditions, and nitrate (NO_3^-) or nitrite (NO_2^-) are sequentially reduced to N_2 via NO and N_2O (Figure 8) [195]. In some denitrifiers or under certain environmental conditions, N_2O is released as the endproduct of denitrification [23, 195]. Denitrification is considered to be the major source of N_2O in wetlands, as the high degree of water-saturation in wetlands soils promotes anoxic conditions [23].

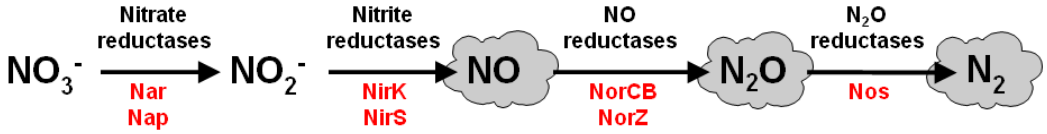

Figure 8: Reductive steps and responsible enzymes in the denitrification pathway of *Bacteria* and *Archaea*. NO, N_2O and N_2 are gaseous products that can be emitted by denitrifiers. Nar=membrane-bound nitrate reductase; Nap=periplasmatic nitrate reductase; NirK=copper-containing nitrite reductase; NirS=cytochrome-cd_1-containing nitrite reductase; NorCB=nitric oxide reductase, short variant; NorZ=nitric oxide reductase, long variant; NosZ=nitrous oxide reductase (based on [195]).

1.3.3.1 Denitrifying microorganisms

The ability to completely or partly denitrify is widespread. Denitrifying organisms are found within the *Bacteria*, *Archaea* and *Fungi* [83, 195]. More recently, evidence is accumulating that there are also some denitrifying Foraminifera (protists) [140]. However, it is not yet resolved, whether the foraminifers themselves or symbiotic partners are responsible for the observed denitrification activity [9]. Within the prokaryotes, denitrifier do not form a monophyletic group. Complete or partial denitrification is encountered e.g., within the *Alpha-*, *Beta-*, *Gamma-*, and *Epsilon-Proteobacteria*, the *Firmicutes* and *Flavobacteria*, as well a in the archaeal genera

Haloferax, *Haloarcula*, or *Halobacterium* [195]. Apart from their large phylogenetic diversity denitrifiers show also a high metabolic diversity as there are phototrophic, chemolithotrophic as well as organotrophic denitrifiers [195]. However, denitrifiers are in general facultative aerobes, i.e. they prefer oxygen as the terminal electron acceptor, with only few exceptions (e.g., *Alcaligenes denitrificans*) [45, 195]. On the other hand, denitrification and nitrate respiration occur also in the presence of oxygen in *Bacteria* and *Fungi* [12, 166, 170]. Organotrophic denitrifiers utilize a variety of carbon sources including sugars (e.g. glucose), organic acids (e.g. acetate, formate, succinate), alcohols (e.g. methanol, ethanol), and aromatic compounds [24, 124, 126, 160]. Denitrifying fungi couple nitrate and nitrite reduction to oxidation of malate, pyruvate, formate or succinate [74]. Chemolitotrophic denitrifiers include sulfur oxidizers like *Thiobacillus denitrificans* or H_2-consuming species like *Paracoccus denitrificans* [195]. Denitrification is also found in nitrifying species like *Nitrosomonas europaea* (so-called nitrifier-denitrification) where denitrification is coupled to ammonium oxidation under oxygen-limited conditions [77, 134]. Moreover, nitrification and denitrification can be coupled by the utilization of the nitrification products nitrite and nitrate by denitrifiers [84]. Denitrifiers occur also under extreme environmental conditions, there are examples of thermophilic, psychrophilic and halophilic denitrifiers [195].

1.3.3.2 Enzymes involved in denitrification

The reductive steps in denitrification involve several oxidoreductases, namely nitrate, nitrite, NO and N_2O reductases (Figure 8). Nitrate respiration is mediated by the dissimilatory nitrate reductases Nar and Nap [129]. The membrane-bound nitrate reductase Nar is composed of 3 subunits and is encoded by *narGHI* [108, 129]. The cytoplasmic domain consists of the α- and β-subunits, while the γ-subunit forms a membrane anchor [129]. The α-subunit contains a 4Fe-4S cluster and a pyra-

nopterin cofactor, which is the site of nitrate reduction [129, 195]. The β-subunit is a globular-protein with 4 iron sulfur clusters (1 3Fe-4S, 3 4Fe-4S) which transfers electrons to the α-subunit [129, 195]. The γ-subunit consists of 5 transmembrane α-helices, a periplasmic N-terminus and a cytoplasmic C-terminus. It contains 2 b-type hemes that transfer electrons from the quinol pool to the β-subunit [129]. A proton motive force is generated by consumption of protons in the cytoplasm and by the release of protons from the quinone pool as electrons are transfered to NarI [83, 129, 195]. The periplasmic nitrate reductase Nap is a heterodimer consisting of the 2 subunits NapA and NapB and is encoded by the *napFDAGHBC* operon [108, 129]. *napA* encodes the catalytic 90-kD subunit, which contains a molybdenum cofactor and a [4Fe-4S] center, while *napB* encodes a smaller 13-19-kD subunit with two-heme cytochrome *c* which transfers electrons to the molybdopterin of the catalytic subunit [108].

Two types of respiratory nitrite reductases are used by denitrifiers to reduce nitrite to NO: The copper-containing NirK and the cytochrome-cd_1-containing NirS [195]. The enzymes are evolutionary unrelated and show no clear pattern of taxonomic distribution, until now no organism is known that harbors both types of nitrite reductase [52, 195]. Even though both types seem to perform identical functions, there are indications that NirK- and NirS-type denitrifiers respond differently to changes in environmental conditions, thus allowing for niche differentiation of denitrifiers [60]. NirK is a homotrimeric periplasmatic enzyme that contains a type 1 and a type 2 copper center in each monomer. Nitrite binds to the type 2 copper center and is reduced to NO by electrons transfered from the type 1 copper center [83, 112, 195]. Several of NirK-type nitrite reductases are known, the NirK I is most intensively studied and often detected in denitrifying isolates. However, additional forms of NirK exist, and it is so far unresolved how widespread those forms are

in denitrifiers [33, 61, 112]. NirS is a homodimeric periplasmatic enzyme with a prosthetic heme *c* and heme *d*1 in each cytochrome cd_1 monomer [83, 195]. Nitrite binds to the *d*1-heme and is reduced to NO by electrons from cytochrome c_{551} via the *c*-heme [83, 195]. NirK and NirS are found in *Bacteria* as well as in *Archaea* [83, 195].

Bacterial respiratory NO reductases are membrane-bound heme-nonheme Fe enzymes and can be devided into two groups: a about 450 amino acid long variant scNor (also called cNor) receives electrons from cytochrome *c*, while the about 760 amino acid long variant lcNor (also called qNor) receives electrons from quinol [196]. Both scNor and lcNOR coexist with NirK or NirS [61, 196]. scNor is a heterodimer consisting of the catalytic subunit NorB and a *c*-type cytochrome NorC, which are encoded by *norCB* [196]. The long chain lcNOR is encoded by *norZ*. Compared to NorB it contains an N-terminal extension that acts as a quinol oxdiase [196]. NO reductases are homologues of heme-copper oxidases, however the NO reductase itself does not translocate protons, even though the electron flow to the reductase is linked to proton extrusion [196]. In *Archaea*, all known NO reductases belong to the scNor type [83, 196]. However, in many archaeal genomes, no *norZ* homologues are detected, thus there might be alternative NO reductases in *Archaea* [83].

Bacterial N_2O reductases (NosZ) are multicopper homodimeric enzymes encoded by *nosZDFY* that are periplasmatic or membrane-bound in gram-negative and gram-positive *Bacteria*, respectively [83]. Each monomer of the NosZ homodimer contains two multi-copper centers Cu_A and Cu_Z [83, 132, 197]. Cu_Z is a [4Cu-2S] copper-sulfur cluster that binds N_2O while Cu_A is a mixed-valent binuclear copper-center that transfers electrons to the bound N_2O molecule [132]. Some *Bacteria* cannot use exogenous N_2O as the induction of N_2O reductase genes requires NO to be present in the cell [197]. Some *Archaea* possess N_2O reductases that are homo-

logues of the bacterial NosZ. In contrast to their bacterial conterparts these N_2O reductases are membrane-bound and receive electrons from menaquinol [83, 197]. However, there are archaeal denitrifiers capable of N_2O reduction whose genomes do not harbor *nosZ* homologues, thus there is likely a second archaeal type of N_2O reductase [197]. Some denitrifiers lack N_2O reductases, thus N_2O is the endproduct of denitrification in those organisms [61, 197]. On the other hand, there are organisms like *Wolinella succinogenes* that reduce N_2O using a slightly modified N_2O reductase without being capable of complete denitrification [197]. *W. succinogenes* reduces nitrate to nitrite and N_2O to N_2, but the intermediate steps are missing [197].

In fungal denitrification, the reductive enzymes are located in the mitochondria and are coupled to the mitochondrial electron transport chain to produce ATP [74]. The membrane-bound fungal nitrate reductase (Nar) reduces nitrate utilizing ubiquinol as electron donor [107, 165]. Properties of fungal Nar resemble those of the bacterial conterpart. However, no orthologues of the bacterial *nar* genes have so far been detected in fungal genomes, indicating that fungal Nar is evolutionary distinct from bacterial Nar [165]. In contrast, the fungal nitrite reductase gene *nirK* detected in the genomes of many denitrifying fungi is an orthologue to the bacterial *nirK* [68]. Fungal NirK is a copper-containing nitrite reductase loctaed in the mitochondrion [75, 107]. Fungal NO reductases are clearly distinct from prokaryotic NO reductases. Fungal Nor is a member of the cytochrome P450 superfamily and is thus called P450nor [107, 165]. The reduction of NO is very different from the oxidation reactions of other P450 proteins, as those require further electron donating proteins [165]. P450nor is a soluble enzyme that utilizes NADH or NADPH to reduce NO to N_2O [165]. N_2O is the major end product in fungal denitrification, even though some fungi are also capable of N_2 production [107, 151, 195]. So far, no

N_2O reductase has been isolated from fungi or identified in fungal genomes [107].

1.3.3.3 Factors influencing denitrification in soils

Denitrification rates in soils as well as the relative contribution of N_2O to the total emitted N gases depend the composition of the denitrifier community as well as on a variety of environmental factors. Soil pH is one of the most important parameters affecting denitrification activities in soil [152]. In general, low pH impairs denitrification and increases the contribution of N_2O to total N_2 gases [25, 152]. However denitrification rates of denitrifier communities from acidic soils are higher at *in situ* pH than at more neutral pH [117, 123]. Acidic pH has a more pronounced effect on N_2O reductase than on the other reductases involved in denitrification [8, 91]. This results in higher N_2O:N_2 ratios in acidic soils. The exact mechanism of inhibition is not yet resolved, but it is likely due to post-transcriptional effects during translation or protein assembly [8, 91].

In general, denitrification activities increase with increasing temperatures, e.g. pure cultures of *Pseudomonas denitrificans* have a temperature optimum of 38°C [178]. In soils, the temperature optimum of denitrification is often higher than the observed *in situ* temperatures [97, 117, 143]. Even though total denitrification activity is increased at higher temperatures, N_2O emissions can be lower, as mostly complete denitrification to N_2 occurs at those temperatures [93]. N_2O reductases are more sensitive to cold temperatures than the other reductases involved in denitrification, thus the N_2O:N_2 ratios are higher at colder temperatures, and winter-time denitrification activities significantly impact on the annual N_2O emissions [53, 93, 110]. However, there are also differences between soil types, and some soils do not show increased N_2O:N_2 ratios at low temperatures, indicating that denitrifiers and N_2O reductases can be adapted to cold temperatures [31]. Freeze-thaw cycles lead to enhanced N_2O emissions from soil, and N_2O emission peaks are often observed in the end of winter [46, 79, 78, 131].

Apart from soil pH and temperature, denitrification in soils is also affected by soil water content, carbon and nitrate availability, and soil C:N ratios [23, 21]. Soil nitrate content often limits denitrification in soils, while carbon sources are not limiting [5, 50, 7, 175]. Due to the influences of the mentioned environmental parameters, denitrification activities and N_2O emissions are highly variable in soils, and even so-called "hot spots" for denitrification exist [50, 138, 189].

1.3.4 Dissimilatory nitrate reduction

Dissimilatory nitrate reduction to ammonium (DNRA) reduces nitrate via nitrite to ammonium [83]. DNRA competes with denitrification for nitrate and can be the dominant process in some soils [142]. High carbon content and low nitrate availability favor DNRA over denitrification [83, 172]. The process occurs in *Bacteria* as well as in fungi [83, 164]. In *Bacteria*, the reduction of nitrate to nitrite is catalyzed by the periplasmic nitrate reductase NapAB (1.3.3.2) [83, 129, 195]. The reduction of nitrite to ammonium is catalyzed by the pentaheme cytochrome *c* nitrite reductase NrfA [83, 195]. In fungal DNRA (also called ammonia fermentation), nitrate reduction to ammonium is coupled to the oxidation of ethanol to acetate [163, 164, 165]. The NADH-dependent assimilatory nitrate and nitrite reductases encoded by *niaD* and *niiA* are utilized for the stepwise reduction of nitrate to ammonium [163, 165]. The electrons for the reduction originate from the oxidation of ethanol to acetate [163, 165]. The oxidation is coupled to ATP-production by acetate kinase [163, 165]. Even though assimilatory enzymes are used for the reduction of nitrate and nitrite, there is no indication that the produced ammonium is actually assimilated by the fungus [165].

2 List of publications and manuscripts included in the dissertation

2.1 Published articles in peer-reviewed journals

1. Palmer, K., Drake, H. L., Horn, M. A. (2009). Genome-derived criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units of nitrate reducers. Applied and Environmental Microbiology 75: 5170-5174. Reference number [**116**]. Own contribution: Concept (50%, together with M. Horn), experimental work (100%), part of the writing (50%).

2. Palmer, K., Biasi, C., Horn, M. A. (2012). Contrasting denitrifier communities relate to contrasting N_2O emission patterns from acidic peat soils in arctic tundra. ISME Journal 6: 1058-1077. Reference number [**115**]. Own contribution: Experimental design (60%, together with M. Horn) and practical work (100%), data analysis (100%), part of the writing (60%).

3. Palmer, K., Horn, M. A. (2012). Putative *Actinobacterial* nitrate reducers and *Proteobacterial* denitrifiers are abundant in permafrost affected N_2O metabolizing acidic palsa peat soil. Submitted to Applied and Environmental Microbiology. Reference number [**119**]. Own contribution: Experimental design (70%, together with M. Horn), practical work (100%), data analysis (100%), part of the writing (60%). This manuscript was under revision when the thesis was handed in.

2.2 Manuscripts in preparation

1. Palmer, K., Horn, M. A. (2012). Denitrification activity of a new and diverse denitrifier community in a pH neutral fen soil in Finnish Lapland is nitrate lim-

ited. Reference number [**120**]. Own contribution: Experimental design (75%, together with M. Horn) and practical work (100%), data analysis (100%), part of the writing (70%).

2. Palmer, K., Drake, H. L., Horn, M. A. (2012). Denitrifier communities in an acidic fen are stable during experimental drought. Reference number [**118**]. Own contribution: Practical work (100%), data analysis (100%), part of the writing (70%).

3. Palmer, K., Schulz, K., Mundinger, A., Mertel, R., Horn, M. A., Drake, H. L. (2012). Stability of methanogenic diversity in an acidic fen under the influence of experimental drought. Reference number [**121**]. Own contribution: Practical work (40%, together with K. Schulz, A. Mundinger, R. Mertel), data analysis (90%), part of the writing (70%).

2.3 Previous peer-reviewed publications not included in the dissertation

1. Palmer, K., Drake, H. L., Horn, M. A. (2010). Association of novel and highly diverse acid-tolerant denitrifiers with N_2O fluxes of an acidic fen. Applied and Environmental Microbiology 76: 1125-1134. Reference number [**117**].

2.4 Published abstracts at national and international conferences

1. Palmer, Horn, M.A. 2012. Unknown denitrifier diversity in a pH neutral fen soil in Finnish Lapland. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract SMV003, p. 210.

2. Palmer, K., Horn, M.A. 2012. Palsa peats represent hitherto underappreciated reservoirs of new denitrifier diversity associated with N_2O fluxes. International Polar Year 2012 Conference. Abstract online.

3. Palmer, K., Biasi, C., Drake, H.L., Horn, M.A. 2011. Cryoturbation affects denitrifier communities in N_2O-emitting arctic permafrost peat soil. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract EMP104, p. 116.

4. Palmer, K., Biasi, C., Drake, H.L., Horn, M.A. 2011. Impact of cryoturbation on denitrifier community structure and activity in N_2O-emitting arctic permafrost peat soil. Ecology of Soil Microorganisms. Abstract 99.

5. Palmer, K., Schulz, K., Horn, M.A., Drake, H.L. 2010. Stability of the methanogenic community in an acidic fen to experimental drought. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract ECV02, p. 81.

6. Palmer, K., Schulz, K., Horn, M.A., Drake, H.L. 2010. Effects of enhanced drought on the diversity of methanogens in an acidic fen. Bayreuth Center of Ecology and Environmental Research (BayCEER) Workshop 2010, Abstract O 1.5.

7. Palmer, K., Schulz, K., Horn, M.A., Drake, H.L. 2010. Impact of artificial drought on diversity, abundance, and gene expression of methanogens in an acidic fen. 13th International Symposium on Microbial Ecology (ISME-13), Abstract on disk.

2.5 Additional presentations of parts of the work at international meetings

1. Palmer, K., Biasi, C., Horn, M.A. 2011. Denitrifier communitiy composition impacts N_2O emission patterns in acidic tundra permafrost soils. Nordic Network for Stable Isotope Research (NordSIR) Meeting.

2. Palmer, K., Drake, H.L., Horn, M.A. 2011. Effect of high latitude on denitrification-dependent N_2O-fluxes and denitrifier community structure in peatlands. Gordon Research Conference on Applied and Environmental Microbiology.

3 Greenhouse gas production in pristine peatlands

Contributions to the synopsis

Data presented in this section are partly represented in the manuscripts (see references in **bold face**). Additional data not represented in the manuscripts are derived from the bachelor thesis of Sonja Perras (part of the work on fermentation and methanogenesis in pH-neutral Puukkosuo fen soil) or from own research (part of the work on fermentation and methanogenesis in pH-neutral Puukkosuo fen soil, work on the comparison of denitrifier communities, manipulation experiments).

Wetlands including pristine peatlands are sources and sinks of the greenhouse gases CH_4 and N_2O [18, 37, 76, 100, 161]. However, greenhouse gas fluxes from peatlands vary between peatlands and are dependent on many environmental factors such as temperature and pH [19, 23, 161]. N_2O emissions from pristine peatlands have been less intensively studied than CH_4 emissions, and environmental parameters affecting source or sink strenght of peatlands for N_2O are largely unresolved. Thus, five peatlands belonging to different peatland types were selected as model systems to assess methanogenesis as well as denitrification-associated N_2O turnover and the possible influence of environmental parameters (Table 6). The five peatlands differed in both CH_4 and N_2O emissions as well as in environmental parameters such as nitrate, ammonium or water content, pH, mean annual temperature or mean annual precipitation (Table 6, Figure 9; more detailed information about sampling sites is given in **[115, 117, 120, 119]**). Some peatlands emitted large amounts of CH_4 (fen soils), some peatlands emitted large amounts of N_2O (Schlöppnerbrunnen fen, cryoturbated peat circles), and some peatlands showed only low emissions of either gas (Peat plateau permafrost tundra, Skalluvaara palsa peat; Figure 9).

Table 6: Peatlands used in the studies.

Site name	Abbreviation	Peatland type	pH [1]	water content (%)	nitrate [1] content (μM)	ammonium [1] content (μM)	MAT [2] (°C)	MAP [3] (mm)
Puukkosuo	PS	Fen	6.8	90	50-150	77	−0.4	772
Schlöppnerbrunnen	SB	Fen	5.0	85	$0-500$	10	5.3	1162
Permafrost tundra	PT	Peat plateau permafrost tundra	4.0	80	< 1	300	−5.6	505
Skalluvaara	SV	Palsa peat	4.5	73	10	125	−1.6	415
Peat circles	PC	Cryoturbated permafrost peat	4.0	70	1500	77	−5.6	505

[1] Determined in watery extracts.

[2] Mean annual temperature.

[3] Mean annual precipitation.

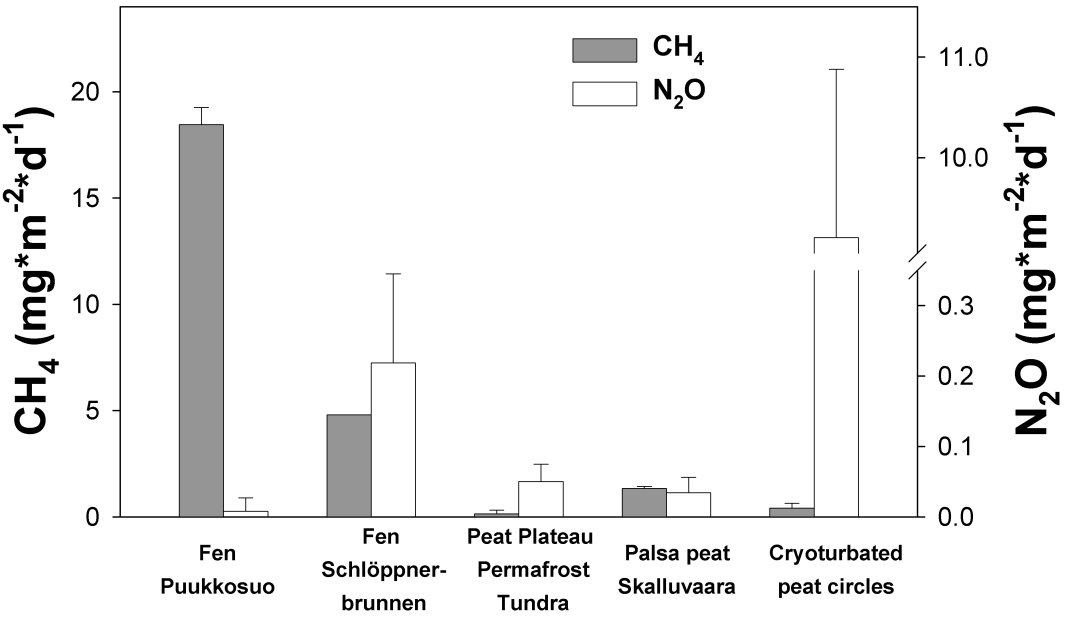

Figure 9: *In situ* emissions of CH_4 and N_2O from different peatland soils. Sources of emission data: own measurements with closed chamber method (CH_4 and N_2O emissions from Puukkosuo fen and Skalluvaara palsa peat soil **[120, 119]**), Julia Köpp (Laboratory of Isotope Biogeochemistry, University of Bayreuth), pers. comm. (CH_4 emissions from Schlöppnerbrunnen fen soil), [42] (N_2O emissions from Schlöppnerbrunnen fen soil), [90] (CH_4 emissions from permafrost tundra and cryoturbated peat soil), [100] (N_2O emissions from permafrost tundra and cryoturbated peat soil).

3.1 Hypotheses tested in this work

The following hypotheses were tested:

1. Methanogens and fermenters are trophically linked in pH-neutral fen soil.

2. Nitrate avalaibility is the main factor regulating N_2O emissions from pristine peatlands.

3. Denitrifier communities in pristine peatland soils are composed of a certain portion of common peatland denitrifiers that are found in all peatland soils as well as of more habitat-specific species. Differences in denitrifier community

composition can be explained by environmental parameters such as pH, mean annual temperature and soil water content.

4. Observed differences in N_2O emissions of different pristine peatlands are correlated with the occurence of specific denitrifying groups.

5. Pristine peatlands harbor stable microbial communities and their potential activities react rapidly to short-term water table manipulations.

Based on the postulated hypotheses the main objectives of the present study were (i) to assess fermentative and methanogenic potentials and associated microbial diversity in a model pristine peatland, (ii) to assess denitrification-associated N_2O production and consumption and associated denitrifier diversity in five model pristine peatlands, (iii) to determine environmental factors that shape denitrifier communities and influence N_2O production and consumptions potentials in pristine peatland soils, and (iv) to assess the effect of water table manipulations on fermentative, methanogenic, and denitrifying microbial populations in a model peatland soil.

3.2 Processes leading to formation of CH_4

3.2.1 Fermentative and methanogenic processes in a pH-neutral fen

Methanogenesis is a major anaerobic reductive pathway in peatland ecosystems, especially when alternative electron acceptors like nitrate or sulfate are scarce [30, 82]. Fermentative processes degrade carbon compounds and thus provide substrates for peatland methanogens [30, 82]. Fermentative and methanogenic potentials of peat soil from the CH_4-emitting pH-neutral fen Puukkosuo (Table 6, Figure 9) were assessed in anoxic microcosms with fen soil from 0 to 20 cm depth. Triplicate microcosms were left untreated or supplemented with N-acetylglucosamine (NAG), Bromoethanesulfonate (BES) (to inhibit methanogenesis [114]) or both NAG and BES. NAG was used as a model carbon compound, as it is abundant in nature (building block of chitin and bacterial cell walls), used as substrate by many fermenters and easily detectable by HPLC [48]. Only minor CH_4 production was observed in unsupplemented microcosms (Figure 10). Approximately 0.2 μmol $CH_4 \cdot g_{DW}^{-1}$ were produced within 10 days of anoxic incubation. Supplemental NAG greatly stimulated CH_4 production, and approximately 8 μmol $CH_4 \cdot g_{DW}^{-1}$ were produced within 10 days of anoxic incubation (Figure 10). In NAG-supplemented microcosms, various fermenation products (mainly acetate, formate, ethanol, and H_2/CO_2) were detected (Figure 11). Inhibition of methanogenesis by BES in NAG-supplemented microcosms resulted in the increased accumulation of fermentation products (Figure 11). Acetate was the most prominent fermentation product, and approximately 600 μM and 900 μM of acetate accumulated within 7 days of anoxic incubation in microcosms supplemented with NAG and with NAG and BES, respectively (Figure 11). Moreover, transient accumulation of ethanol and formate was observed. Approximately 100 μM and 200 μM of both ethanol and formate were detected after 4 days of anoxic incubation in microcosms supplemented with NAG and with NAG

and BES, respectively, and were subsequently consumed (Figure 11). The observed fermentation products indicate the occurence of diverse fermentation processes such as mixed acid fermentation, alcoholic fermentation or propionic acid fermentation [168]. Acetate is a dominant fermentation product in many wetland ecosystems such as acidic fens or taiga pond sediments [48, 82]. Acetate is also used as substrate in acetoclastic methanogenesis [92], thus the absence of acetate in microcosms without BES indicates that Puukkosuo fen soil might indicate the potential to produce CH_4 acetoclastically. On the other hand, acetate can be used by acetate-oxidizing fermenters in a syntrophic association with hydrogenotrophic methanogenesis [49], thus it remains unresolved whether acetate is directly or indirectly converted to CH_4 in Puukkosuo fen soil.

The effect of acetate and various other methanogenic substrates (i.e., formate, methanol, H_2/CO_2) on CH_4 production was assessed in anoxic microcosms with fen soil from 0 to 20 cm depth that was preincubated under anoxic conditions for 120 days to reduce alternative electron acceptors and activate fen methanogens. Triplicate microcosms were supplemented with either acetate, formate, methanol or H_2/CO_2, unsupplemented microcosms served as controls. 20 μmol $CH_4 \cdot g_{DW}^{-1}$ accumulated in unsupplemented microcosms within 11 days of incubation (Figure 12). All tested substrates stimulated CH_4 production. Supplemental H_2/CO_2 led to a 6-fold increase in CH_4 production (Figure 12). Supplemental formate, acetate, and methanol led to 4-fold, 1.6-fold and 1.4-fold increased CH_4 production, respectively, indicating that fen methanogens are able to utilize a variety of substrates. Moreover, the greater stimulation by hydrogen and formate as compared to stimulation by acetate or methanol indicates that methanogenesis in pH-neutral fen soil is dominated by hydrogenotrophic rather than acetoclastic or methylotrophic methanogenesis. The contribution of methanogenic precursors to overall methanogenesis varies

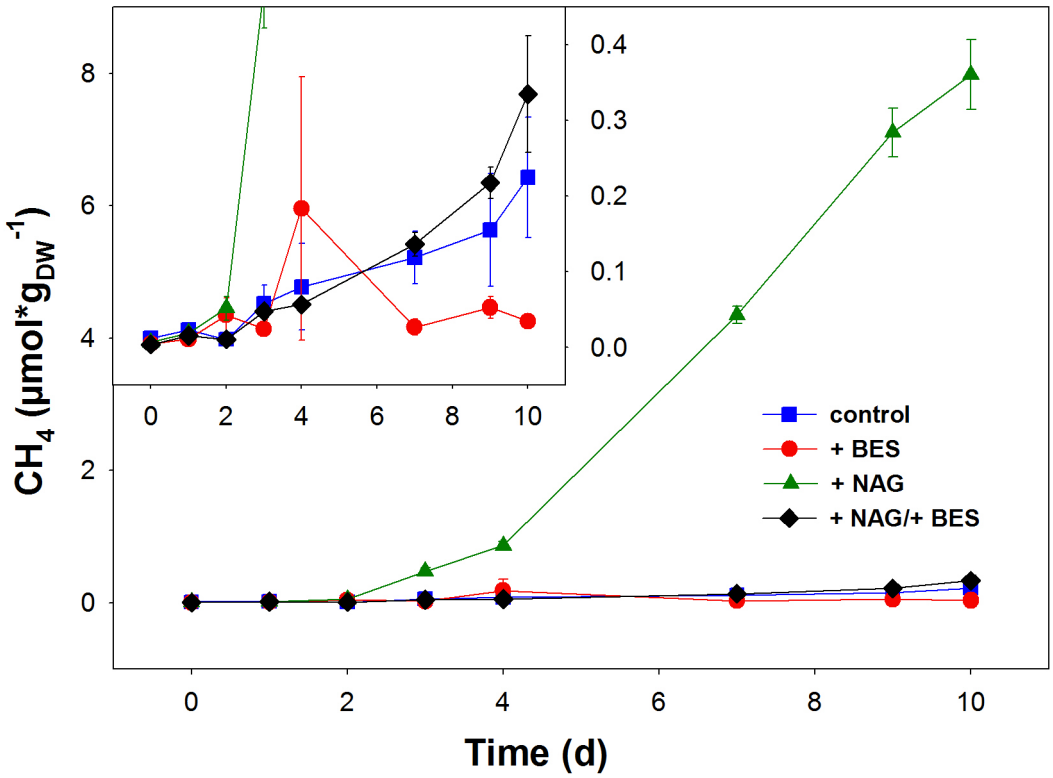

Figure 10: Methanogenic potentials in anoxic microcosms with Puukkosuo fen soil. Microcosms were incubated without substrates (control), with 500 μM NAG (+NAG), with 20 mM BES (+BES), or 500 μM NAG and 20 mM BES (+NAG/+BES). Mean values of three replicate microcosms and standard errors are displayed. The inset in represents an enlargement to allow better visualization of minor CH_4 production in control and +NAG/+BES microcosms.

between different peatlands. Hydrogenotrophic methanogenesis is predominant in many acidic peat and fen soils, however dominance of acetoclastic methanogenesis is observed in mesotrophic fen soil [41, 71, 185]. Methanol stimulates methanogenesis in pH-neutral but not in acidic bog and fen soil microcosms, and stimulation by methanol can be in the same range as stimulation by acetate [14, 54, 185]. The results obtained from microcosm studies with Puukkosuo fen soil likewise indicate a rather small stimulation of methanogenesis by methanol which is in the same range as stimulation by acetate. The collective data indicate that CH_4 in pH-neutral

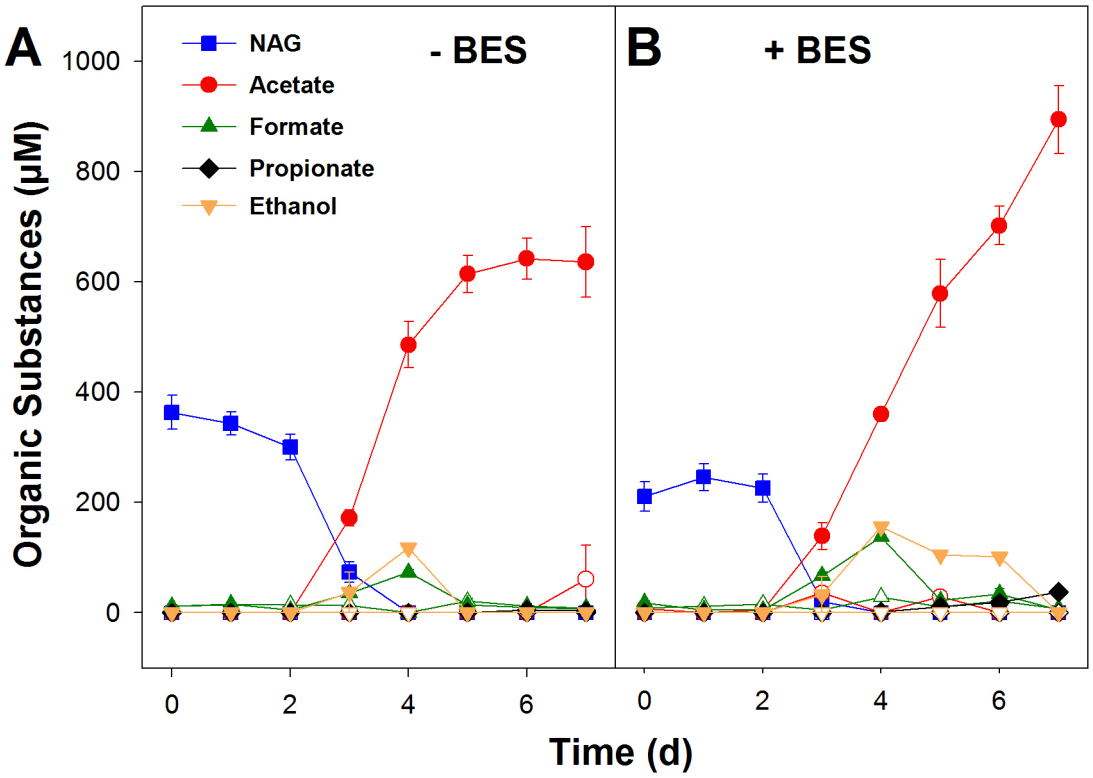

Figure 11: Main fermentation products in anoxic microcosms with Puukkosuo fen soil. Microcosms were incubated without (A) or with (B) 20mM BES to inhibit methanogenesis. Open symbols represent microcosms supplemented with 0 μM NAG, closed symbols represent microcosms supplemented with 500 μM NAG. Mean values of three replicate microcosms and standard errors are displayed. Additionally, production of H_2/CO_2 was detected in all microcosms (data not shown).

fen soil is mainly produced hydrogenotrophically, however, a small contribution of acetoclastic and methylotrophic methanogenesis is also feasible.

3.2.2 Diversity of prokaryotes putatively associated with fermentations and methanogenesis in a pH-neutral fen

Diversity of *Bacteria* and methanogenic *Archaea* in pH-neutral fen soil was assessed by amplicon pyrosequencing of bacterial 16S rRNA genes and *mcrA* as a structural gene marker for methanogenic *Archaea* (1.3.2), respectively. Sequences were qual-

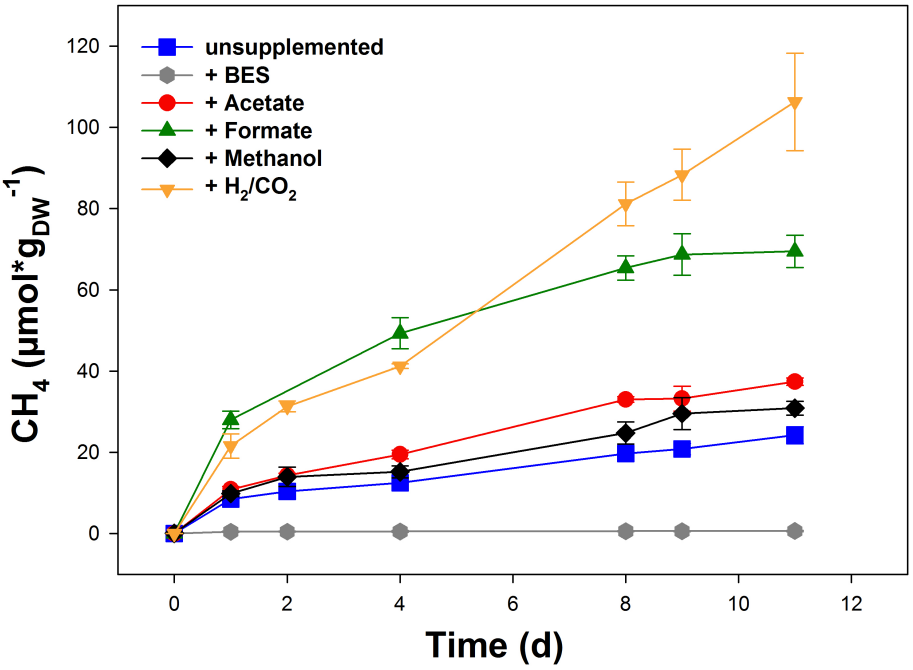

Figure 12: Effect of supplemental substrates on methanogenic potentials in anoxic microcosms with Puukkosuo fen soil. Microcosms were preincubated for 120 days before substrate supplementation. Argon was used as headspace gas. 1 mM of acetate, formate, or methanol or 8% H_2/2% CO_2 (v/v) in the atmosphere. Mean values of three replicate microcosms and standard errors are displayed.

ity filtered (i.e., pyrosequencing and PCR-based errors were removed by denoising with PyroNoise and SeqNoise algorithms; method described in [**119**]) and clustered at 87% (family level) and 84% (species level) similarity for 16S rRNA genes and *mcrA*, respectively, using the Needleman Wunsch algorithm (method described in [**119**]). Phylogenetic trees were generated to assign detected sequences to phyla. In total, 159 and 352 family-level bacterial 16S rRNA OTUs were detected in amplicon libraries from forward and reverse reads, respectively. OTUs mainly affiliated to *Firmicutes* and *Proteobacteria* (approximately 60% of all sequences, Figure 13). Among the *Proteobacteria*, *Alpha-* and *Delta-Proteobacterial* sequences were most

abundant. Additionally, families affiliated to *Actinobacteria*, *Acidobacteria*, *Bacteriodetes*, *Chloroflexi*, *Cyanobacteria*, and *Nitrospiraceae* were detected (Figure 13). 15% of all sequences (i.e., 66 families) were not closely related to known bacterial families and might thus represent novel families. In acidic bog and fen soil, *Proteobacteria*, *Firmicutes*, *Acidobacteria* and *Actinobacteria* are also frequently detected [27, 48, 55, 62, 106, 122, 185]. Sequences and isolates of bacteria in pH-neutral permafrost soil consisted mainly of *Actinobacteria*, *Firmicutes* and *Alpha-* and *Delta-Proteobacteria* [157]. Some *Delta-Proteobacteria* are important sulfate reducers, indicating that pH-neutral Puukkosuo fen soil harbors the potential for sulfate reduction. Additionally, many genera within the *Delta-Proteobacteria* and *Firmicutes* such as *Syntrophobacter* spp. or *Clostridium* spp. are capable of syntrophic interactions with hydrogenotrophic methanogens and are frequently detected in wetlands [30, 48, 145, 146, 156, 185], indicating that those syntrophic organisms might provide substrates to hydrogenotrophic methanogens in Puukkosuo fen soil.

mcrA sequences from Puukkosuo fen were assigned to 9 and 9 species-level OTUs based on forward and reverse reads, respectively. OTU distributions obtained with sequences from forward and reverse reads were similar, thus only results from forward reads are described in more detail. Three species-level OTUs had a relative abundance of $> 1\%$ of all *mcrA* sequences. OTU 1, 2, and 3 accounted for 48.3%, 48.2%, and 1.1% of detected *mcrA* sequences and were related to uncultured *mcrA* as well as to *mcrA* of *Methanoregula* spp., *Methanocella paludicola*, and *Methanoregula* spp., respectively (Figure 14), indicating that both *Methanomicrobiales* and *Methanocellales* are important players in the CH_4 production of Puukkosuo fen. Methanogens of both groups produce CH_4 hydrogenotrophically [6, 92], and higher stimulation with substrates useable by hydrogenotrophic methanogens was observed in microcosm experiments (Figure 12). None of the detected *mcrA* affiliated with *mcrA* of

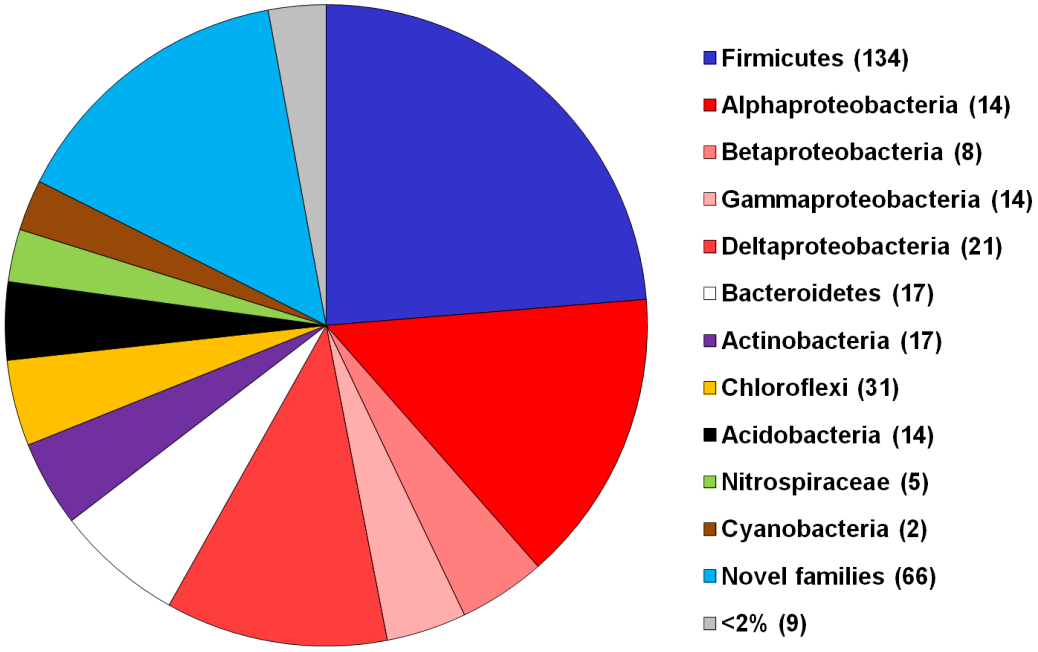

Figure 13: Bacterial families detected in pH-neutral fen soil by amplicon pyrosequencing of bacterial 16S rRNA genes. In total, 2 375 quality filtered sequences were derived from reverse reads, which were assigned to 352 OTUs based on a family-level threshold similarity of 87 %. Results obtained with forward read sequences were similar and are thus not shown. Affiliation of families to major groups and number of detected families per group (in parentheses) are displayed.

the *Methanosarcinaceae*, indicating that this group and thus acetoclastic methanogenesis is of minor importance in Puukkosuo fen soil. Indeed, the stimulatory effect of acetate on methanogenic potentials in fen soil microcosms was much lower than of H_2/CO_2 (Figure 12). 16S rRNA gene sequences and *mcrA* sequences affiliated with hydrogenotrophic methanogenic groups are frequently observed as the predominant groups in peatland soils, even though sequences affiliated with *Methanosarcinales* are also detected [40, 54, 55, 191],[**121**]. *Methanosarcinales* seem to be more abundant in wetter than in drier sites in an acidic mesotrophic fen in Lapland, and were identified as CO_2 consumers in slightly acidic Schlöppnerbrunnen fen soil [55, 191].

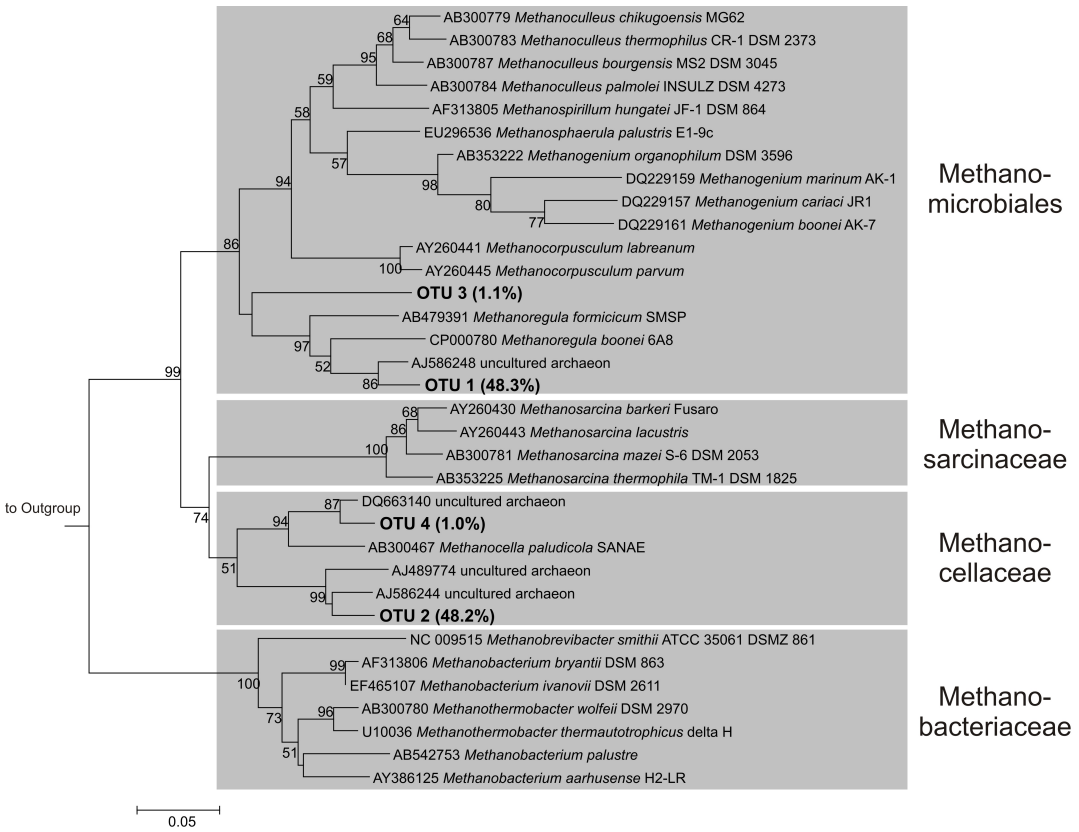

Figure 14: Phylogenetic tree of representative *mcrA* sequences (forward reads) from Puukkosuo fen soil. The tree is based on *in silico* translated amino acid sequences obtained by amplicon-pyrosequencing. OTUs were grouped at species-level phylogenetic similarity of 84% after ampliconnoise quality-filtering. Values in parenthese represent relative abundances of the OTUs. In total, 1 785 quality filtered sequences were used for OTU calculations. Gray boxes indicate branches where the majority of sequences group into a certain phylogenetic class. The percentage of replicate trees in the bootstrap analysis (10 000 replicates), in which the associated taxa clustered together, are shown next to the branches (values below 50% have been omitted). *mcrA* of *Methanococcus maripaludis* S2 was used as outgroup.

3.2.3 Conclusions: Fermentation and methanogenesis in pH-neutral fen soil

pH-neutral Puukkosuo fen soil showed the potential to produce acetate, formate, ethanol, and H_2/CO_2 from NAG and thus provide precursors for fen methanogenesis. Methanogenic potentials were stimulated mainly by H_2/CO_2 and formate, indicating a predominance of hydrogenotrophic methanogenesis in pH-neutral fen

soil. This finding was supported by analysis of *mcrA* as a structural gene marker for methanogens, as all detected sequences affiliated with *mcrA* of hydrogenotrophic taxa. The bacterial community in Puukkosuo fen soil was dominated by *Firmicutes*, *Alpha-* and *Delta-Proteobacteria* including syntrophic genera as well as by novel families, indicating high bacterial diversity and novelty as well as diverse metabolic capacities in pH-neutral fen soil.

3.3 Processes involved in turnover of N_2O

3.3.1 N_2O production and consumption in pristine peatlands

N_2O-emissions from pristine peatlands are highly variable (Figure 9). Water-saturated fen soils containing minor amounts of nitrate emit only small amounts of N_2O and often can display also sink-functions for N_2O (Figure 9) [43, 42, 93, 141, 161],[**117, 120**], while permafrost-affected tundra soils like palsa peats or cryoturbated peat circles can emit high amounts of N_2O (Figure 9) [100, 138],[**119**]. Especially in palsa peats, N_2O emissions are highly variable depending e.g., on the thickness of plant cover and nitrate availability, and nitrate-limited palsas also act as N_2O sinks [100],[**119**].

Statistical analyses were conducted to assess the correlation between environmental parameters and *in situ* N_2O emission of five contrasting peatland soils (Table 6, Figure 9). Spearman rank correlation revealed positive ($R = 0.9$, $P = 0.08$) and negative ($R = -0.8$, $P = 0.08$) correlations between soil nitrate and ammonium content and *in situ* N_2O emission, respectively (Table 7), indicating that denitrification rather than nitrification is the process responsible for observed N_2O emission from peatlands (Figure 9). Indeed, peatland soil has a high degree of water-saturation, and denitrification is the main source of N_2O in water-saturated and thus mainly anoxic soils [23, 130]. The amount of total carbon in the studied peatland soils decreased with increasing water content and increasing mean annual temperature ($R = -0.9$ and $R = -0.8$, respectively), indicating that wet and warm conditions facilitate the mineralization of soil carbon. Interestingly, *in situ* N_2O emissions did not correlate with soil pH (Table 7), indicating that nitrate availability rather than soil pH is the main driver of N_2O emissions from peat soil.

N_2O production in unsupplemented anoxic microcosms with peatland soil ranged from < 1 to 1200 nmol$\cdot g_{DW}^{-1}$ (Figure 15). N_2O production was highest in microcosms

Table 7: Correlation of soil parameters and observed N_2O emissions. Spearman rank correlations were calculated based on mean values for environmental parameters. Values for N_2O emissions were based on Figure 9, values for environmental parameters were taken from site descriptions [100, 119, 120, 185].

Parameter	pH	water content	MAT [1]	NO_3^-	NH_4^+	Total C [2]	Total N [3]	C:N
Correlation (R)	0.10	-0.20	0.21	0.87	-0.82	0.10	0.50	-0.60
Significane (P)	0.95	0.78	0.78	0.08	0.08	0.95	0.45	0.35

[1] Mean annual temperature.

[2] Total carbon.

[3] Total nitrogen.

with cryoturbated peat soil and lowest in microcosms with fen soil (Figure 15). In microcosms with palsa and cryoturbated peat soil, N_2O that was produced within the first 50 to 100 hours of incubation was subsequently consumed (Figure 15), demonstrating the capacity of peatland soils for N_2O reduction. N_2O production was always higher in microcosms that were amended with acetylene to block N_2O reduction [190] than in unamended microcosms (Figure 15), indicating that N_2O was not the sole endproduct of denitrification in either of the systems, but that complete denitrification to N_2 occured. In acetylene-amended microcosms, highest N_2O production (1800 nmol$\cdot g_{DW}^{-1}$) was observed in microcosms with cryoturbated peat soil (Figure 15), reflecting the high nitrate content of the soil and the observed high *in situ* emissions (Figure 9) [100, 138],**[115]**. N_2O production in acetylene-amended microcosms with palsa peat soil and with pH-neutral fen soil was about 10 fold lower than in microcosms with cryoturbated peat soil (approximately 200 nmol$\cdot g_{DW}^{-1}$). Lowest N_2O production was observed in acetylene-amended microcosms with permafrost tundra soil (< 14 nmol$\cdot g_{DW}^{-1}$) (Figure 15).

Spearman rank correlation of environmental parameters and observed N_2O to (N_2O+N_2) in anoxic microcosms with peat soil revealed that the contribution of N_2O to total N-gases decreased with increasing pH ($R = -0.9$, $P = 0.08$) and

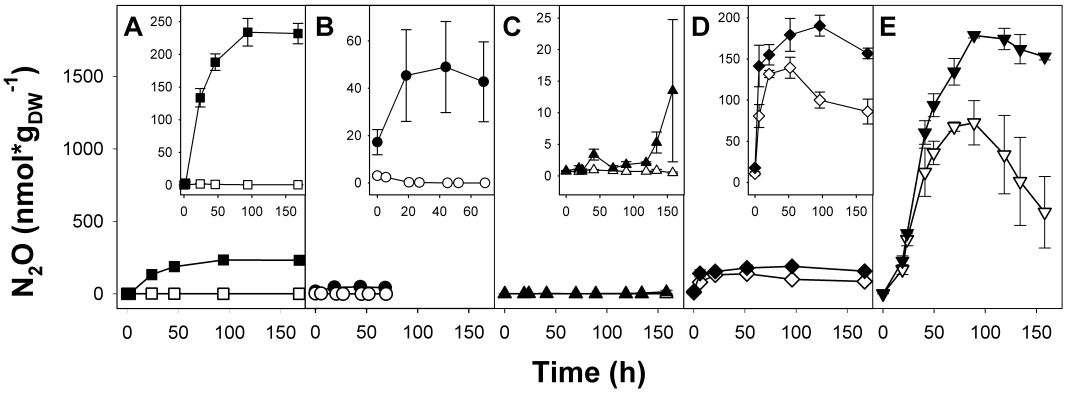

Figure 15: Production of N_2O in unsupplemented anoxic microcosms with peatland soil from 0 to 20 cm depth. Open symbols represent microcosms without addition of acetylene, closed symbols represent microcosms with addition of acetylene to block N_2O reductase [190]. The insets in (A), (B), (C), and (D) represent enlargements to allow better visualization of relatively small amounts of N_2O produced in those microcosms. A: Puukkosuo fen (pH 6.8), B: Schlöppnerbrunnen fen (pH 5.0), C: Permafrost tundra (pH 4.0), D: Skalluvaara palsa peat (pH 4.5), E: Cryoturbated peat circles (pH 4.0).

increasing mean annual temperature ($R = -1.0$, $P = 0.02$), indicating that under warmer and pH-neutral conditions N_2 is the favoured endproduct of denitrification. Indeed, the relative contribution of N_2O to total N-gases is higher under more acidic conditions in soils and pure cultures as N_2O reductase is more severely affected by acidic pH than the other N-reductases involved in denitrification [8, 39, 91, 170, 171]. Moreover, N_2O-reduction is more strongly affected by low temperatures than the preceeding reductive steps, thus N_2O release from soil is often high also in winter [31, 53, 110]. However, denitrifier communities can be adapted to low pH and low temperatures as well [31, 117].

Supplemental nitrate (0 to 500 μM) stimulated N_2O production in nitrate-depleted acetylene-amended anoxic microcosms with peatland soil to different extents (Figure 16). Maximal initial N_2O production rates (v_{max}) were highest in microcosms with Schlöppnerbrunnen fen soil (pH 5), while v_{max} was lowest in microcosms with

Skalluvaara palsa peat soil (pH 4.5) (Figure 16, Table 8). In microcosms with permafrost tundra soil, N_2O production rates were highest with 10 μM nitrate and decreased with increasing nitrate concentrations, indicating that denitrification in permafrost tundra soil is saturated at low nitrate concentrations. Supplemental nitrite (0 to 500 μM) likewise stimulated N_2O production in nitrate-depleted acetylene-amended anoxic microcosms with peatland soil (Figure 16). v_{max} were highest in microcosms with Puukkosuo fen soil (52 nmol$\cdot g_{DW}^{-1}\cdot h^{-1}$), intermediate in microcosms with Schlöppnerbrunnen fen and cryoturbated peat soil ($32 - 33$ nmol$\cdot g_{DW}^{-1}\cdot h^{-1}$), and lowest in permafrost tundra and Skalluvaara palsa peat soil (18 and 15 nmol$\cdot g_{DW}^{-1}\cdot h^{-1}$, respectively) (Table 8). v_{max} values were in the same range in nitrate- and nitrite-supplemented microcosms with Schlöppnerbrunnen fen soil, while v_{max} values were 2- to 9-fold higher in nitrite- than in nitrate-supplemented microcosms with soil from the other peatlands (Table 8).

v_{max}/K_M (indicative for a soils nitrate/nitrite affinity) were highest for nitrate- and nitrite-dependent denitrification in microcosms with cryoturbated peat soil (Table 8). Nitrate-dependent v_{max}/K_M were positively correlated with the nitrate content of the soil and *in situ* N_2O emissions as well as negatively correlated with the ammonium content of the soil (Spearman rank correlation: $R = 0.9$, $P = 0.08$; $R \approx 1.0$, $P < 0.0001$; $R = -0.8$, $P = 0.08$ for nitrate content, *in situ* emissions, and ammonium content, respectively).

Table 8: Parameters of apparent nitrate- and nitrite-dependent Michaelis-Menten kinetics in anoxic acetylene-amended microcosms with peatland soil.

	Nitrate amended					Nitrite amended				
Soil	Puukkosuo fen	Schlöppnerbrunnen fen	Permafrost tundra	Skalluvaara palsa peat	Cryoturbated peat circles	Puukkosuo fen	Schlöppnerbrunnen fen	Permafrost tundra	Skalluvaara palsa peat	Cryoturbated peat circles
v_{max} (nmol$\cdot h^{-1}\cdot g_{DW}^{-1}$) [1]	18	25	2	3	17	52	33	18	15	32
K_M (μM) [1]	29	11	n.a. [2]	17	3	62	7	21	39	6
v_{max}/K_M ($10^{-3}\cdot h^{-1}$) [1]	17	53	n.a. [2]	11	466	22	105	41	25	407

[1] Kinetic parameters calculated from Figure 16.

[2] Not applicable.

The ratio of N_2O to total N-gases (i.e., the amount of N_2O produced in the absence to the amount of N_2O produced in the presence of acetylene) at different

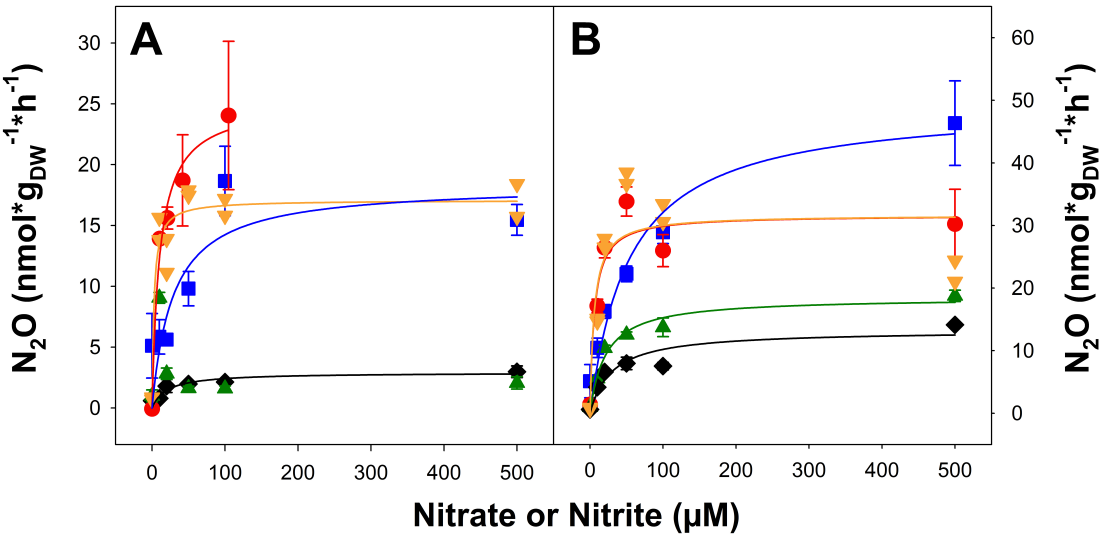

Figure 16: Apparent Michaelis-Menten kinetics of nitrate- (A) and nitrite-dependent (B) denitrification in acetylene-amended anoxic microcosms with peatland soil from 0 to 20 cm depth. Blue squares: Puukkosuo fen (pH 6.8), Red circles: Schlöppnerbrunnen fen (pH 5.0), Green upward triangles: Permafrost tundra (pH 4.0), Black diamonds: Skalluvaara palsa peat (pH 4.5), Orange downward triangles: Cryoturbated peat circles (pH 4.0). Individual values of duplicate microcosms (cryoturbated peat circles) or mean values and standard errors of three replicate microcosms (all other soils) are displayed. Michaelis-Menten curves were fitted to the data where applicable (solid lines).

concentrations of supplemented nitrate and nitrite differed between the soils (Figure 17). In pH-neutral fen soil, $N_2O/(N_2O+N_2)$ was below 10% when 10 μM nitrate or nitrite were supplied and did not exceed 30% at 100 μM supplied nitrate/nitrite. In contrast, $N_2O/(N_2O+N_2)$ was 100% at all supplied concentrations of nitrate or nitrite in microcosms with permafrost tundra soil. Indeed, $N_2O/(N_2O+N_2)$ was negatively correlated with soil pH (Spearman rank correlation: $R = -0.9$, $P = 0.08$ for 100 μM supplied nitrate). Moreover, there was a positive correlation between $N_2O/(N_2O+N_2)$ when nitrate and when nitrite were supplied (Spearman rank correlation: $R \approx 1.0$, $P < 0.0001$ for 10 μM supplied nitrate/nitrite). Nitrate concentrations generally influence the ratios of N_2O/N_2+N_2O produced from denitrification

in soils and sediments [10, 17, 65].

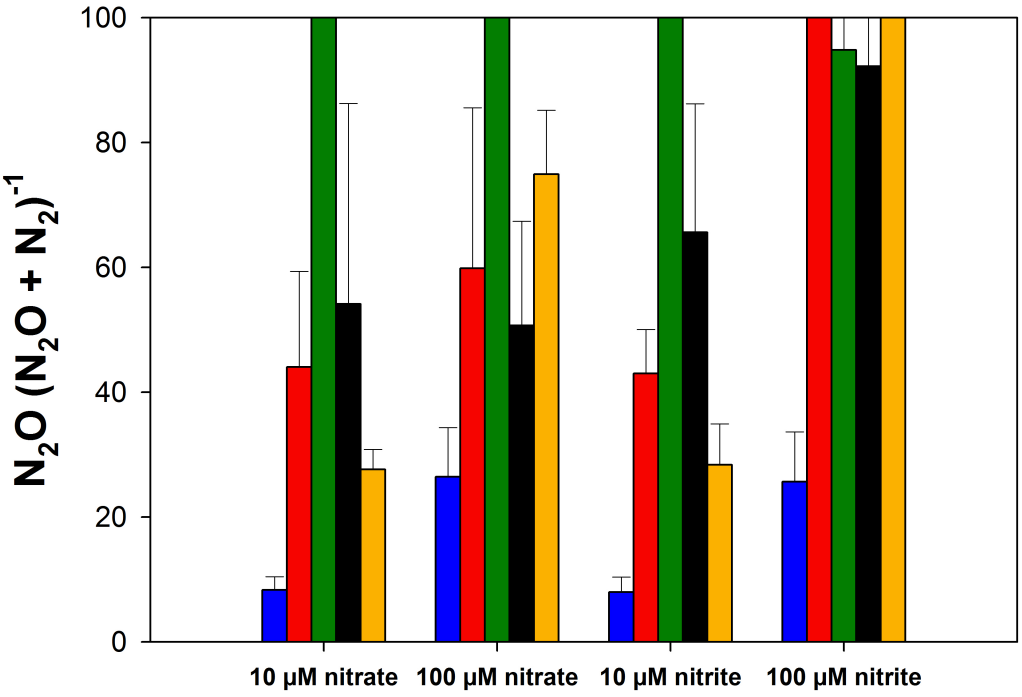

Figure 17: Effect of supplemental nitrate and nitrite on the ratio of N_2O to total N-gases in anoxic peatland soil microcosms. Blue: Puukkosuo fen (pH 6.8), Red: Schlöppnerbrunnen fen (pH 5.0), Green: Permafrost tundra (pH 4.0), Black: Skalluvaara palsa peat (pH 4.5), Orange: Cryoturbated peat circles (pH 4.0). Mean values and standard errors of three replicate microcosms are displayed.

The collective data indicate that (i) *in situ* N_2O emissions from pristine peatland soils depend mainly on the amount of available nitrate, (ii) denitrification and N_2O consumption potentials are present in all studied peatlands, and (iii) *in situ* pH and nitrate content are the main factors determing the ratio of N_2O to total N-gases.

3.3.2 Diversity of peatland denitrifiers

Diversity of peatland denitrifiers was assessed by amplicon pyrosequencing of the structural genemarkers *narG*, *nirK*, *nirS*, and *nosZ* from 0 to 20 cm soil [**115, 119, 120, 118**]. In total, 8 385, 19 292, 5 218, and 5 920 quality-filtered sequences of *narG*, *nirK*, *nirS*, and *nosZ*, respectively, were used for analyzing phylogenetic diversity of peatland denitrifiers at species-level (67%, 83%, 82%, and 80% for *narG*, *nirK*, *nirS*, and *nosZ*, respectively). 13 to 35 species-level operational taxonomic units (OTUs) were detected per genemarker.

Three major species-level OTUs (i.e., with a relative abundance > 1% in at least one soil) of *narG* were obtained, which occured in all peatland soils in varying relative abundances, indicating that the major *narG* OTUs are ubiquitous in peatland soils (Figure 18 A). The relative abundances of OTU 1 ranged from 35% in pH-neutral fen soil to 98% in cryoturbated permafrost soil. Sequences belonging to OTU 1 were related to *Actinobacterial narG* (e.g., *Actinosynnema mirum*, *Streptomyces coelicolor*) (Figure 19). The relative abundance of OTU 1 was greater in acidic than in pH-neutral soils, moreover OTU 1 was especially abundant in the severly frost-affected soils of the Skalluvaara palsa peat and the cryoturbated peat circles (Figure 18 A), indicating that *Actinobacteria* are the predominantly detectable nitrate-reducers in acidic and more specifically in frost-affected systems. *Actinobacterial* predominance has also been detected in Canadian permafrost soil [158]. Moreover, *Actinobacteria* are common in many soil types including peatland soils [27, 56, 86, 87, 104, 188]. *Actinobacteria* frequently occur in more extreme habitats like acidic soils or cold environments, as they often show a higher range of tolerance against environmental stresses [104, 193]. The relative abundance of OTU 2 ranged from 2% in cryoturbated permafrost soil to 59% in pH-neutral fen soil (Figure 18). Sequences of OTU 2 were related to *Beta-Proteobacterial narG* (e.g.,

Table 9: Species-level diversity of denitrification associated-genes in peatland soils. OTU tables were rarified with 100 iterations at sampling depth of 500, 1000, 100, and 100 for *narG*, *nirK*, *nirS*, and *nosZ*, respectively.

Gene marker	Soil	No. of OTUs observed [1]	No. of OTUs estimated estimated [2]	H [3]	E [4]
narG	Puukkosuo	6.77 ± 0.14	10.81 ± 0.50	1.30 ± 0.004	0.48 ± 0.006
	Schlöppnerbrunnen	5.76 ± 0.05	8.30 ± 0.14	1.32 ± 0.001	0.53 ± 0.002
	Permafrost tundra	5.19 ± 0.11	7.06 ± 0.29	0.91 ± 0.004	0.40 ± 0.005
	Skalluvaara	2.46 ± 0.06	2.53 ± 0.08	0.55 ± 0.004	0.47 ± 0.010
	Peat circles	3.34 ± 0.06	3.48 ± 0.08	0.28 ± 0.005	0.17 ± 0.004
nirK	Puukkosuo	21.28 ± 0.17	30.20 ± 0.82	1.55 ± 0.003	0.35 ± 0.001
	Schlöppnerbrunnen	12.18 ± 0.09	13.05 ± 0.22	1.95 ± 0.004	0.54 ± 0.002
	Permafrost tundra	6.67 ± 0.12	8.70 ± 0.36	1.42 ± 0.003	0.53 ± 0.005
	Skalluvaara	4.00 ± 0.00	4.00 ± 0.00	1.49 ± 0.003	0.75 ± 0.001
	Peat circles	4.05 ± 0.10	4.70 ± 0.19	0.07 ± 0.002	0.03 ± 0.001
nirS	Puukkosuo	10.37 ± 0.15	12.98 ± 0.43	2.09 ± 0.016	0.62 ± 0.003
	Schlöppnerbrunnen	7.01 ± 0.09	10.24 ± 0.35	0.75 ± 0.011	0.27 ± 0.003
	Permafrost tundra	7.80 ± 0.07	7.89 ± 0.09	2.51 ± 0.010	0.85 ± 0.003
	Skalluvaara	7.91 ± 0.07	8.00 ± 0.08	2.43 ± 0.012	0.82 ± 0.004
	Peat circles	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	* [5]
nosZ	Puukkosuo	7.20 ± 0.10	7.84 ± 0.17	2.01 ± 0.013	0.71 ± 0.004
	Schlöppnerbrunnen	3.71 ± 0.08	4.30 ± 0.15	0.68 ± 0.011	0.37 ± 0.005
	Permafrost tundra	2.75 ± 0.07	2.88 ± 0.09	0.25 ± 0.010	0.18 ± 0.005
	Skalluvaara	3.99 ± 0.05	4.09 ± 0.08	1.30 ± 0.011	0.66 ± 0.006
	Peat circles	3.54 ± 0.07	3.77 ± 0.11	0.46 ± 0.013	0.26 ± 0.006

[1] Number of OTUs observed in rarified OTU tables ± standard error.

[2] Chao1 richness estimate of rarified OTUs ± standard error.

[3] Shannon diversity index of rarified OTUs ± standard error.

[4] Species Evenness of rarifierd OTUs ± standard error.

[5] Could not be calculated because only 1 OTU was detected.

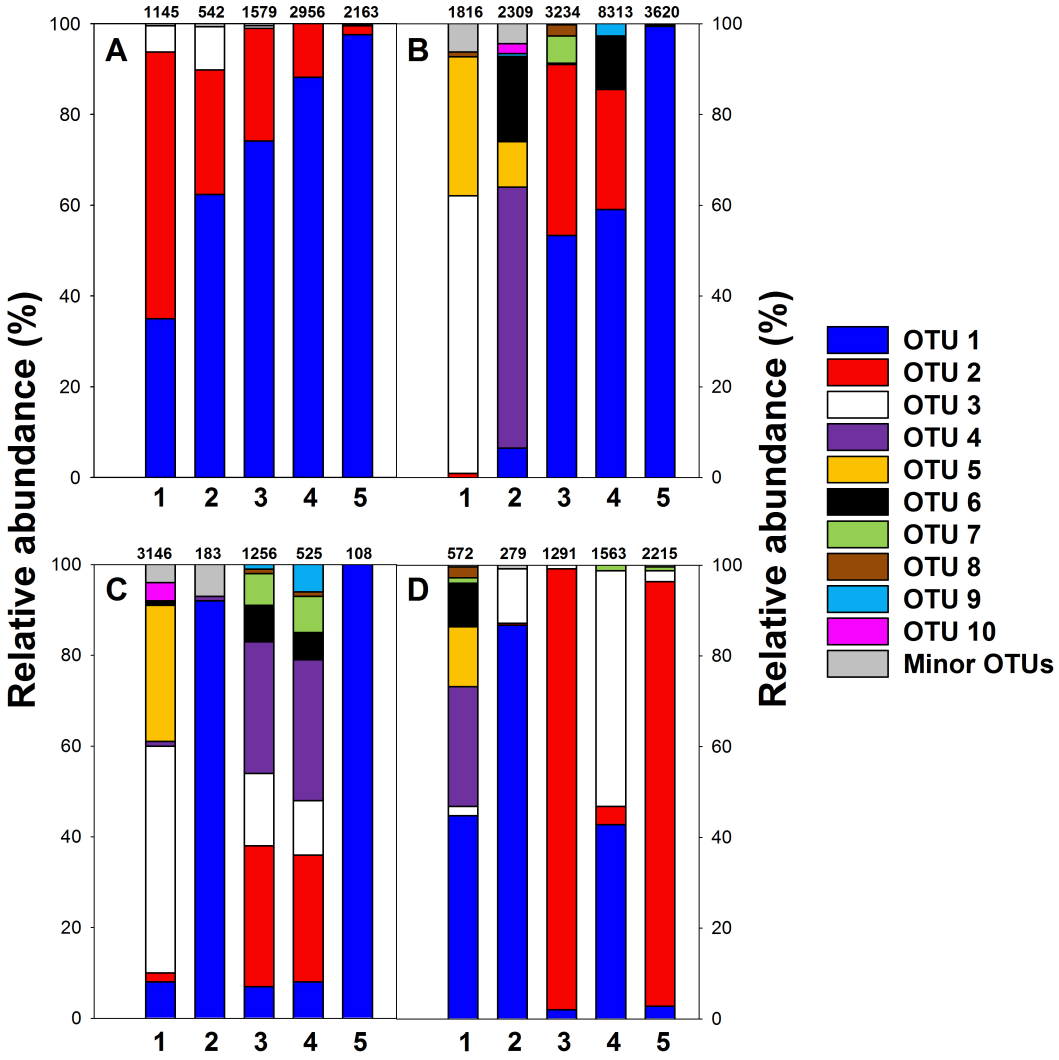

Figure 18: Relative abundances of *narG* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) species-level OTUs derived from amplicon pyrosequencing libraries of peatland soils. Sequences were obtained from 0 to 20 cm soil of Puukkosuo fen (1), Schlöppnerbrunnen fen (2), permafrost tundra (3), Skallu-vaara palsa peat (4), and cryoturbated peat circles (5). Numbers above the bars give the total number of quality filtered sequences in each library. Minor OTUs were below 1%, 2%, 3%, and 1% in *narG*, *nirK*, *nirS*, and *nosZ* amplicon libraries.

Ralstonia spp., *Burkholderia* spp.) (Figure 19). *Beta-Proteobacteria* occur in a variety of peatland soils, including acidic peatlands in Russia and Finland [86, 105].

The relative abundance of OTU 2 was in general smaller in acidic than in pH-neutral soil, and even smaller in permafrost-affected palsa peat and cryoturbated permafrost soil (11% and 2%, respectively), indicating that *Beta-Proteobacteria*-related organisms might be negatively affected by acidic pH as well as by the mixing action of the permafrost. However, *Beta-Proteobacteria* have been detected in cold environments like the Greenland ice sheet and were also detected in greater abundance in unturbated permafrost tundra, indicating that cold temperatures alone were not limiting their abundance [187],[**115**]. Relative abundances of OTU 3 were smaller than those of OTUs 1 and 2, indicating that this *narG* phylotype is of lesser importance in nordic peatland soils. OTU 3 had a relative abundance of 6% and 10% in pH-neutral and acidic fen soil, respectively, while its relative abundance was <1% in all permafrost-affected peatland soils (Figure 18). Sequences of OTU 3 were related to *narG* of *Deinococci* like *Thermus thermophilus* and *Marinithermus hydrothermalis* (Figure 19). Species-level diversity of *narG* was highest in Puukkkosuo fen soil as indicated by the number of observed and estimated OTUs, Shannon diversity index and Species Evenness (Table 9). Shannon diversity index was likewise high in Schlöppnerbrunnen fen soil but decreased in permafrost affected soils (Table 9).

Detected *nirK* sequences grouped into 10 major species-level OTUs (i.e., OTUs with a relative abundance of > 2% in at least one of the soils), all of which affiliated with *Proteobacterial nirK* (Figure 18 B, Figure 20). *nirK* community composition differed between the different soils. OTUs 1 and 2, related to *nirK* of *Alcaligenes* sp. and *Rhodopseudomonas* sp., respectively, dominated *nirK* communities in frost-affected soils, while they only accounted for < 1% and 6% of detected *nirK* in Puukkosuo and Schlöppnerbrunnen fen soil, respectively (Figure 18 B, Figure 20). This indicates that OTUs 1 and 2 might be more resistant to the cold and more acidic conditions that occur in permafrost soils. OTU 3, related to *nirK* of *Rhizobium etli*

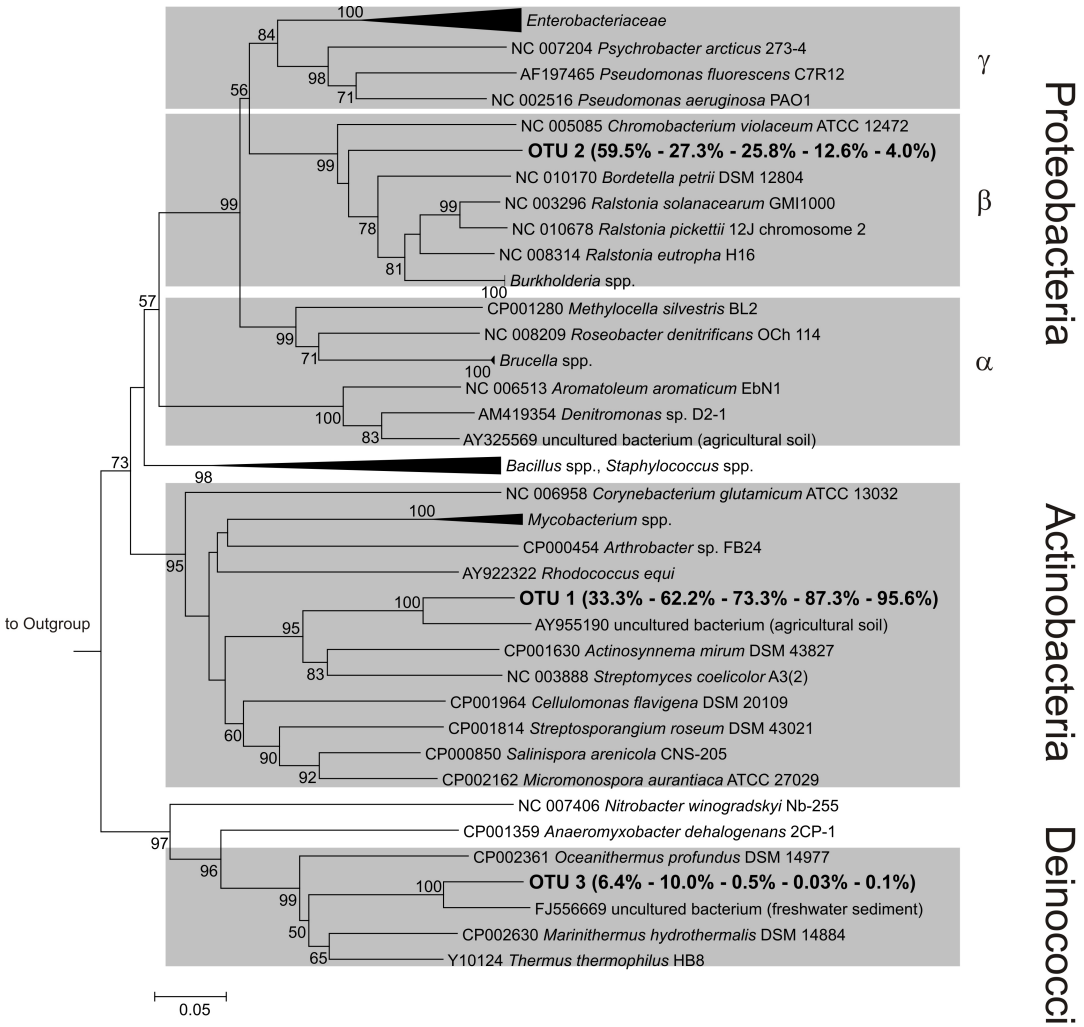

Figure 19: Phylogenetic tree of representative *narG* sequences (forward reads) from different peatland soils. The tree is based on *in silico* translated amino acid sequences. Sequences were obtained via amplicon-pyrosequencing from peatland soils. OTUs were grouped at species-level phylogenetic similarity of 67% after ampliconnoise quality-filtering. Values in parenthese represent relative abundances of the OTUs in Puukkosuo fen, Schlöppnerbrunnen fen, permafrost tundra, Skalluvaara palsa peat, and cryoturbated peat circles. In total, 8 385 quality filtered sequences were used for OTU calculations. Gray boxes indicate branches where the majority of sequences group into a certain phylogenetic class. The percentage of replicate trees in the bootstrap analysis (10 000 replicates), in which the associated taxa clustered together, are shown next to the branches (values below 50% have been omitted). *narG* of *Haloarcula marismortui* ATCC 43049 was used as outgroup.

and *Methylobacterium* sp., was detected almost exclusively in pH-neutral fen soil from Puukkosuo, where it accounted for > 60% of detected *nirK*, indicating that this OTU might be adapted to neutral pH and thus unable to tolerate higher levels of acidity (Figure 18 B). OTU 4 was only detected in slightly acidic fen soil from Schlöppnerbrunnen and was only distantly related to known *nirK* (Figure 18 B, Figure 20). Thus, there might be novel and specialized NirK-type denitrifiers in this fen, that can tolerate slightly acidic conditions but are more sensible to soil freezing. Detected and estimated species-level OTU numbers were highest in Puukkosuo and Schlöppnerbrunnen fen soils and lowest in permafrost-affected palsa peat and peat circle soil (Table 9). Shannon diversity and Species Evenness indices were similar in all soils except for cryoturbated peat circle soil, which showed very low Shannon diversity and Species Evenness (Table 9).

nirS sequences likewise grouped into 10 major species-level OTUs (i.e., OTUs with a relative abundance of > 3% in at least one of the soils). Three different types of *nirS* communities were detected: The first type was detected in the pH-neutral fen Puukkosuo and consisted mainly of OTUs 3 and 5 (approximately 80% of all sequences), which affiliated with uncultured soil and sediment bacteria in the phylogenetic tree (Figure 18 C, Figure 21). Thus, this community type is likely dominated by hitherto unknown NirS-type denitrifiers. The second *nirS* community type was detected in Schlöppnerbrunnen fen and cryoturbated peat circle soil. This community type was dominated by OTU 1 (Figure 18 C), which affiliated with *nirS* of *Bradyrhizobium* sp., *Rhodanobacter* sp., and *Magnetospirillum magneticum* in the phylogenetic tree (Figure 21). The third type was detected in unturbated permafrost tundra and Skalluvaara palsa peat soil and was dominated by OTUs 2, 3, and 4 (Figure 18 C). OTUs 2 and 3 were related to *nirS* of uncultured sediment bacteria, while OTU 4 was related to *nirK* of *Azoarcus tolulyticus* (Figure 21). Species-level

nirS diversity as expressed by number of observed and estimated OTUs as well as by Shannon diversity and Species Evenness indices was high in Puukkosuo fen soil and low in cryoturbated peat circle soil, while it was at a similar level in all other studied soils (Table 9).

nosZ sequences grouped into 8 major species-level OTUs (i.e., OTUs with a relative abundance of >1% in at least one of the soils). *nosZ* OTU distribution differed between the 5 soils (Figure 18 D). 7 major OTUs were detected in Puukkosuo fen soil, OTUs 1, 4, 5, and 6 were most prominent with relative abundances of 45%, 26%, 13%, and 10%, respectively (Figure 18 D). OTU 1 was also detected in the 4 other soils, it dominated detected *nosZ* in Schlöppnerbrunnen fen soil (87%), but had a very low abundance in permafrost tundra and cryoturbated peat circle soil (2% and 3% of detected *nosZ*, respectively). OTU 2 was almost exclusively detected in permafrost tundra and cryoturbated peat circle soil, where it accounted for 97% and 94% of detected *nosZ*, respectively (Figure 18 D). OTU 3 was the predominant OTU in Skalluvaara palsa peat soil (52%) and was also abundant in Schlöppnerbrunnen fen soil (12%). OTUs 1, 2, 3, 4, 5, and 6 were affiliated with *nosZ* of *Azospirillum lipoferum*, *Mesorhizobium* sp., *Bradyrhizobium japonicum*, *Bosea* sp., *Azospirillum largimobile*, and *Herbaspirillum* spp., respectively (Figure 22), indicating that detected peatland *nosZ* are diverse and dominated by *Alpha-* and *Beta-Proteobacterial nosZ*. *Beta-Proteobacterial nosZ* were almost exclusively detected in Puukkosuo fen soil (Figure 22), indicating that *Beta-Proteobacteria* might be more severely affected by the more acidic pH in the other soil types, confirming the effect observed for *narG* (Figure 18 D, Figure 19). Species-level *nosZ* diversity as given by the number of observed and estimated OTUs and Shannon diversity and Species Evenness indices was highest in Puukkosuo fen soil (Table 9). This finding is in line with the results obtained with the other gene markers.

The collective data indicate that (i) *Actinobacterial*, *Alpha-* and *Beta-Proteobacterial* nitrate reducers and denitrifiers are common in peatlands, (ii) some species occur in all peatlands and might thus contribute to a core denitrifier community in peatlands, (iii) nitrate reducer and denitrifier community compositions differ between different peatland types, and (iii) highest diversity is associated with pH-neutral fen soil.

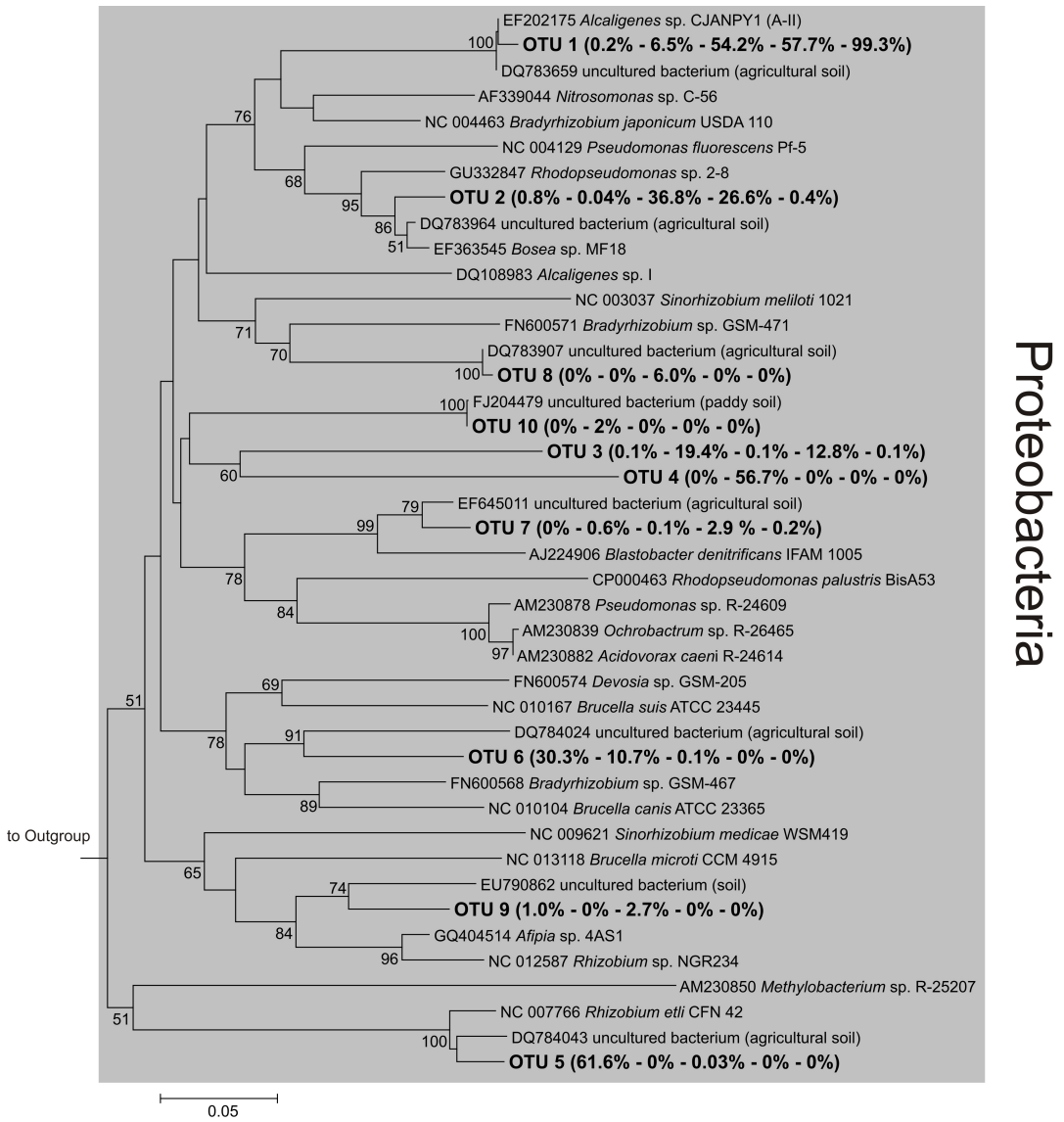

Figure 20: Phylogenetic tree of representative *nirK* sequences from different peatland soils. The tree is based on *in silico* translated amino acid sequences. Sequences were obtained via amplicon-pyrosequencing from peatland soils. OTUs were grouped at species-level phylogenetic similarity of 83% after ampliconnoise quality-filtering. Values in parenthese represent relative abundances of the OTUs in Puukkosuo fen, Schlöppnerbrunnen fen, permafrost tundra, Skalluvaara palsa peat, and cryoturbated peat circles. In total, 19 292 quality filtered sequences were used for OTU calculations. Gray boxes indicate branches where the majority of sequences group into a certain phylogenetic class. The percentage of replicate trees in the bootstrap analysis (10 000 replicates), in which the associated taxa clustered together, are shown next to the branches (values below 50% have been omitted). *nirK* of *Haloarcula marismortui* ATCC 43049 was used as outgroup.

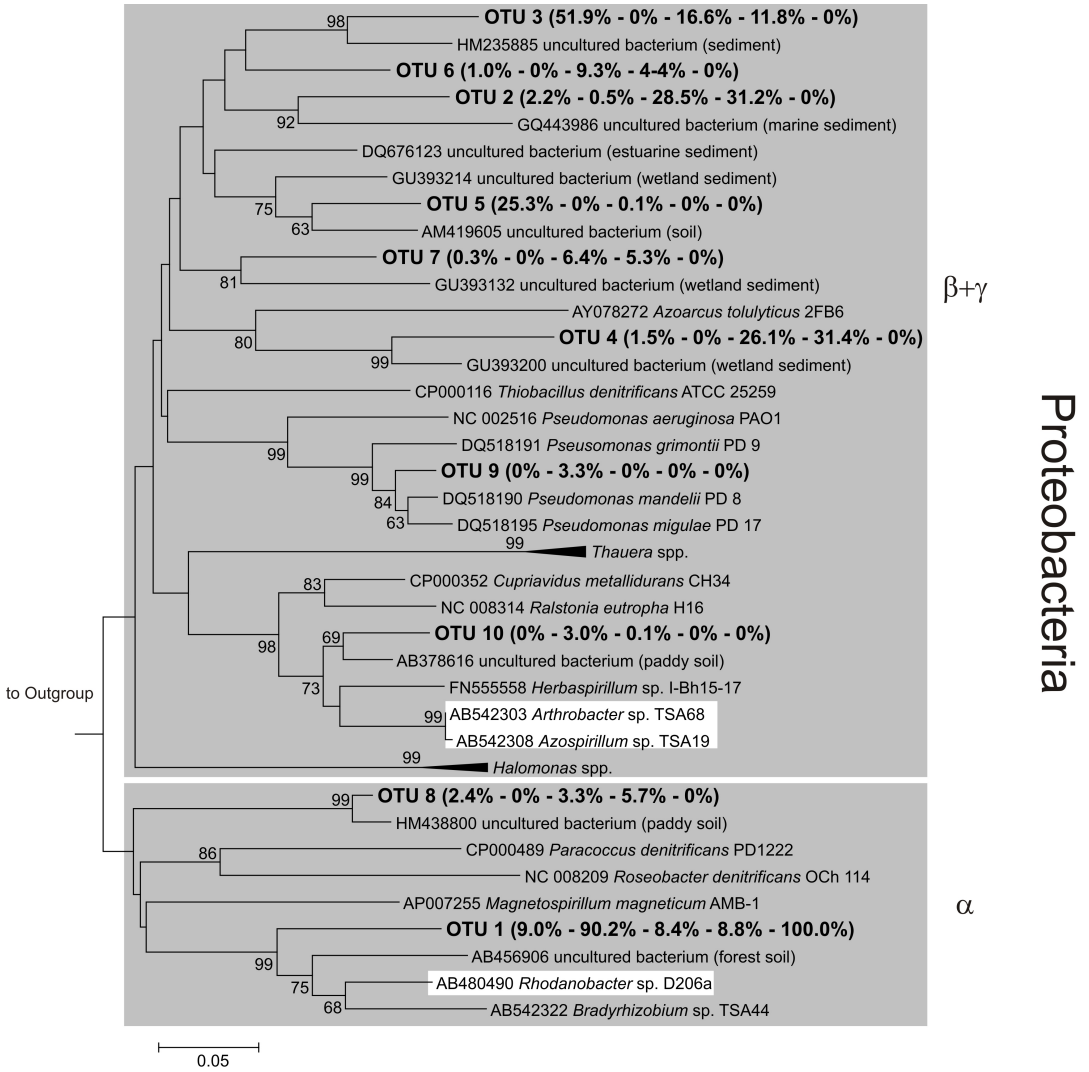

Figure 21: Phylogenetic tree of representative *nirS* sequences (forward reads) from different peatland soils. The tree is based on *in silico* translated amino acid sequences. Sequences were obtained via amplicon-pyrosequencing from peatland soils. OTUs were grouped at species-level phylogenetic similarity of 82% after ampliconnoise quality-filtering. Values in parenthese represent relative abundances of the OTUs in Puukkosuo fen, Schlöppnerbrunnen fen, permafrost tundra, Skalluvaara palsa peat, and cryoturbated peat circles. In total, 5 218 quality filtered sequences were used for OTU calculations. Gray boxes indicate branches where the majority of sequences group into a certain phylogenetic class. The percentage of replicate trees in the bootstrap analysis (10 000 replicates), in which the associated taxa clustered together, are shown next to the branches (values below 50% have been omitted). *nirS* of *Rhodothermus marinus* DSM 4252 was used as outgroup.

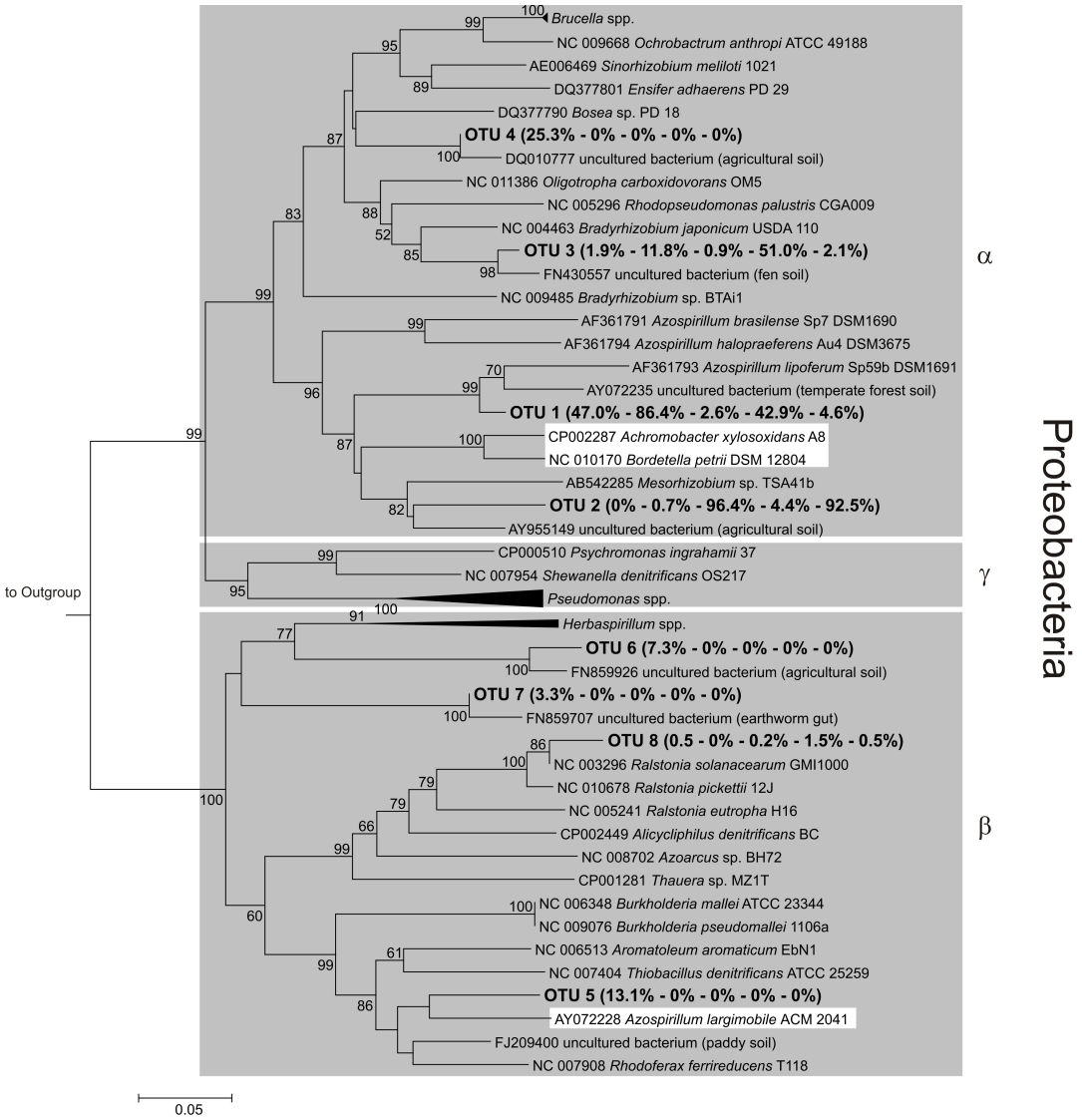

Figure 22: Phylogenetic tree of representative *nosZ* sequences (forward reads) from different peatland soils. The tree is based on *in silico* translated amino acid sequences. Sequences were obtained via amplicon-pyrosequencing from peatland soils. OTUs were grouped at species-level phylogenetic similarity of 80% after ampliconnoise quality-filtering. Values in parenthese represent relative abundances of the OTUs in Puukkosuo fen, Schlöppnerbrunnen fen, permafrost tundra, Skalluvaara palsa peat, and cryoturbated peat circles. In total, 5 920 quality filtered sequences were used for OTU calculations. Gray boxes indicate branches where the majority of sequences group into a certain phylogenetic class. The percentage of replicate trees in the bootstrap analysis (10 000 replicates), in which the associated taxa clustered together, are shown next to the branches (values below 50% have been omitted). *nosZ* of *Haloarcula marismortui* ATCC 43049 was used as outgroup.

3.3.3 Environmental factors shaping denitrifier communities in peatlands

Statistical analyses based on the community composition of sequences received from pyrosequencing of *narG*, *nirK*, *nirS*, and *nosZ* from different peatland soils were conducted to assess the effect of different environmental parameters on community composition of nitrate reducers and denitrifiers. Environmental parameters were classified as primary environmental parameters (i.e., pH, temperature, precipitation, nitrate and ammonium content, total carbon, total nitrogen, C/N ratio) or derived parameters (i.e., N_2O emission, ratio of N_2O to total N-gases, relative abundance of *narG* or *nosZ*). Canonical correspondence analyses (CCA) were conducted for each gene marker on the basis of rarified species-level OTU tables to cluster the communities according to their habitat and to elucidate the primary or derived environmental parameters that influence community composition.

CCA of *narG* revealed a clear separation of Puukkosuo and Schlöppnerbrunnen fen soil communities from each other and from the permafrost affected communities (Figure 23 A), as was already observed for direct comparison of OTU relative abundances (Figure 18 A). Community composition was affected by pH, total soil carbon and mean annual precipitation (Figure 23 A). The relative abundance of OTU 1 was negatively correlated with pH and soil water content (Spearman rank correlations with $R = -0.8$, $P = 0.08$ and $R \approx -1.0$, $P = 0.02$, respectively), and positively correlated with total soil carbon ($R = 0.9$, $P = 0.08$), while OTU 2 was positively and negatively correlated with soil water content and total soil carbon, respectively ($R \approx 1.0$, $P < 0.001$ and $R = -0.9$, $P = 0.08$, respectively).

CCA of *nirK* showed a clear separation of all studied peatland communities (Figure 23 B). The *nirK* community of Puukkosuo fen was most dissimilar to the communities in the other peatland soil, as it was also indicated earlier (Figure 18 B, Table 9). pH and total soil carbon determined the community composition of detected

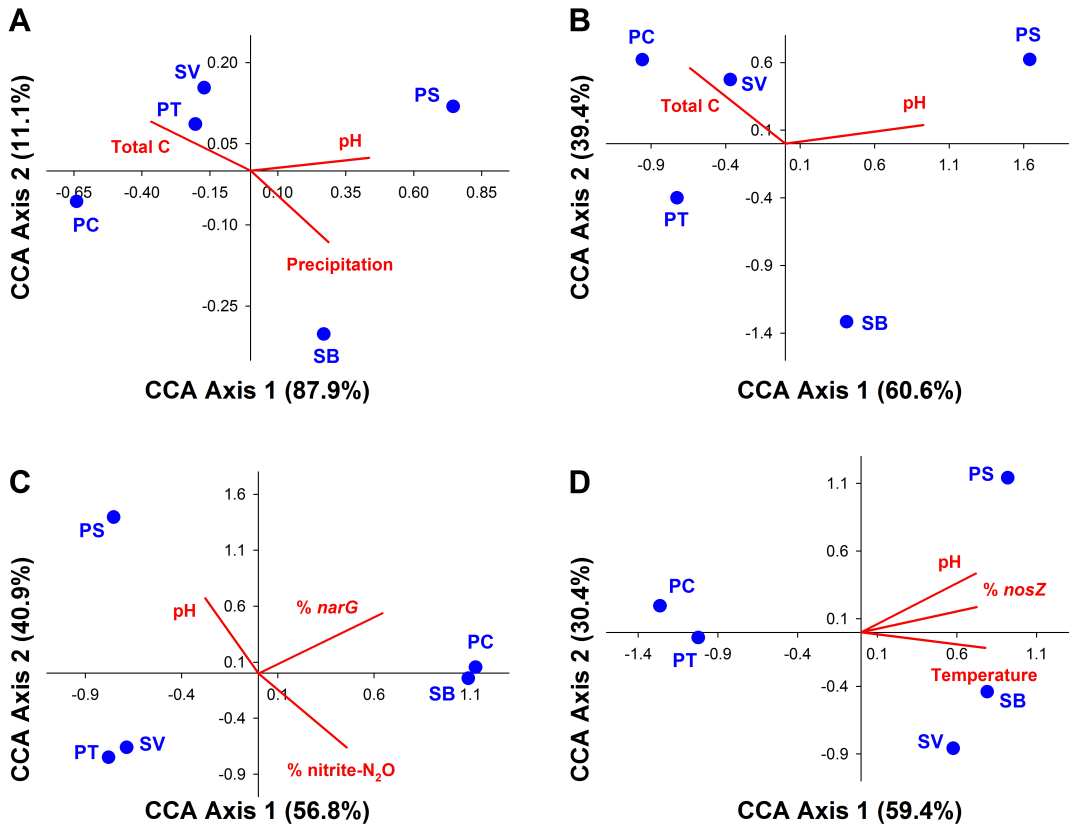

Figure 23: Canonical correspondence analyses (CCA) based on relative species-level OTU abundances of *narG* (A), *nirK* (B), *nirS* (C), and *nosZ* (D). Blue circles represent the different sampling sites (Table 6). Primary and derived environmental parameters affecting the community composition of denitrification-associated genes are displayed in red. OTU abundances from rarified datasets(100 iterations at sampling depths of 500, 1000, 100, and 100 for *narG*, *nirK*, *nirS*, and *nosZ*, respectively) were used for the calculation of CCAs.

nirK (Figure 23 B). Correlations between environmental parameters and individual OTUs were observed for OTU 1, which was predominant in frost-affected soils and was positively, negatively and negatively correlated with total soil carbon, soil water content and soil pH, respectively (Spearman rank correlations with $R = 0.9$, $P = 0.08$, $R \approx -1.0$, $P = 0.02$, and $R = -0.8$, $P = 0.08$, respectively). On the other hand, the relative abundance of OTU 5 which was abundant in Puukkosuo and

Schlöppnerbrunnen fen soil was positively and negatively correlated with soil water content and total soil carbon, respectively ($R = 0.98$, $P = 0.02$ and $R = -0.87$, $P = 0.08$, respectively), indicating that total soil carbon does not only affect the *nirK* community composition in total but also more directly the relative abundance of individual OTUs. OTU 2 was the only OTU that showed a correlation with *in situ* N_2O emission, the relative abundance of OTU 2 was negatively correlated with N_2O emission ($R = -0.9$, $P = 0.08$), indicating that denitrifiers carrying this type of *nirK* might be important for N_2O reduction *in situ*.

CCA based on relative abundances of detected *nirS* revealed 3 distinct clusters of *nirS* communities, thus supporting the visual comparison (Figure 23 C, Figure 18 C). The *nirS* community from Puukkosuo fen was clearly distinct from the community in the other peatland soils, and Skalluvaara palsa peat and peatland tundra as well as Schlöppnerbrunnen fen and cryoturbated peat circle communities clustered together (Figure 23 C). Community composition of detected *nirS* was influenced by pH as a primary environmental factor and by the relative abundance of *narG* and the ratio of N_2O to total N-gases (when 10 μM nitrite were supplied) as derived factors (Figure 23 C). The relative abundance of several OTUs was correlated to observed *in situ* N_2O emissions: OTU 1 was positively correlated with *in situ* N_2O emissions (Spearman rank correlation with $R = 0.98$, $P = 0.02$), while OTUs 2, 4 and 6 were negatively correlated with *in situ* N_2O emissions ($R = -0.98$, $P = 0.02$, $R = -0.87$, $P = 0.08$, and $R = -0.98$, $P = 0.02$, respectively), indicating that denitrifiers harboring those types of *nirS* might be involved in production and consumption of N_2O in peatland soils, respectively.

CCA analysis based on relative abundances of *nosZ* in peatland soils likewise supported the observed grouping of peatland soils (Figure 23 D, Figure 18 D), as the *nosZ* community of Puukkosuo fen was clearly separated from the other soils, while

nosZ communities of Schlöppnerbrunnen fen and Skalluvaara palsa peat as well as *nosZ* communities of permafrost tundra and cryoturbated peat circles grouped together (Figure 23 D). Community composition of *nosZ* was influenced by the primary environmental parameters pH and temperature as well as by the relative abundance of *nosZ* in peatland soils as a secondary parameter (Figure 23 D). Unlike OTUs of *nirK* and *nirS*, OTUs of *nosZ* were not correlated with observed *in situ* N_2O emissions. However, OTUs 1 and 2 were negatively and positively correlated with the ratio of N_2O to total N-gases in unsupplemented anoxic microcosms, respectively (Spearman rank correlations with $R \approx -1.0$, $P = 0.02$ and $R = 0.9$, $P = 0.08$, respectively), indicating that denitrifiers harboring *nosZ* of OTU 1 might be highly efficient in N_2O consumption and cause the observed differences in N_2O emissions.

The collective data indicate that (i) nitrate reducer and denitrifier communities in different peatland soils are clearly distinct, (ii) the primary and derived environmental factors affecting community composition of denitrification associated genes differ between the genes, and (iii) several OTUs of *nirK*, *nirS*, and *nosZ* are likely determining N_2O emissions from peatland soils.

3.3.4 Conclusions: Denitrification in peatland soils

All studied peatland soils were capable of N_2O production and consumption. However, N_2O emission was only observed from acidic soils with high nitrate content, indicating that soil nitrate content is one of the main drivers of N_2O emissions from peatland soils. N_2O consumption was especially efficient in pH-neutral fen soil, but also the other soils consumed N_2O once nitrate or nitrite were depleted, indicating that peatland soils can be permanent or temporary sinks for N_2O when other N-oxides are scarce. The capacity of peatland soils to produce or consume N_2O is reflected in the community composition of denitrification-associated genes from peatlands, and several OTUs of *nirK*, *nirS*, and *nosZ* are indicators for N_2O production or consumption. Several OTUs of denitrification-associated genes were detected in all sampled peatlands, indicating that a core denitrifier community might exist in all types of peatlands.

3.4 Effect of water table manipulations on anaerobic processes in a model peatland

Prolonged drought periods and more severe precipitation events are anticipated due to climate change [174]. Those events are predicted to severely affect the degree of water saturation in northern hemisphere peatlands and thus the availability of oxygen in the soil, which might in turn affect the release or uptake of greenhouse gases by those peatlands [44, 154, 174]. Thus, the effect of water table manipulations on anaerobic CO_2, CH_4, and N_2O producing or consuming processes and the involved microbial populations (i.e., fermenters, methanogens, and denitrifiers) was assessed in the fen Schlöppnerbrunnen as a model system. Experimental short-term drought was established by artificially lowering the water table for 6 to 12 weeks in the summers of 2006, 2007, and 2008 by 30 to 50 cm in three treatment plots. Treatment plots were rewetted at the end of the drought period (for details concerning the experimental setup refer to [111]). The effect on fermentative and methanogenic potentials in microcosms with fen soil as well as on the community composition of methanogens and denitrifiers were assessed before and after the drought period [**118, 121**]. Fermenters were not targeted by molecular approaches in this study. In 2009, treatment plots were artificially flooded by constant irrigation from May until September. The effect on fermentative, methanogenic and denitrifying potentials in microcosms with fen soil as well as on the community composition of methanogens and denitrifiers were assessed at the beginning and at the end of the flooding period.

3.4.1 Effect of water table manipulations on fermentation potentials in acidic fen soil

Fermentation potentials were assessed in anoxic NAG supplemented microcosms with fen soil taken from control and drought plots (0 to 10 cm and 30 to 40 cm soil)

before and after a 42 day drought period in 2006 (3.2.1). BES was added to inhibit methanogenesis [114]. NAG was always consumed, and acetate, CO_2, and ethanol accumulated. Apparent Michaelis-Menten kinetics based on initial CO_2 production rates yielded higher maximal reaction velocities (v_{max}) in 0 to 10 cm than in 30 to 40 cm soil (Figure 24 A, B). v_{max} was similar in control and drought plots in 30 to 40 cm soil depth before the drought period, while it was higher in drought than in control plots in 0 to 10 cm soil depth (Figure 24 A, B). After the drought period, v_{max} decreased in both plot types in 30 to 40 cm depth soil, while it increased in 0 to 10 cm depth soil, indicating that there was a seasonal shift in fermentation potential in both soil layers rather than an effect of the drought treatment.

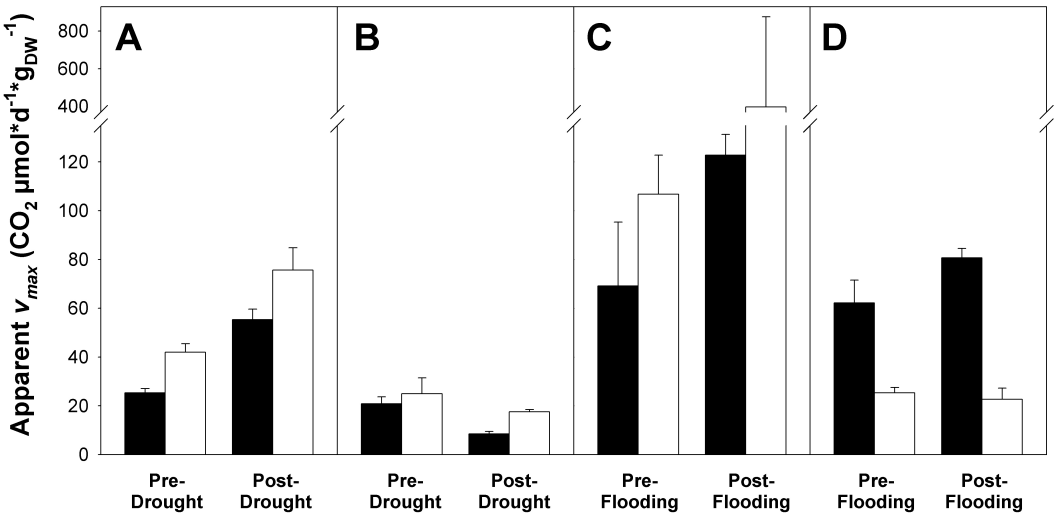

Figure 24: Effect of water table manipulations on NAG-dependent fermentation in anoxic microcosms with fen soil. Microcosms were set up before and after a 42 day long experimental drought in 2006 with soil from 0 to 10 cm depth (A) and 30 to 40 cm depth (B) as well as before and after 6 month of experimental flooding in 2009 with soil from 0 to 20 cm depth (C) and 20 to 40 cm depth (D). Microcosms were supplemented with 0 to 1 mM NAG after 10 (A, B) or 7 (C, D) days of anoxic preincubation to reduce alternative electron acceptors. v_{max} are based on initial CO_2 production rates in microcosms. Black bars represent microcosms with soil from control plots, white bars represent microcosms with soil from treatment plots. Mean values of three replicate microcosms and standard errors are displayed.

Fermentation potentials were likewise assessed in anoxic microcosms with fen soil taken from control and treatment plots before and after the 6 month flooding of treatment plots in 2009. NAG-dependent v_{max} values were slightly higher in 0 to 20 cm than in 20 to 40 cm depth soil before the flooding period and were similar in control and treatment plots (Figure 24 C, D). Prolonged flooding of fen soil increased v_{max} in 0 to 20 cm depth soil, while v_{max} decreased in 20 to 40 cm depth soil from both control and treatment plots by a similar degree, and were thus essentially unaffected by flooding (Figure 24 C, D).

The collective data obtained during the manipulation experiments indicate that fermentation potentials in fen soil are largely unaffected by a lowered or raised water table, but are rather affected by soil depth and seasonal variations. Indeed, *in situ* CO_2 fluxes of the acidic fen are likewise unaffected by experimental drought [111]. The fen Schlöppnerbrunnen harbors a large diversity of facultatively anaerobic fermenters whichs are likely largely unaffected be altering of the water table and the subsequent alteration of oxygen penetration into the soil [30, 48, 185].

3.4.2 Effect of water table manipulations on methanogenesis in an acidic fen soil

Methanogenic potentials were assessed in unsupplemented anoxic microcosms with fen soil from control and drought plots before and after 42 days of experimental drought. Before the drought, methanogenic potentials were essentially the same in control and drought plots. Prolonged drought decreased the total amount of CH_4 produced and prolonged the initial lag phase before the onset of methanogenesis (Figure 25). This indicates that the higher drought-induced soil aeration inhibits methanogenesis and might restore alternative electron acceptors like nitrate, sulfate or iron(III) in fen soil. Indeed, increased concentrations of sulfate and iron(III) are detected after experimental drought in Schlöppnerbrunnen fen soil [72]. Alternative

electron acceptors are preferentially reduced upon the onset of anoxia in microcosms [95], which likely causes to the observed prolonged lag phase. The inhibitory effect of alternative electron acceptors on methanogenesis is also frequently observed in peatlands as sulfate or iron accumulated during oxid periods suppresses methanogenesis in peatland soil for long time periods even after the water saturation [29, 58]. This effect was also observed *in situ* in the fen Schlöppnerbrunnen [71]. Prolonged drought periods decrease methanogenic potentials in peatland soils as the water table is considerably lowered, even though methanogenesis may continue in anoxic microenvironments [72, 73, 191].

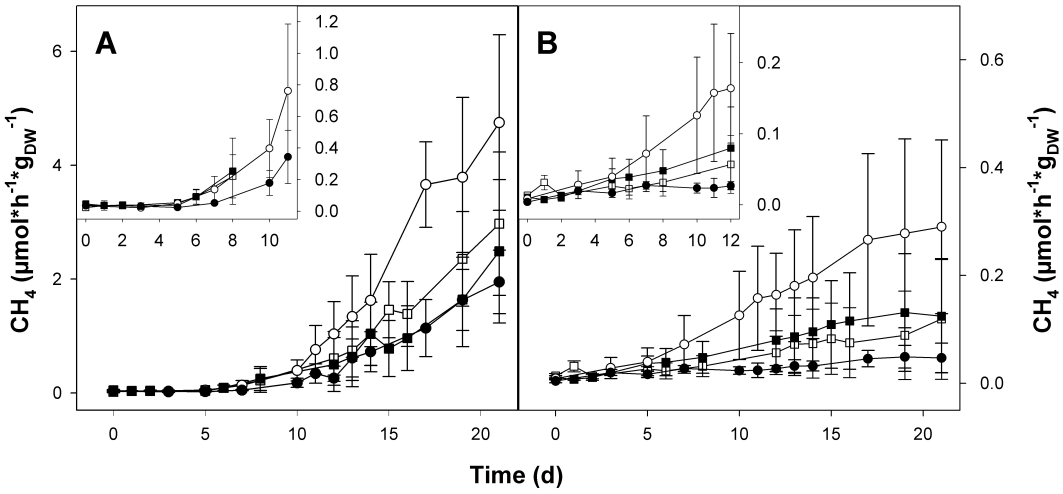

Figure 25: Effect of 42 days of experimental drought on methanogenic potentials in anoxic microcosms with fen soil from 0 to 10 cm (A) and 30 to 40 cm depth (B). Squares and circles represent microcosms with fen soil sampled before and after 42 days of experimental drought, respectively. Open and closed symbols represent control and drought plots, respectively. Mean values of three replicate microcosms and standard errors are displayed.

In 2009, the methanogenic potential of fen soil before and after 6 month of experimental flooding from 0 to 20 cm and 20 to 40 cm was assessed in formate-supplemented anoxic microcosms. Apparent Michaelis-Menten kinetics were calculated based on initial CH_4 production rates. v_{max} were higher in 0 to 20 cm

than in 20 to 40 cm depth soil (Figure 26), indicating higher methanogenic potentials in the upper soil. Indeed higher cell numbers of methanogens and higher methanogenic potentials are generally found in the upper layers of peatland soils [40, 62, 73, 71, 185]. Experimental flooding increased v_{max} in both soil depths in treatment plots, while v_{max} remained constant or decreased in 0 to 20 cm and 20 to 40 cm fen soil from control plots (Figure 26), indicating that higher water saturation of fen soil increases methanogenic potentials likely by promoting anoxia in the soil. Indeed, methanogenic potentials and CH_4 release are higher in water-saturated peatlands and are decreased in drained peatland systems [73, 80, 113].

The effect of water table manipulations on the structure of the methanogenic community was assessed during the experimental drought in 2008 as well as during the experimental flooding by combined quantitative PCR and terminal restriction fragment length polymorphism (TRFLP) analysis of the structural gene marker *mcrA* in 3 control and 3 treatment plots (for methodic details see [**121**]). *mcrA* copy numbers ranged between 1% and 5% of archaeal 16S rRNA gene copy numbers in all plots and sampling timepoints (Figure 27). Experimental drought decreased *mcrA* abundances, while experimental flooding increased *mcrA* abundances (Figure 27). In control plots, *mcrA* abundances were similar at the start and the end of the treatment periods (Figure 27).

Methanocellaceae, *Methanomicrobiales/-bacteriales*, and *Methanosarcinaceae* were detected by TRFLP analysis of *mcrA* genes and transcripts from the fen Schlöppnerbrunnen (Figure 28). TRFs affiliated with *Methanocellaceae* and *Methanomicrobiales/-bacteriales* accounted for more than 70% of detected TRFs from *mcrA* genes, while *Methanosarcinaceae*-affiliated TRFs were below 30%. Relative abundances of the detected groups did not significantly change after experimental drought or experimental flooding, however there was a slight difference in the *mcrA* community

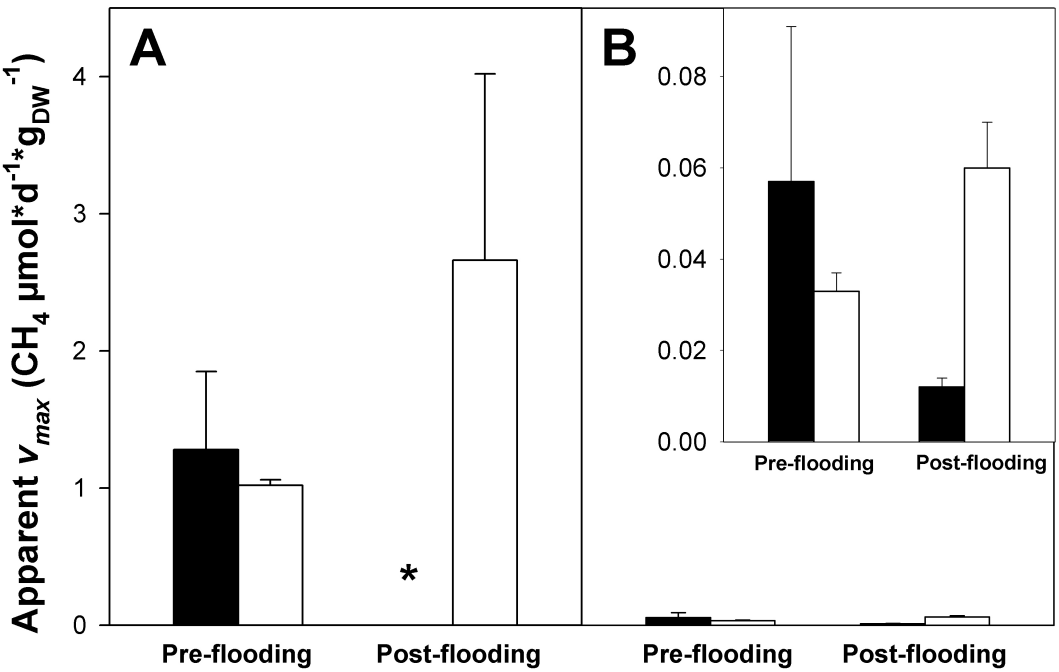

Figure 26: Effect of 6 month flooding on formate-dependent methanogenesis in anoxic microcosms with fen soil from 0 to 20 cm (A) and 20 to 40 cm depth (B). Microcosms were supplemented with 0 to 1 mM formate after 7 days of anoxic preincubation to reduce alternative electron acceptors. v_{max} are based on initial CH_4 production rates in microcosms. Black bars represent microcosms with soil from control plots, white bars represent microcosms with soil from flooded plots. Mean values of three replicate microcosms and standard errors are displayed. Post-flooding CH_4 production rates in microcosms with 0 to 20 cm soil from control plots did not follow apparent Michaelis-Menten kinetics (*). The insert in (B) represents a magnification of the y-axis to better visualize the low observed v_{max}.

composition in 2008 and 2009 (Figure 28 A and B, respectively), with *Methanocellaceae* being to most abundant group in 2008 and *Methanomicrobiales/-bacteriales* being the most abundant group in 2009. This indicates that the composition of the methanogenic community is rather stable when subjected to short-term changes such as experimental drought or flooding but might be influenced by annual variations.

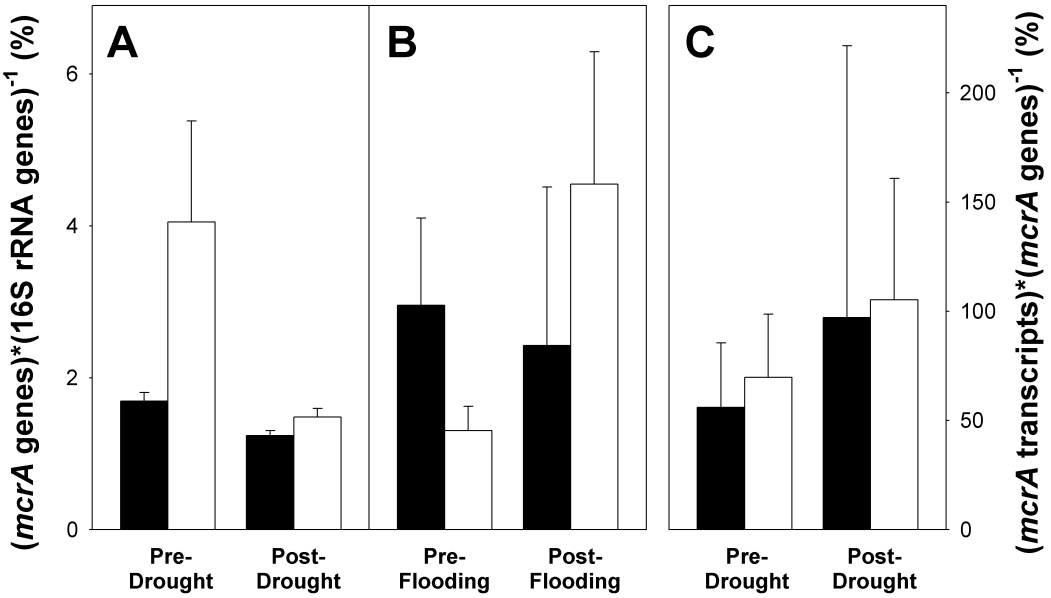

Figure 27: Effect of water table manipulations on the abundance of *mcrA* genes and transcripts in an acidic fen. *mcrA* gene copy numbers in relation to archaeal 16S rRNA genes were determined before and after a 42 day long experimental drought in 2006 with soil from 0 to 40 cm depth (A) as well as before and after 6 month of experimental flooding in 2009 with soil from 0 to 40 cm depth (B). *mcrA* transcript numbers were determined before and after a 42 day long experimental drought in 2006 with soil from 0 to 40 cm depth (C). Black bars represent abundances in control plots, white bars represent abundances in treatment plots. Mean values of three replicate plots (four sampling depths per plot) and standard errors are displayed.

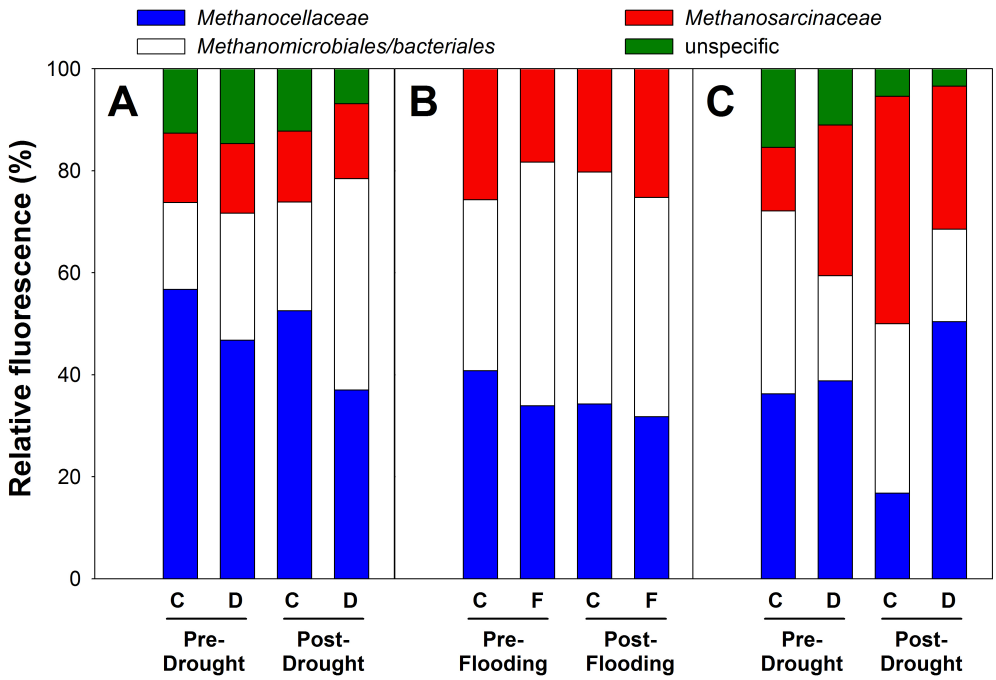

Figure 28: Effect of water table manipulations on the community composition of *mcrA* genes and transcripts in an acidic fen. Community composition was determined by TRFLP analysis before and after a 42 day long experimental drought in 2006 with soil from 0 to 40 cm depth on gene (A) and transcript (C) level as well as on gene level before and after 6 month of experimental flooding in 2009 with soil from 0 to 40 cm depth (B). All TRFs that could be assigned to a certain order/family of methanogens were grouped. Mean values of three replicate plots (four sampling depths per plot) are displayed.

3.4.3 Effect of water table manipulations on denitrification in an acidic fen

The effect of water table manipulations on the abundance of nitrate reducers and denitrifiers in acidic fen soil was assessed by qPCR of *narG* and *nosZ* in control and treatment plots before and after the experimental drought in 2008 as well as before and after experimental flooding in 2009 (qPCR assay described in [118]). *narG* and *nosZ* copy numbers ranged from 2% to 14% and from 0.02% to 0.2% of bacterial 16S rRNA gene copy numbers, respectively (Figure 29). After 42 days of experimental drought, relative abundances of *narG* and *nosZ* in drought plots were 2x and 1.5 as high as before the drought period, respectively, while they were slightly lower in control plots (0.75x and 0.6x as high as before the drought period, respectively) (Figure 29), indicating that growth of nitrate reducers and denitrifiers was stimulated by the experimental drought. Experimental drought leads to increased oxygen penetration into the soil and might thus promote the growth of facultatively aerobic denitrifiers. The observed effect of experimental flooding on *narG* and *nosZ* abundances was less pronounced than the effect of experimental drought. Relative abundances of *narG* were lower post- than pre-flooding in flooded and control plots, while relative abundances of *nosZ* were similar post- than pre-flooding in both plot types (Figure 29), indicating that increased water saturation does not influence the relative abundance of nitrate reducers or denitrifiers in fen soil. Moreover, the relative abundance of nitrate reducers undergoes likely stronger seasonal variations than the relative abundance of denitrifiers, as the relative abundance of *narG* was lower in the end of the experimental period in 2008 and 2009 (August and November, respectively), while the variation in *nosZ* relative abundance was less pronounced (Figure 29).

The effect of water table manipulations on the community composition of nitrate reducers and denitrifiers was assessed by TRFLP analysis of *narG* and *nosZ* in con-

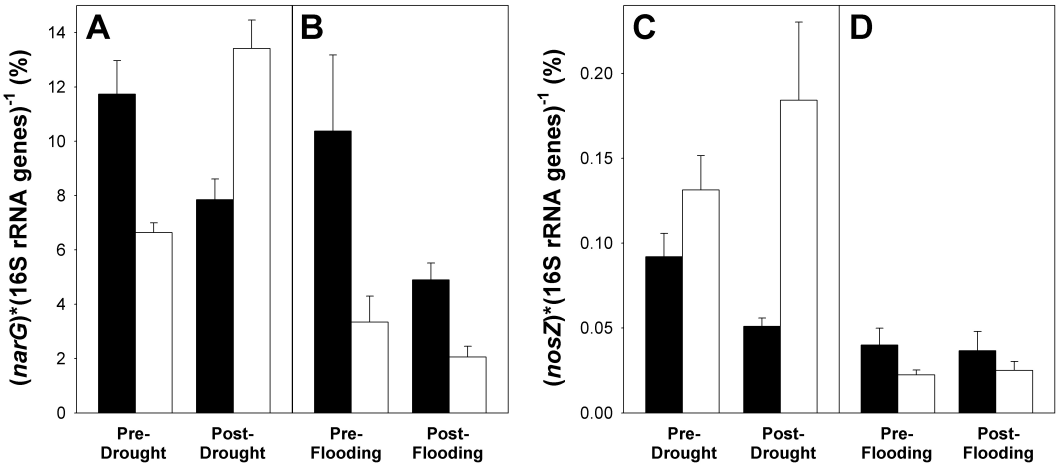

Figure 29: Effect of water table manipulations on the abundance of *narG* (A,B) and *nosZ* (C,D) in an acidic fen. *narG* and *nosZ* gene copy numbers in relation to bacterial 16S rRNA genes were determined before and after a 42 day long experimental drought in 2006 with soil from 0 to 40 cm depth (A,C) as well as before and after 6 month of experimental flooding in 2009 with soil from 0 to 40 cm depth (B,D). Black bars represent abundances in control plots, white bars represent abundances in treatment plots. Mean values of three replicate plots (four sampling depths per plot) and standard errors are displayed.

trol and treatment plots before and after the experimental drought in 2008 as well as before and after experimental flooding in 2009 (experimental procedure of TRFLP described in [**118**]). Up to 8 and 9 TRFs were observed for *narG* and *nosZ*, respectively (Figure 30, Figure 31). Community composition of *narG* and *nosZ* was similar in control and treatment plots at all sampling timepoints (Figure 30,Figure 31), indicating that the composition of the nitrate reducer and denitrifier community is likely unaffected by short-term water table manipulations or by seasonal variability. Stability of denitrifier communities to water table fluctuations is observed in many wetland systems. Denitrifier communities in a constructed wetland vary between individual sites but are rather unaffected by hydrological pulsing (i.e., short-term drought and subsequent flooding) [155]. Moreover, the community composition of *nosZ* in fen grassland soil does not change significantly in response to variations in

water content [159].

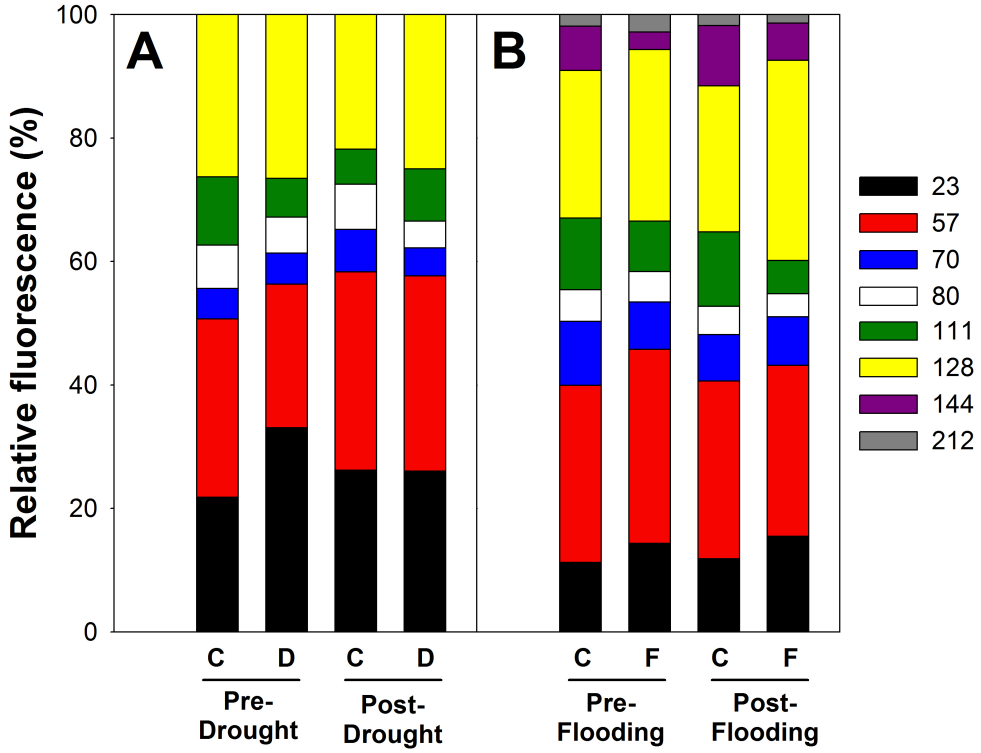

Figure 30: Effect of water table manipulations on the community composition of *narG* genes in an acidic fen. Community composition was determined by TRFLP analysis before and after a 42 day long experimental drought in 2006 with soil from 0 to 40 cm depth (A) as well as before and after 6 month of experimental flooding in 2009 with soil from 0 to 40 cm depth (B). Mean values of three replicate plots (four sampling depths per plot) are displayed.

The effect of prolonged flooding of the fen soil on nitrate-dependent denitrification was assessed in anoxic microcosms with fen soil taken before and after a 6 months flooding period in 2009 from 0 to 20 cm and 20 to 40 cm depth from control and treatment plots. Apparent Michaelis-Menten kinetics were determined based on initial N_2O production rates in acetylene-amended nitrate-supplemented microcosms. v_{max} were higher in 0 to 20 cm than in 20 to 40 cm depth soil, thus denitrification potentials like fermentative and methanogenic potentials are mainly located in the upper soil layers (Figure 32). Prolonged flooding increased v_{max} in

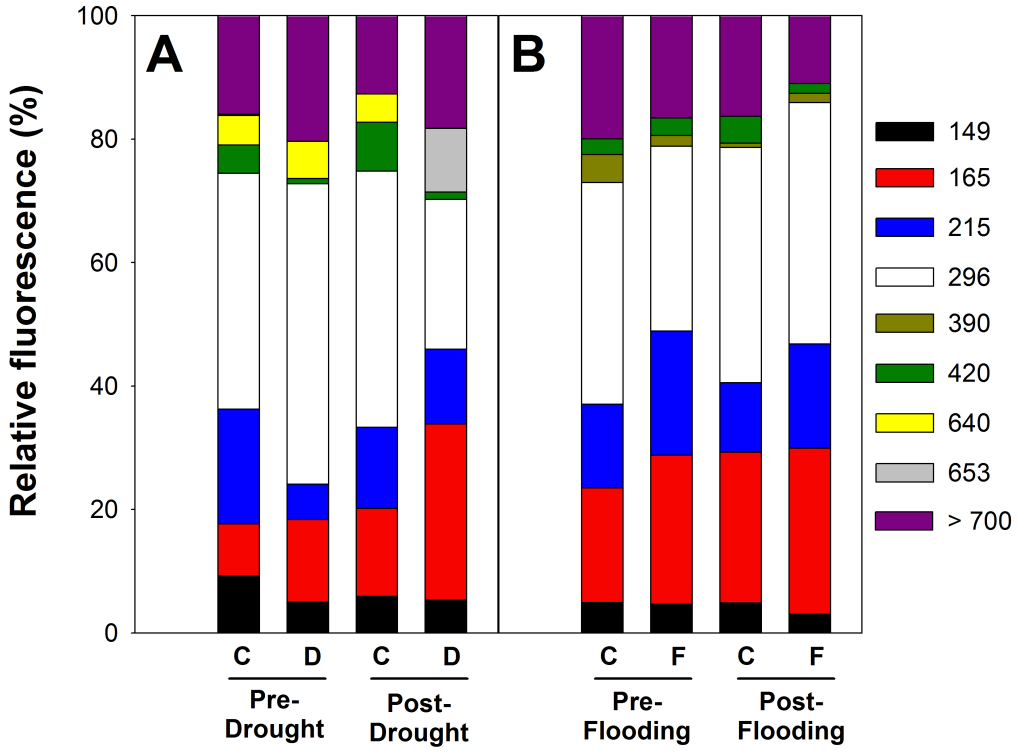

Figure 31: Effect of water table manipulations on the community composition of *nosZ* genes in an acidic fen. Community composition was determined by TRFLP analysis before and after a 42 day long experimental drought in 2006 with soil from 0 to 40 cm depth (A) as well as before and after 6 month of experimental flooding in 2009 with soil from 0 to 40 cm depth (B). Mean values of three replicate plots (four sampling depths per plot) are displayed.

both depths in treatment plots, while v_{max} was similar in control plots pre- and post-flooding (Figure 32 A, B). The ratio of N_2O to total N-gases was lower post- than pre-flooding in treatment plots, while it was similar in control plots at both time points (Figure 32 C, D). Thus, the higher water-saturation of fen soil as induced by prolonged flooding likely leads to increased denitrification and higher N_2O consumption potentials. Higher *in situ* denitrification rates are detected in a constructed wetland when soil is flooded after an initial drought period [155]. N_2O consumption is strongly affected by the amount of available oxygen in the system, and under water-saturated, mostly anoxic conditions the end-product of denitrifi-

cation is mainly N_2 [3, 26]. Water-saturated peat soil can be a sink for N_2O rather than a source, while peat soil with a lowered water table water table is a source of N_2O [42, 93, 136, 137, 141]. Moreover, drying of nutrient-rich peat soil increases the amount of emitted N_2O [99, 136, 137]. Thus, a raised water table due to increased precipitation might enhance the N_2O sink strength in peatland ecosystems.

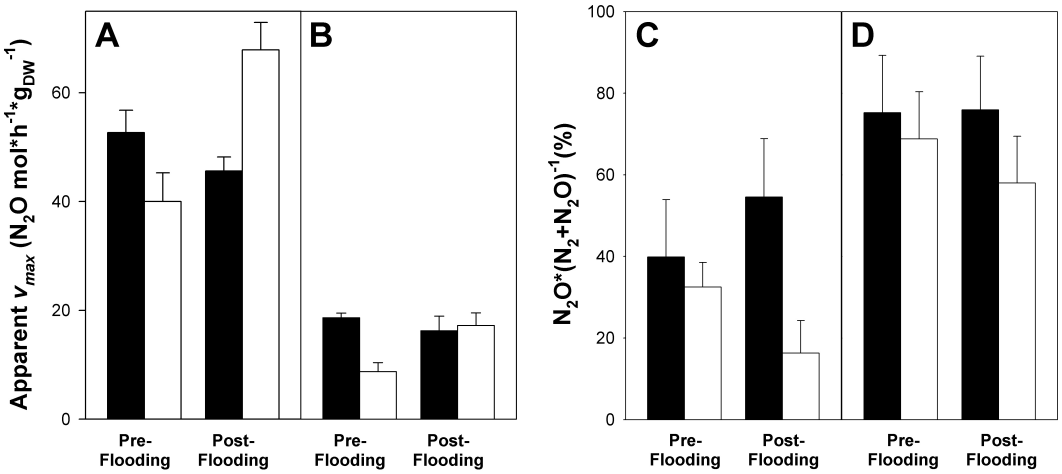

Figure 32: Effect of 6 month flooding on nitrate-dependent N_2O production and the ratio of N_2O to total N-gases in anoxic microcosms with fen soil from 0 to 20 cm (A, C) and 20 to 40 cm depth (B, D). Microcosms were supplemented with 0 to 100 μM nitrate and incubated with or without acetylene (15% vol/vol) in the gasphase. v_{max} are based on initial N_2O production rates in microcosms with acetylene. The ratio of N_2O to total N-gases equals the ratio of N_2O production in microcosms without and with acetylene. Black bars represent microcosms with soil from control plots, white bars represent microcosms with soil from flooded plots. Mean values of three replicate microcosms and standard errors are displayed.

3.4.4 Conclusions: water table manipulations

The short-term water table manipulations in the model fen Schlöppnerbrunnen affected methanogenic and denitrifying potentials in microcosms but had no obvious effect on fermentation potentials, thus future changes in water table height are likely to affect the turnover and release of greenhouse gases from the fen. Observed changes

in physiological potentials were concomitant with small but detectable changes in relative abundances of the involved microbial groups, thus highlighting the capacity of fen microorganism to respond to changing environmental conditions. The community composition of methanogens and denitrifiers was not affected by the manipulation experiments, thus indicating a rather stable microbial community in fen soil. The collective data thus indicate that the acidic fen harbors a rather stable microbial community that is capable of adapting its activity to changing hydrological conditions quite rapidly.

3.5 General conclusions

CH_4 is produced from polymers and sugars under anoxic conditions in wetlands, and different groups of organisms are involved in the conversion of sugars to CH_4 (Figure 4; [30]). In Puukkosuo fen soil, the model sugar NAG is first converted to H_2/CO_2, acetate, and formate, which can then be used directly or indirectly by methanogens, leaving CH_4 as the end product (Figure 33). Methanogens in fen soil are thus trophically linked to fen soil fermenters, which supports the postulated **Hypothesis 1** (3.1).

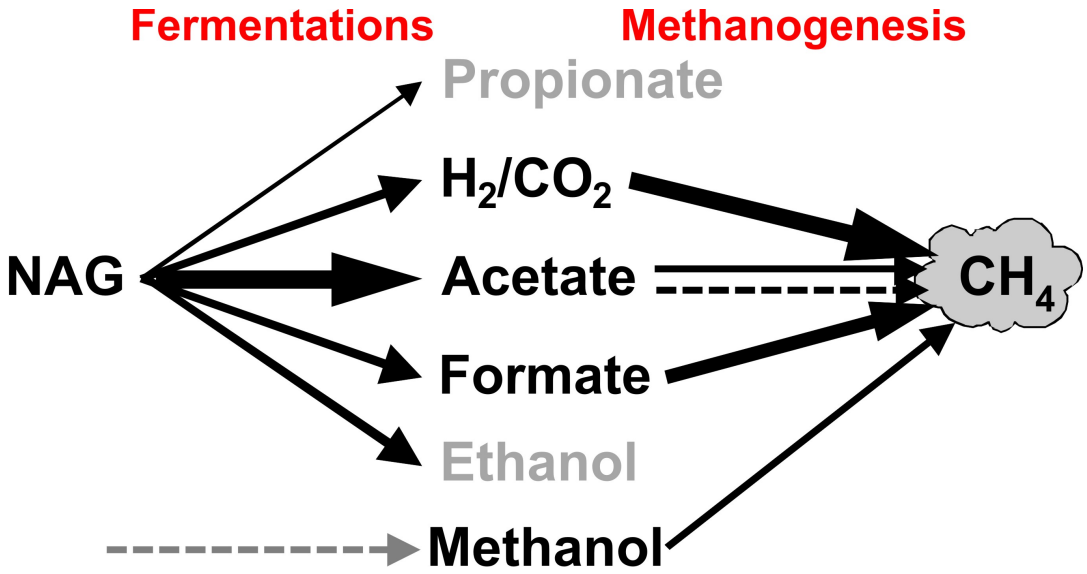

Figure 33: Schematic model of processes contributing to methanogenesis in pH-neutral fen soil. Sugars like NAG are fermented to a variety of fermentation products, mostly H_2/CO_2 and acetate, which in turn are used by methanogens. The relative contribution of each process is indicated by its arrow size. Dotted arrows indicate that more than one step/organism might be involved in the conversion of the compound. Methanol was not measured in microcosm experiments, and possible sources of methanol in fen soil were not investigated.

Denitrification was investigated in five model peatlands, revealing nitrate availability as the major environmental factor determining differences in observed *in situ* N_2O emissions (Figure 34), which is in agreement with the postulated **Hypothe-**

sis 2 (3.1). Analysis of denitrifier community composition indicated that several species-level OTUs of the nitrite reductases *nirK* and *nirS* were related to the observed N_2O emission patterns. One OTU of *nirS* (OTU 2) was positively correlated with N_2O emissions while several OTUs of *nirK* and *nirS* were negatively correlated with N_2O emissions, indicating that N_2O emissions from peatland soils might be regulated by specific groups of nitrite reducers (Figure 34), thus confirming the postulated **Hypothesis 4** (3.1). Denitrifier community composition differed between the peatlands, even though some species-level OTUs were detected in all five peatland soils, indicating the presence of common peatland denitrifiers, as it was postulated in **Hypothesis 3** (3.1). pH was determined as the major environmental factor affecting community composition, further environmental factors that affect denitrifier community composition include soil carbon content, mean annual temperature, and mean annual precipitation (Figure 34). Thus, differences in denitrifier community composition are likely caused by differences in environmental parameters, especially pH, as was postulated in **Hypothesis 3** (3.1). Future research should be directed towards further identification and isolation of key denitrifiers associated with N_2O fluxes in peatlands and to the specific effects of environmental parameters on those key denitrifiers, especially in the context of global warming.

Changes in water table level due to predicted extreme weather events caused by global warming affect the fermentative, methanogenic and denitrifying potentials in fen soil (Figure 35). The involved microorganisms adapt rapidly to the changes in environmental parameters by changes in their activity, while changes in population size of methanogens and denitrifiers are minor. The composition of the methanogenic and denitriyfing community is largely unaffected by the changes in water table, indicating that the composition of the microbial community is not impacted by short-term water table fluctuations, which is in agreement with **Hypothesis 5** (3.1).

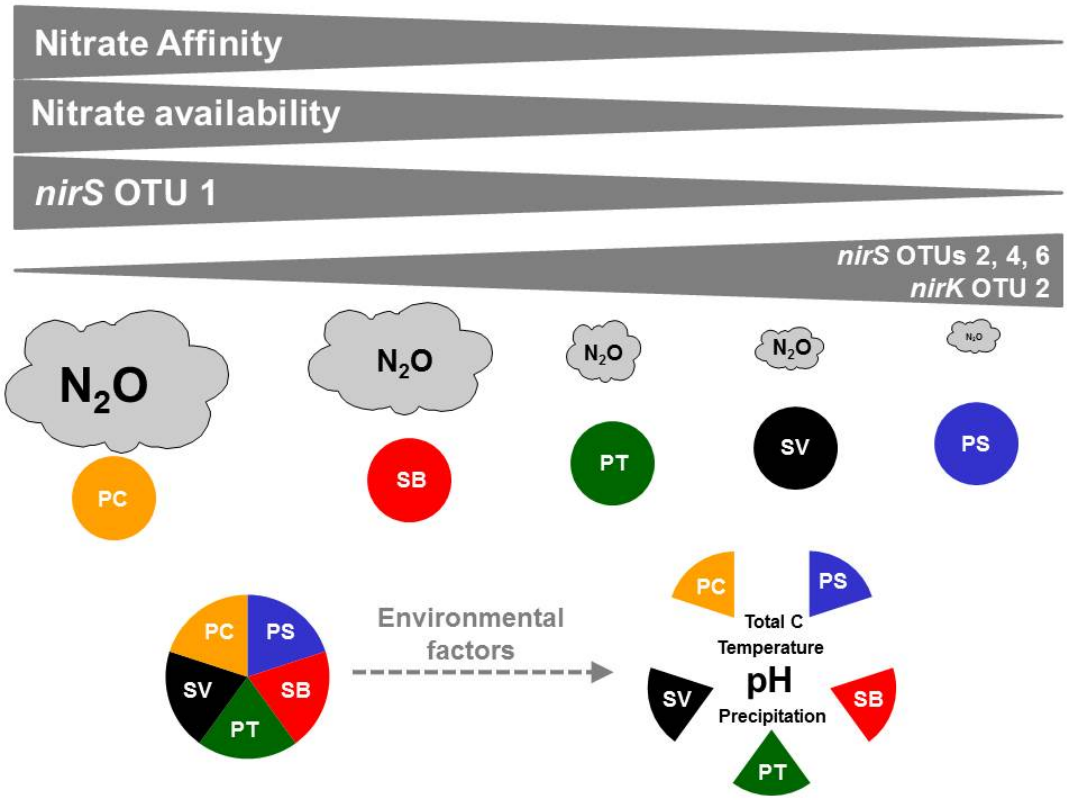

Figure 34: Proposed model of environmental factors determining N_2O emissions from and denitrifier community composition in peatland soils. Cloud size indicates the magnitude of *in situ* N_2O emissions from peatland soil. Grey triangles indicate relative importance of environmental parameters for *in situ* N_2O emissions. The peatlands under investigation are represented by colored spheres, identity of spheres as in Table 6. Denitrifier communities in peatland soils are based on a common core, as indicated by the left bottom circle. Denitrifier communities develop differently due to differences in the environmental factors present. The most important primary environmental factors affecting denitrifier community composition are indicated in the center of the right bottom circle, the depicted size of a factor is related to the relative importance of a factor in shaping denitrifier communities in peatland soils.

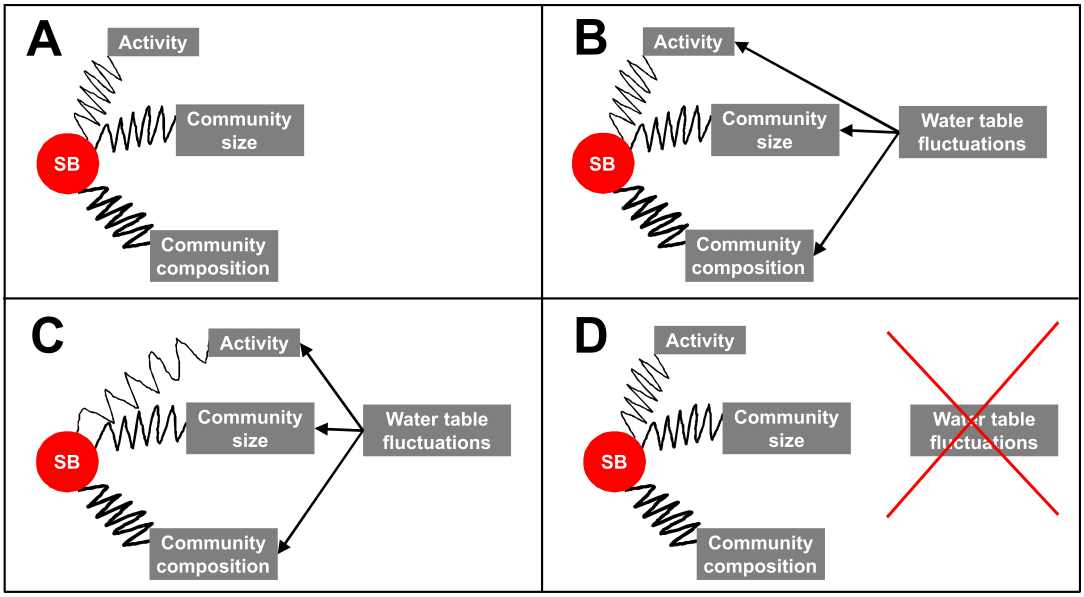

Figure 35: Proposed model to demonstrate the effects of changing water tables on microorganisms in fen soil. Microbial communities in Schlöppnerbrunen fen soil are depicted as the red sphere on the left of each picture. Microbial communities are characterized by their activity, their size, and their composition, as indicated by the grey boxes. The characteristics of the system are attached to it by springs, the spring strengths are indicated by the thickness of the line. In (A), the initial state of the system (i.e., the fen under normal hydrological conditions, prior to drought or excess flooding) characterized by relaxed springs is displayed. Water table fluctuations such as excess drought or flooding target microbial activity, community size and community composition (B). The magnitude of the observed effect of water table fluctuations is dependent on the spring strength. As springs for microbial activity are weaker than of community size or community composition, effects of water table manipulations are mainly observed on the activity level (C). When the water table fluctuation vanishes, the springs return the system to its inital state (D).

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4 Manuscripts

4.1 Published articles in peer-reviewed journals

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Genome-Derived Criteria for Assigning Environmental *narG* and *nosZ* Sequences to Operational Taxonomic Units of Nitrate Reducers▽†

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Ninety percent of cultured bacterial nitrate reducers with a 16S rRNA gene similarity of ≥97% had a *narG* or *nosZ* similarity of ≥67% or ≥80%, respectively, suggesting that 67% and 80% could be used as standardized, conservative threshold similarity values for *narG* and *nosZ*, respectively (i.e., any two sequences that are less similar than the threshold similarity value have a very high probability of belonging to different species), for estimating species-level operational taxonomic units. Genus-level tree topologies of *narG* and *nosZ* were generally similar to those of the corresponding 16S rRNA genes. Although some genomes contained multiple copies of *narG*, recent horizontal gene transfer of *narG* was not apparent.

Nitrate reducers (i.e., both dissimilatory nitrate reducers and denitrifiers) reduce nitrate to nitrite, which can then be reduced to ammonium by dissimilatory nitrate reducers or sequentially reduced to nitric oxide, nitrous oxide, and dinitrogen by denitrifiers (29). *narG* codes for the alpha subunit of the dissimilatory nitrate reductase, which reduces nitrate to nitrite and is thus common to both dissimilatory nitrate reducers and denitrifiers (29). *nosZ* codes for nitrous oxide reductase, which reduces nitrous oxide to dinitrogen and is common to denitrifiers but not dissimilatory nitrate reducers (29). Both *narG* and *nosZ* are commonly used as gene markers for community level analysis of nitrate reducers (2, 8, 9, 16, 18, 19, 20, 25). However, standardized criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units (OTUs) are required so that diverse data sets on nitrate-reducing communities can be normalized. The widespread ability of bacteria and archaea to denitrify (29) complicates the development of such criteria for genes involved in denitrification. Some closely related *narG* and closely related *nosZ* genes occur in distantly related taxa, and *narG* or *nosZ* phylogenies do not always reflect 16S rRNA phylogenies (17). However, *nosZ*-based phylogenies in general have a high degree of congruency with 16S rRNA gene-based phylogenies (3, 10, 30), and recent horizontal gene transfer of *nosZ* seems unlikely (10), indicating that denitrifier structural genes might be used for estimating the species-level novelty, as well as species-level diversity, of denitrifiers in environmental samples. The limited amount of data on horizontal gene transfer of *narG* (4, 24) identifies a need to extend such an approach to this gene. The limited number of studies that have compared 16S rRNA with *narG* or *nosZ* phylogenies accentuates the need for a more thorough analysis of the phylogenetic relatedness of these three genes (3, 4, 7). Thus, the main objectives of this study were to (i) resolve criteria for standardizing OTU assignment of environmental *narG* and *nosZ* sequences, (ii) determine whether those criteria can be used as indicators of novel species, and (iii) investigate the impact of horizontal gene transfer on *narG*.

Analysis of *narG* and *nosZ* sequence data. One hundred fourteen *narG* and 85 *nosZ* sequences from pure cultures were retrieved from GenBank along with the associated 16S rRNA gene sequences (see Table S1 in the supplemental material). The fragments of *narG*, *nosZ*, and the 16S rRNA gene that were analyzed correspond to regions amplified by the commonly used primers narG1960f/-2650r (18), nosZF/-R (19), and 27F/1492R (14), respectively. Alignments of 16S rRNA gene fragments and in silico-translated *narG* and *nosZ* fragments were performed with ClustalW implemented in MEGA 4.0 (13) and manually refined. The number of base or amino acid differences per site (D) was calculated for pairwise comparisons of all *narG*, *nosZ*, and 16S rRNA gene fragments. The similarity (S) was expressed as $S = 1 - D$. The percent similarity of *narG* or *nosZ* from nucleic acid- and amino acid-based comparisons was plotted against the percent similarity of the corresponding 16S rRNA gene. This approach is similar to that used for derivation of 16S rRNA gene-based criteria for species delineation, which is based on DNA/DNA similarities (as a valid criterion to distinguish two species) (28).

Phylogenetic trees based on nucleotide sequences of 16S rRNA gene, *narG*, and *nosZ* fragments were calculated in MEGA 4.0 (13) using the neighbor-joining algorithm (21) and 500 bootstrap replications (5). Tree topologies were compared to evaluate the use of *narG* and *nosZ* as indicators of new species.

The impact of horizontal gene transfer on *narG* diversity was assessed by locating putatively horizontally transferred genes in whole-genome sequences of species using the score-based identification of genomic islands–hidden Markov model algorithm (SIGI-HMM) as implemented in Colombo (http://www.tcs.informatik.uni-goettingen.de/colombo-sigihmm), which identifies putatively alien genes based on differences in codon usage (27), and using SeqWord, which can be used to identify putatively alien genes based on oligonucleotide biases (6).

Correlations between structural gene sequences, species diversity, and horizontal gene transfer. A 16S rRNA gene similarity of ≥97% (a conservative threshold similarity for assign-

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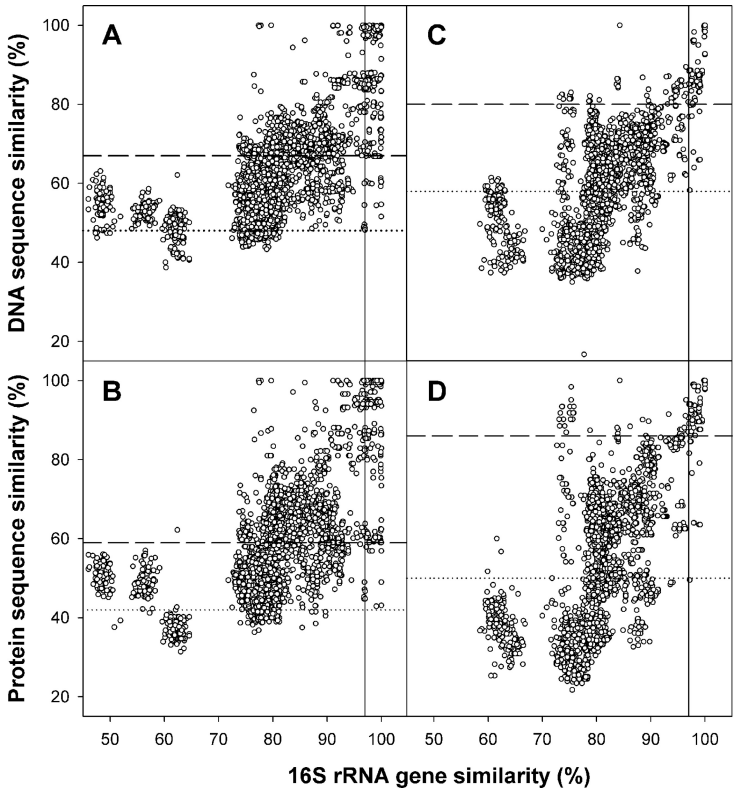
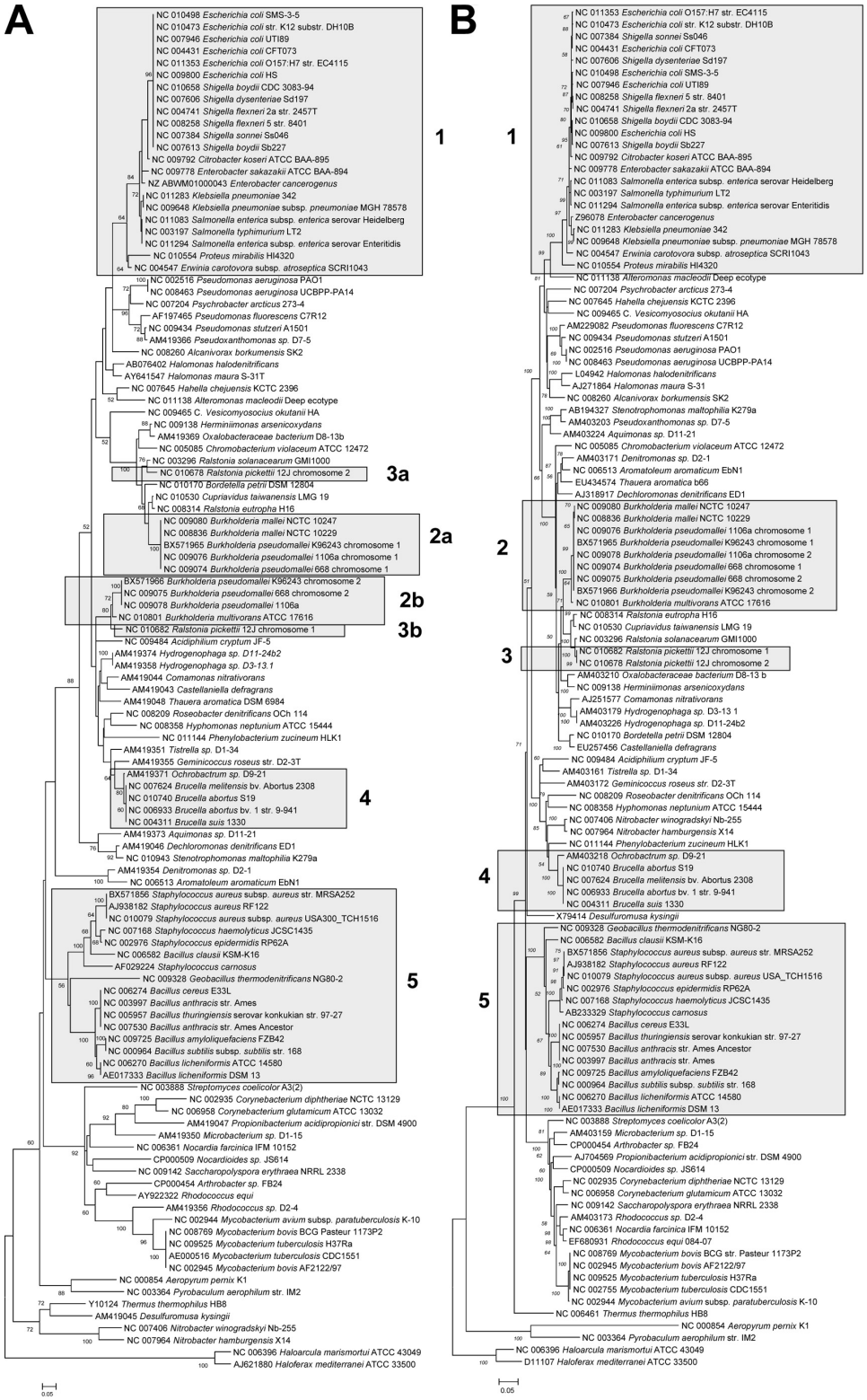


FIG. 1. Correlation of DNA and in silico-translated amino acid sequence similarities of *narG* (A, B) and *nosZ* (C, D) versus 16S rRNA gene similarity. Dotted lines represent the similarity values, below which two sequences always had less than 97% 16S rRNA gene sequence similarity. The dashed lines represent the 90% quantile of pairwise sequence comparisons with a 16S rRNA gene sequence similarity of 97% (i.e., threshold similarity). The solid lines mark the 97% 16S rRNA gene similarities.

ing two organisms to different species [22, 23]) corresponded to an average *narG* sequence similarity of 85% (86% for translated amino acids). Nitrate reducers that had a <48% *narG* sequence similarity (or <42% for translated amino acids) always had a 16S rRNA gene similarity of <97% (Fig. 1) and therefore belonged to different species. Ninety percent of the nitrate reducers with a 16S rRNA gene similarity of ≥97% had a *narG* sequence similarity of ≥67% and a *narG* translated amino acid similarity of ≥59%. The same gene sequence and translated amino acid similarities were obtained for 90% of nitrate reducers with a 16S rRNA gene similarity of ≥98.7%, which has recently been suggested as a threshold similarity for species differentiation (22). The correlation analysis suggests that a *narG* sequence similarity of 67% and a *narG* amino acid similarity of 59% could be used as threshold similarities (i.e., any two sequences that are less similar than the threshold similarity value have a very high probability of belonging to different species) for estimating *narG*-derived species-level OTUs. These relationships are indicative of different evolutionary rates for *narG* and the 16S rRNA gene. Tree topologies of *narG* and 16S rRNA phylogenies were generally similar at the genus level, since species of the same genus mostly clustered together in both trees (Fig. 2). However, multiple copies of *narG* were present in some species. In such cases, the multiple *narG* copies clustered separately (Fig. 2), while the multiple copies of the 16S rRNA genes from the organisms analyzed clustered closely together (data not shown).

The percentage of putatively alien genes found in whole-genome sequences ranged from 0% to 20.6% based on codon usage analysis, and the Nar operon was predicted to be putatively alien only in *Pseudomonas stutzeri* A1501 and was estimated to be transferred from the *Gammaproteobacteria* (see Table S1 in the supplemental material). In *Nitrobacter winogradskyi* Nb-255, *narI* and *narJ* were identified as putatively alien genes transferred from the *Gammaproteobacteria* (see Table S1 in the supplemental material). It appeared to be unlikely, based on oligonucleotide bias, that the Nar operon was horizontally transferred in any species (e.g., see Fig. S1 to S3 in the supplemental material).

A 16S rRNA gene similarity of ≥97% (a conservative threshold similarity for assigning two organisms to different species [22, 23]) corresponded to an average *nosZ* sequence similarity of 89% (92% for translated amino acids). Denitrifiers with a <58% *nosZ* similarity (or <50% similarity of translated amino acids) always had a 16S rRNA gene similarity of <97% (Fig. 1) and therefore belonged to different species.


A
NC 010498 Escherichia coli SMS-3-5
NC 010473 Escherichia coli str. K12 substr. DH10B
NC 007946 Escherichia coli UTI89
NC 004431 Escherichia coli CFT073
NC 011353 Escherichia coli O157:H7 str. EC4115
NC 009800 Escherichia coli HS
NC 010658 Shigella boydii CDC 3083-94
NC 007606 Shigella dysenteriae Sd197
NC 004741 Shigella flexneri 2a str. 2457T
NC 008258 Shigella flexneri 5 str. 8401
NC 007384 Shigella sonnei Ss046
NC 007613 Shigella boydii Sb227
NC 009792 Citrobacter koseri ATCC BAA-895
NC 009778 Enterobacter sakazakii ATCC BAA-894
NZ ABWM01000043 Enterobacter cancerogenus
NC 011283 Klebsiella pneumoniae 342
NC 009648 Klebsiella pneumoniae subsp. pneumoniae MGH 78578
NC 011083 Salmonella enterica subsp. enterica serovar Heidelberg
NC 003197 Salmonella typhimurium LT2
NC 011294 Salmonella enterica subsp. enterica serovar Enteritidis
NC 010554 Proteus mirabilis HI4320
NC 004547 Erwinia carotovora subsp. atroseptica SCRI1043
1
NC 002516 Pseudomonas aeruginosa PAO1
NC 008463 Pseudomonas aeruginosa UCBPP-PA14
NC 007204 Psychrobacter arcticus 273-4
AF197465 Pseudomonas fluorescens C7R12
NC 009434 Pseudomonas stutzeri A1501
AM419366 Pseudoxanthomonas sp. D7-5
NC 008260 Alcanivorax borkumensis SK2
AB076402 Halomonas halodenitrificans
AY641547 Halomonas maura S-31T
NC 007645 Hahella chejuensis KCTC 2396
NC 011138 Alteromonas macleodii Deep ecotype
NC 009465 C. Vesicomyosocius okutanii HA
NC 009138 Herminiimonas arsenicoxydans
AM419369 Oxalobacteraceae bacterium D8-13b
NC 005085 Chromobacterium violaceum ATCC 12472
NC 003296 Ralstonia solanacearum GMI1000
NC 010678 Ralstonia pickettii 12J chromosome 2
3a
NC 010170 Bordetella petrii DSM 12804
NC 010530 Cupriavidus taiwanensis LMG 19
NC 008314 Ralstonia eutropha H16
NC 009080 Burkholderia mallei NCTC 10247
NC 008836 Burkholderia mallei NCTC 10229
BX571965 Burkholderia pseudomallei K96243 chromosome 1
NC 009076 Burkholderia pseudomallei 1106a chromosome 1
NC 009074 Burkholderia pseudomallei 668 chromosome 1
2a
BX571966 Burkholderia pseudomallei K96243 chromosome 2
NC 009075 Burkholderia pseudomallei 668 chromosome 2
NC 009078 Burkholderia pseudomallei 1106a
NC 010801 Burkholderia multivorans ATCC 17616
2b
NC 010682 Ralstonia pickettii 12J chromosome 1
3b
NC 009484 Acidiphilium cryptum JF-5
AM419374 Hydrogenophaga sp. D11-24b2
AM419358 Hydrogenophaga sp. D3-13.1
AM419044 Comamonas nitrativorans
AM419043 Castellaniella defragrans
AM419048 Thauera aromatica DSM 6984
NC 008209 Roseobacter denitrificans OCh 114
NC 008358 Hyphomonas neptunium ATCC 15444
NC 011144 Phenylobacterium zucineum HLK1
AM419351 Tistrella sp. D1-34
AM419355 Geminicoccus roseus str. D2-3T
AM419371 Ochrobactrum sp. D9-21
NC 007624 Brucella melitensis bv. Abortus 2308
NC 010740 Brucella abortus S19
NC 006933 Brucella abortus bv. 1 str. 9-941
NC 004311 Brucella suis 1330
4
AM419373 Aquimonas sp. D11-21
AM419046 Dechloromonas denitrificans ED1
NC 010943 Stenotrophomonas maltophilia K279a
AM419354 Denitromonas sp. D2-1
NC 006513 Aromatoleum aromaticum EbN1
BX571856 Staphylococcus aureus subsp. aureus str. MRSA252
AJ938182 Staphylococcus aureus RF122
NC 010079 Staphylococcus aureus subsp. aureus USA300_TCH1516
NC 007168 Staphylococcus haemolyticus JCSC1435
NC 002976 Staphylococcus epidermidis RP62A
NC 006582 Bacillus clausii KSM-K16
AF029224 Staphylococcus carnosus
NC 009328 Geobacillus thermodenitrificans NG80-2
NC 006274 Bacillus cereus E33L
NC 003997 Bacillus anthracis str. Ames
NC 005957 Bacillus thuringiensis serovar konkukian str. 97-27
NC 007530 Bacillus anthracis str. Ames Ancestor
NC 009725 Bacillus amyloliquefaciens FZB42
NC 000964 Bacillus subtilis subsp. subtilis str. 168
NC 006270 Bacillus licheniformis ATCC 14580
AE017333 Bacillus licheniformis DSM 13
5
NC 003888 Streptomyces coelicolor A3(2)
NC 002935 Corynebacterium diphtheriae NCTC 13129
NC 006958 Corynebacterium glutamicum ATCC 13032
AM419047 Propionibacterium acidipropionici str. DSM 4900
AM419350 Microbacterium sp. D1-15
NC 006361 Nocardia farcinica IFM 10152
CP000509 Nocardioides sp. JS614
NC 009142 Saccharopolyspora erythraea NRRL 2338
CP000454 Arthrobacter sp. FB24
AY922322 Rhodococcus equi
AM419356 Rhodococcus sp. D2-4
NC 002944 Mycobacterium avium subsp. paratuberculosis K-10
NC 008769 Mycobacterium bovis BCG Pasteur 1173P2
NC 009525 Mycobacterium tuberculosis H37Ra
AE000516 Mycobacterium tuberculosis CDC1551
NC 002945 Mycobacterium bovis AF2122/97
NC 000854 Aeropyrum pernix K1
NC 003364 Pyrobaculum aerophilum str. IM2
Y10124 Thermus thermophilus HB8
AM419045 Desulfuromusa kysingii
NC 007406 Nitrobacter winogradskyi Nb-255
NC 007964 Nitrobacter hamburgensis X14
NC 006396 Haloarcula marismortui ATCC 43049
AJ621880 Haloferax mediterranei ATCC 33500
0.05
B
NC 011353 Escherichia coli O157:H7 str. EC4115
NC 010473 Escherichia coli str. K12 substr. DH10B
NC 007384 Shigella sonnei Ss046
NC 004431 Escherichia coli CFT073
NC 007606 Shigella dysenteriae Sd197
NC 010498 Escherichia coli SMS-3-5
NC 007946 Escherichia coli UTI89
NC 008258 Shigella flexneri 5 str. 8401
NC 004741 Shigella flexneri 2a str. 2457T
NC 010658 Shigella boydii CDC 3083-94
NC 009800 Escherichia coli HS
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NC 009792 Citrobacter koseri ATCC BAA-895
NC 009778 Enterobacter sakazakii ATCC BAA-894
NC 011083 Salmonella enterica subsp. enterica serovar Heidelberg
NC 003197 Salmonella typhimurium LT2
NC 011294 Salmonella enterica subsp. enterica serovar Enteritidis
Z96078 Enterobacter cancerogenus
NC 011283 Klebsiella pneumoniae 342
NC 009648 Klebsiella pneumoniae subsp. pneumoniae MGH 78578
NC 004547 Erwinia carotovora subsp. atroseptica SCRI1043
NC 010554 Proteus mirabilis HI4320
1
NC 011138 Alteromonas macleodii Deep ecotype
NC 007204 Psychrobacter arcticus 273-4
NC 007645 Hahella chejuensis KCTC 2396
NC 009465 C. Vesicomyosocius okutanii HA
AM229082 Pseudomonas fluorescens C7R12
NC 009434 Pseudomonas stutzeri A1501
NC 002516 Pseudomonas aeruginosa PAO1
NC 008463 Pseudomonas aeruginosa UCBPP-PA14
L04942 Halomonas halodenitrificans
AJ271864 Halomonas maura S-31
NC 008260 Alcanivorax borkumensis SK2
AB194327 Stenotrophomonas maltophilia K279a
AM403203 Pseudoxanthomonas sp. D7-5
AM403224 Aquimonas sp. D11-21
NC 005085 Chromobacterium violaceum ATCC 12472
AM403171 Denitromonas sp. D2-1
NC 006513 Aromatoleum aromaticum EbN1
EU434574 Thauera aromatica b66
AJ318917 Dechloromonas denitrificans ED1
NC 009080 Burkholderia mallei NCTC 10247
NC 008836 Burkholderia mallei NCTC 10229
NC 009076 Burkholderia pseudomallei 1106a chromosome 1
BX571965 Burkholderia pseudomallei K96243 chromosome 1
NC 009078 Burkholderia pseudomallei 1106a chromosome 2
NC 009074 Burkholderia pseudomallei 668 chromosome 1
NC 009075 Burkholderia pseudomallei 668 chromosome 2
BX571966 Burkholderia pseudomallei K96243 chromosome 2
NC 010801 Burkholderia multivorans ATCC 17616
2
NC 008314 Ralstonia eutropha H16
NC 010530 Cupriavidus taiwanensis LMG 19
NC 003296 Ralstonia solanacearum GMI1000
NC 010682 Ralstonia pickettii 12J chromosome 1
NC 010678 Ralstonia pickettii 12J chromosome 2
3
AM403210 Oxalobacteraceae bacterium D8-13 b
NC 009138 Herminiimonas arsenicoxydans
AJ251577 Comamonas nitrativorans
AM403179 Hydrogenophaga sp. D3-13 1
AM403226 Hydrogenophaga sp. D11-24b2
NC 010170 Bordetella petrii DSM 12804
EU257456 Castellaniella defragrans
NC 009484 Acidiphilium cryptum JF-5
AM403161 Tistrella sp. D1-34
AM403172 Geminicoccus roseus str. D2-3T
NC 008209 Roseobacter denitrificans OCh 114
NC 008358 Hyphomonas neptunium ATCC 15444
NC 007406 Nitrobacter winogradskyi Nb-255
NC 007964 Nitrobacter hamburgensis X14
NC 011144 Phenylobacterium zucineum HLK1
AM403218 Ochrobactrum sp. D9-21
NC 010740 Brucella abortus S19
NC 007624 Brucella melitensis bv. Abortus 2308
NC 006933 Brucella abortus bv. 1 str. 9-941
NC 004311 Brucella suis 1330
4
X79414 Desulfuromusa kysingii
NC 009328 Geobacillus thermodenitrificans NG80-2
NC 006582 Bacillus clausii KSM-K16
BX571856 Staphylococcus aureus subsp. aureus str. MRSA252
AJ938182 Staphylococcus aureus RF122
NC 010079 Staphylococcus aureus subsp. aureus USA_TCH1516
NC 002976 Staphylococcus epidermidis RP62A
NC 007168 Staphylococcus haemolyticus JCSC1435
AB233329 Staphylococcus carnosus
NC 006274 Bacillus cereus E33L
NC 005957 Bacillus thuringiensis serovar konkukian str. 97-27
NC 007530 Bacillus anthracis str. Ames Ancestor
NC 003997 Bacillus anthracis str. Ames
NC 009725 Bacillus amyloliquefaciens FZB42
NC 000964 Bacillus subtilis subsp. subtilis str. 168
NC 006270 Bacillus licheniformis ATCC 14580
AE017333 Bacillus licheniformis DSM 13
5
NC 003888 Streptomyces coelicolor A3(2)
AM403159 Microbacterium sp. D1-15
CP000454 Arthrobacter sp. FB24
AJ704569 Propionibacterium acidipropionici str. DSM 4900
CP000509 Nocardioides sp. JS614
NC 002935 Corynebacterium diphtheriae NCTC 13129
NC 006958 Corynebacterium glutamicum ATCC 13032
NC 009142 Saccharopolyspora erythraea NRRL 2338
AM403173 Rhodococcus sp. D2-4
NC 006361 Nocardia farcinica IFM 10152
EF680931 Rhodococcus equi 084-07
NC 008769 Mycobacterium bovis BCG str. Pasteur 1173P2
NC 002945 Mycobacterium bovis AF2122/97
NC 009525 Mycobacterium tuberculosis H37Ra
NC 002755 Mycobacterium tuberculosis CDC1551
NC 002944 Mycobacterium avium subsp. paratuberculosis K-10
NC 006461 Thermus thermophilus HB8
NC 000854 Aeropyrum pernix K1
NC 003364 Pyrobaculum aerophilum str. IM2
NC 006396 Haloarcula marismortui ATCC 43049
D11107 Haloferax mediterranei ATCC 33500
0.05

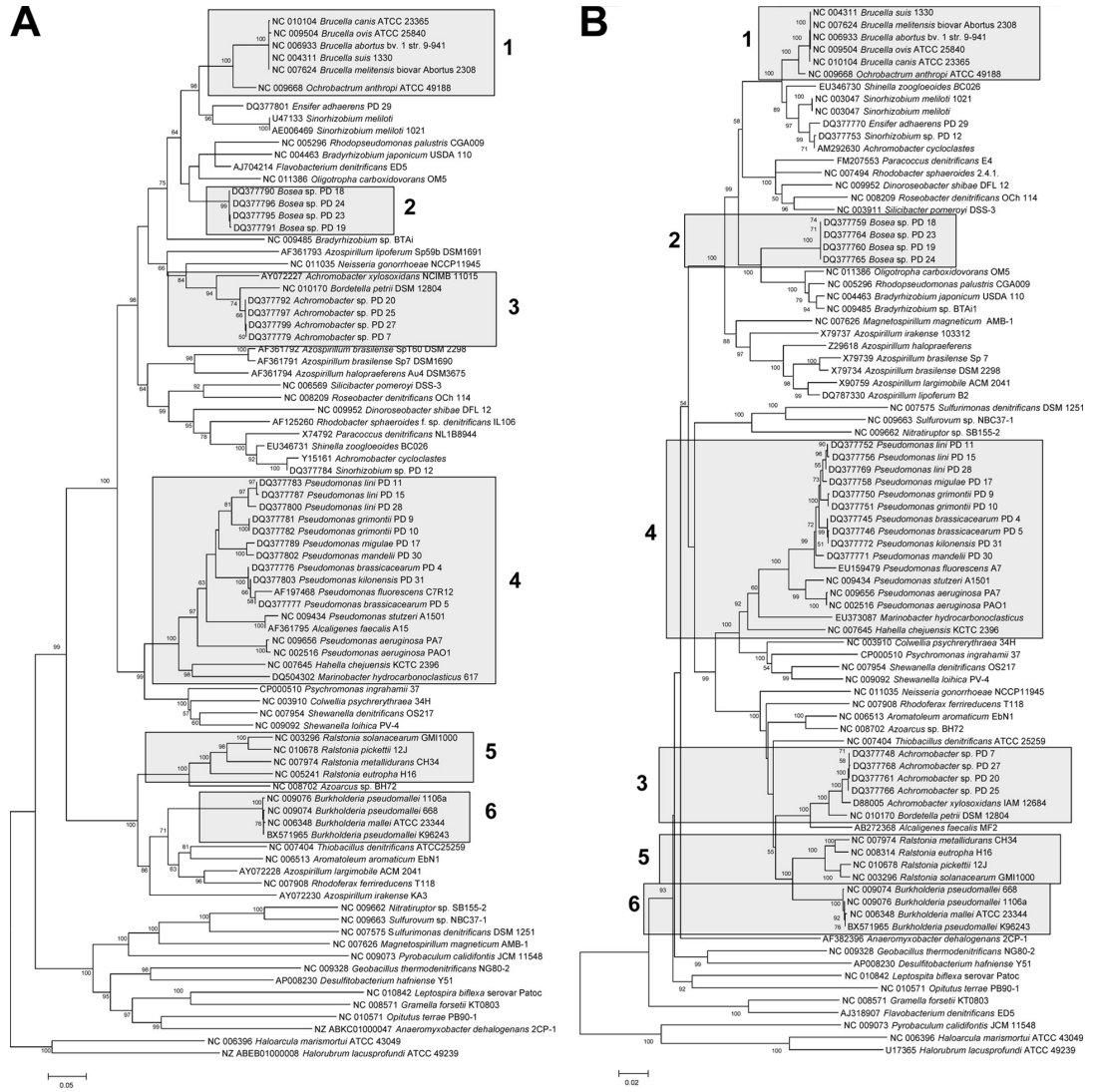

FIG. 3. Comparison of *nosZ* (A) and 16S rRNA (B) phylogenies. Boxes 1 to 6 display examples of closely related species clustering together in the *nosZ* tree. Not all sequences of *Azospirillum* sp. cluster together in the *nosZ* tree. Neighbor-joining trees were constructed from nucleotide sequences of *nosZ* and 16S fragments with approximate lengths of 700 and 1,400 bp, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap supports below 50% are not shown. The bars represent estimated sequence dissimilarities of 5% (A) and 2% (B).

Ninety percent of denitrifiers with a 16S rRNA gene similarity of ≥97% had a *nosZ* sequence similarity of ≥80% and a translated amino acid similarity of ≥86%. Ninety percent of denitrifiers with a 16S similarity of ≥98.7% had a *nosZ* sequence similarity of ≥85% and a translated amino acid similarity of ≥90%. The correlation analysis suggests that a *nosZ* sequence similarity of 80% and a *nosZ* amino acid similarity of 86% could be used as threshold similarities for estimating

FIG. 2. Comparison of *narG* (A) and 16S rRNA (B) phylogenies. Boxes 1, 4, and 5 display examples of closely related species clustering together in the *narG* tree. Boxes 2 and 3 illustrate that multiple copies of *narG* from the same organism cluster separately. Neighbor-joining trees were constructed from nucleotide sequences of *narG* and 16S fragments with approximate lengths of 650 and 1,400 bp, respectively. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Bootstrap supports below 50% are not shown. The bars represent estimated sequence dissimilarities of 5% (A) and 0.1% (B).

nosZ-derived species-level OTUs with a very high probability of estimating correctly. The calculated threshold similarities are in good agreement with those calculated for the *nosZ* fragment amplified by the primers nosZ661F/-1773R (9). Phylogenetic trees of *nosZ* and 16S rRNA genes had similar clustering at the genus level (Fig. 3). Multiple copies of *nosZ* per organism were not found. The different threshold similarities of *nosZ* and *narG* suggest that *nosZ* is more conserved than *narG*, indicating that species-level OTU assignment of *nosZ* might be more reliable than that of *narG*.

Conclusions. The above considerations indicate that *narG* or *nosZ* analyses can be used to estimate species-level diversity of the associated bacteria on the basis of sequence similarities. Such analysis is considered to yield a minimum number of species in a sample (i.e., the true species-level diversity might be much higher). Analyses of gene markers of different functional groups revealed similar threshold similarities indicating species-level OTUs (e.g., for *dsrAB* and *amoA* gene fragments, they were 80 to 90%) (11, 12). Since *narG* or *nosZ* sequences from organisms of the same genus generally form coherent clusters in phylogenetic trees (Fig. 2 and 3), distinct clusters of environmental sequences could provide evidence for new genus-level diversity. Recent horizontal gene transfer does not appear to have occurred for either *narG* (as indicated by the results in this study) or *nosZ* (as indicated by the results in reference 10). The adaptation of genomic features of an alien gene to those of a host genome takes several hundred million years (15), and genes that were horizontally transferred 50 million years ago can be reliably detected by sequence analyses (26). Sequence differences might also be caused by gene duplication and diversification (e.g., in species in which multiple gene copies are present) rather than by horizontal gene transfer (1). However, analysis of codon usage and oligonucleotide bias does not always reveal the transfer of genes from closely related organisms with similar genomic features (27).

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

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Cryoturbated peat circles (i.e., bare surface soil mixed by frost action; pH 3-4) in the Russian discontinuous permafrost tundra are nitrate-rich 'hotspots' of N_2O-emissions in arctic ecosystems, while 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 soil, 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 soil, 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 soil differed. Quantitative PCR (inhibition-corrected per DNA extract) revealed higher abundances of *narG* in cryoturbated than in unturbated peat soil. Copy numbers of *nirS* were up to 1000x higher than those of *nirK* in both soils, and *nirS* $nirK^{-1}$ copy number ratios in cryo- and unturbated peat soil differed. The collective data indicate that the contrasting N_2O emission patterns of cryoturbated and unturbated peat soils are associated with contrasting denitrifier communities.

1 ## Introduction

2 Nitrous oxide (N_2O) is a major ozone depleting substance in the atmosphere and the
3 third most important greenhouse gas on earth (Forster *et al*., 2007; Ravishankara *et al*.,
4 2009). The global warming potential of N_2O is 300-fold higher than that of CO_2 on a 100 year
5 basis, and the atmospheric concentration of N_2O increased from 270 ppb to 319 ppb from
6 1750 to 2005 (Forster *et al*., 2007). Agricultural and pristine tropical soils are well recognized
7 major sources of N_2O, while the importance of arctic peatlands as sources of N_2O is just
8 emerging (Denman *et al*., 2007; Repo *et al*., 2009; Marushchak *et al*., 2011).
9 Areas of bare surface soil mixed by frost action in acidic tundra (pH 3 to 4) are termed
10 cryoturbated peat circles, and emit N_2O at rates documented for tropical and agricultural soils
11 (Werner *et al*., 2007; Maljanen *et al*., 2007; Repo *et al*., 2009). The estimated global N_2O
12 emission from cryoturbated peat circles is about 0.1 Tg N_2O y^{-1}, which is equivalent to 4% of
13 the global warming potential of the arctic methane emissions and to 0.6% of the total global
14 annual N_2O emission (Christensen, 1993; Denman *et al*., 2007; Repo *et al*., 2009).
15 Vegetation is absent from about 12% of the area in the arctic, including cryoturbated peat
16 circles (Walker *et al*., 2005). Nitrate concentrations approximate 2 mM in the pore water of
17 such unvegetated cryoturbated peat soil, and are approximately 1000 x higher than in
18 adjacent vegetated unturbated peat areas where N_2O emissions are negligible (Repo *et al*.,
19 2009). Repeated freezing and thawing of the cryoturbated soil leads to breakdown of soil
20 aggregates, renders decomposable organic carbon more easily accessible to microbes, and
21 may thereby activate the microbial community including N_2O-producers (Mørkved *et al*.,
22 2006; Sharma *et al*., 2006). Thus, cryoturbated peat circles represent acidic 'hotspots' of
23 microbial N_2O emission in tundra (Repo *et al*., 2009; Marushchak *et al*., 2011).
24 The main source of N_2O in water-logged anoxic soils including peatlands is
25 denitrification (Conrad, 1996; Pihlatie *et al*., 2004; Palmer *et al*., 2010). Complete
26 denitrification is the sequential reduction of nitrate or nitrite to dinitrogen (N_2) via nitric oxide
27 (NO) and N_2O; nitrite is likewise an intermediate when nitrate is utilized (Zumft, 1997). The
28 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 & 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 (e.g., Bru *et al*., 2011; Enwall *et al*., 2010; Jones & Hallin, 2010; Palmer *et al*., 2010; Horn *et al*., 2006; Rich *et al*., 2003; Philippot *et al*., 2002; Prieme *et al*., 2002; Braker *et al*., 2000). 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 (e.g., pH, temperature, C-to-N-ratio, as well as the availability of substrates and electron acceptors; van Cleemput, 1998). Acidic pH < 5 impairs denitrification and increases the product ratio of N_2O to N_2 (Simek & 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 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 the present study were (i) to compare ecophysiological traits (i.e., capacities) of acid-tolerant denitrifier communities in cryoturbated and unturbated peat soil, (ii) to develop pyrosequencing-based strategies for the in-depth analysis of denitrifier communities by parallel analysis of multiple denitrification associated genes, (iii) to

1 determine if contrasting and new denitrifier communities occur in cryoturbated and
2 unturbated peat soil by such pyrosequencing-based strategies and quantitative PCR, and (iv)
3 thus to identify potential microbial catalysts of the exceptionally high N_2O emissions from
4 cryoturbated peat soil.
5

Material 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; Supplemental Material and Methods). Cumulative N_2O emissions in the field from the cryoturbated soil are 1.2 ± 0.3 g N_2O m^{-2}, while those of the unturbated soil are negligible (<0.006 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 were 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 soil, respectively.

Assessment of denitrification in soil microcosms

Soil of the three replicate sampling sites was homogenized and pooled prior to microcosm experiments. Soil slurries at *in situ* pH_{H2O} of approximately 4 were prepared with 4-5 g of soil and 3 volumes of deionized water in 125-ml infusion flasks, and sealed with 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 nitrous oxide reductases and thus the reduction of N_2O to N_2 (Yoshinari & 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 approximately 4 days (90 h) in unsupplemented microcosms with cryoturbated peat soil and acetylene, indicating that internal nitrate and nitrite were depleted (Figure 1 A).

For apparent Michaelis-Menten kinetics, soil was pre-incubated for 7 days under anoxic conditions to deplete internal nitrate and nitrite. Such soil was supplemented with 0 to 500 µM of $NaNO_3$ 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 (Segel, 1993; Palmer *et al.*, 2010; Supplemental Material and Methods). Soil that was pre-incubated under anoxic conditions for 9 days was utilized to study the effect of the electron donors acetate, ethanol, formate, propionate, butyrate, and lactate on denitrification in microcosms supplemented with 1 mM nitrite and 0.5 mM of electron donors in the presence of acetylene. After another 47 days of anoxic incubation, 1 mM nitrite and 2 mM of electron donors (0.5 mM for propionate only) were resupplied. N_2O production, nitrite and electron donors were determined regularly after the initial supplementation and after resupplementation. N_2O production rates were calculated from 3-4 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 greater than 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; Supplemental Material 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 prior to cell lysis was applied (Peršoh *et al*., 2008; Supplemental Material and Methods).

narG, *nirK*, *nirS*, and *nosZ* were amplified using the primer pairs narG1960f (TAY GTS GGS CAR GAR AA)/narG2650r (TTY TCR TAC CAB GTB GC; Philippot *et al*., 2002), F1aCu (ATC ATG GTS CTG CCG CG)/R3Cu (GCC TCG ATC AGR TTG TGG TT; Throbäck

et al., 2004), cd3aF (GTS AAC GTS AAG GAR ACS GG)/R3cd (GAS TTC GGR TGS GTC TTG A; Throbäck *et al*., 2004), and nosZF (CGC TGT TCl TCG ACA GYC AG)/nosZR (ATG TGC AKI GCR TGG CAG AA; Rich *et al*., 2003), respectively. Each primer was preceeded by a 6 basepair-long barcode (AGCGTC for unturbated, and ATATAC for cryoturbated soil samples) to separate sequences after pyrosequencing. Eight 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 (i.e., amplicons) of the correct size were pooled prior to subsequent analyses. For detailed PCR protocols refer to Supplemental Material 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 utilize target gene specific primers fused with a barcode and an approximately 30 bp long sequencing adaptor, resulting in primers with more than 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 utilizing 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 (i.e., *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 via isopropanol precipitation. Sequencing from 5' (forward) and 3' (reverse) ends of amplicons was performed after ligation of A (CGT

ATC GCC TCC CTC GCG CCA TCA G) and B (CTA TGC GCC TTG CCA GCC CGC TCA G) sequencing adaptors at the Göttingen Genomics Laboratory employing 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* as well as *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 (i.e., containing sequences from both cryoturbated and unturbated peat soil) were clustered [i.e., assigned to operational taxonomic units (OTUs)] at species-level threshold distances of 33% [*narG* (Palmer *et al*., 2009)], 17% [*nirK*, (Depkat-Jakob *et al*., unpublished)], 18% (*nirS*, Depkat-Jakob *et al*., unpublished), 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*. unpublished (wwwagak.informatik.uni-kl.de/staff/nebel/www_jaguc/www_jaguc.pdf; Figure S1). In brief, JAguc2 generates a pairwise sequence alignment prior to calculation of a distance matrix and clustering with the average similarity method. This approach is more reliable [i.e., 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 (Sun *et al*., 2009; Quince *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 (Quince *et al*., 2011; Behnke *et al*., 2011). The threshold-distances utilized to call OTUs in this study were 17-33%, which is substantially greater than the above reported maximal PCR- and pyrosequencing noise (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 e.g., 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 (Figure S1). Rarefaction curves were generated for each sequence set with aRarefact (http://www.uga.edu/~strata/software/) 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. Stability of tree topologies was assessed by calculating 10 000 bootstrap replicates (Saitou & Nei, 1987). Diversity measures with 95% confidence intervals were calculated as described (Bray & Curtis, 1957; Hill *et al*., 2003; Sørensen, 1948; 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 & Knight, 2005; Lozupone *et al*., 2007).

Quantification of narG*,* nirK*,* nirS*,* nosZ*, and 16S rRNA genes in soil*

Quantitative kinetic real-time PCRs (qPCRs) were performed as described with DNA extracts from 3 replicate cryoturbated and 3 unturbated sites in 6 technical replicates per DNA extract (Zaprasis *et al*., 2010; Supplemental Material and Methods). Thermal protocols and primers were as described (Supplemental Material 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 $\leq 10^1$ gene copy numbers μl^{-1} of DNA extract. 16S rRNA gene copy numbers were determined concomitantly for all environmental samples in order to quantify microorganisms that harbor genes associated with denitrification in soil relative to the total bacterial

population (Muyzer *et al*., 1993; Zaprasis *et al*., 2010). Inhibition of qPCR was assessed per individual DNA extract according to Zaprasis *et al.* (2010) by spiking soil DNA with pure standard DNA. Please refer to Supplemental Material 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 soil 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

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.

Results

Denitrification activities in cryoturbated and unturbated peat soil

Unsupplemented cryoturbated peat soil but not unturbated peat soil produced N_2O under anoxic conditions without apparent delay (Figure 1 A). pH approximated 4 in microcosms with cryoturbated and unturbated peat soil. After 49.5 hours 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 1 A). 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 1 A). Cryoturbated peat soil contained 4.8 µmol NO_3^- g_{DW}^{-1} prior to anoxic incubation. Nitrate was below the detection limit (i.e., <1.5 µmol NO_3^- g_{DW}^{-1}) after 90 h of incubation, and 75% of the initially present NO_3^--N were 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 1 A); 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 1 A).

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 soil; 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 1 B), while 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 Fig. 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 soil (Figure 1 B). Stimulation of N_2O production was higher with nitrite than with nitrate in cryoturbated (P=0.04) and unturbated peat soil (P=0.06) when N_2O production rates were compared. N_2O concentrations were similar after 1 to 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 1 B).

Initial nitrite-dependent N_2O production rates of microcosms with cryoturbated and unturbated peat soil and nitrate-dependent N_2O production rates of microcosms with cryoturbated peat soil followed apparent Michaelis-Menten kinetics, while 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 to 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 subject 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 50 µM and greater (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 (i.e., N_2 plus N_2O) after 8 hours 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 correlation coefficients of 1.0 and 0.9, respectively; 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.

Anaerobic fermentation activities and trophic links to denitrifiers

Organic acids were not detectable in cryoturbated peat soil after the anoxic pre-incubation to deplete nitrate and nitrite (0 h, Figure S3 A). 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 (i.e, 9 days of preincubation plus 49 days of treatment; Figure S3 A). In contrast, approximately 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, Figure S3 A). 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 (i.e, 9 days of preincubation plus 49 days of treatment; Figure S3 A). Formate was below the detection limit after 58 days of incubation, indicating formate consumption. Such data suggest that fermentation potentials are lower in cryoturbated than in unturbated peat soil.

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 in order to identify putative substrates of acid-tolerant permafrost denitrifiers (Table 3; Supplemental 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 (i.e., when electron donors and nitrite where supplemented to the microcosms for the first time; Table 3). Such data suggest that acid-tolerant denitrifiers in cryoturbated and unturbated peat soil 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, 6 115 ± 1 447 sequences per gene marker and soil with an average sequence length of 444 ± 19 bp were obtained (Figure S4). As forward and reverse reads of *nirK* and *nirS* amplicons overlapped almost completely (approximately 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 (approximately 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 (i.e., *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 (e.g., 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 (Figure S1; Behnke *et al*., 2011; Quince *et al*., 2011). However, the number of OTUs stabilized from 10-30 % of threshold distance, indicating that the threshold distances of 17-33 % utilized for diversity analyses in this study rendered the analysis insensitive to such potential inaccuracies (Figure S1; see Materials and Methods for details). Coverages were always greater than 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 (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 reverse reads, although similar number of sequences were obtained, indicating that the utility of forward reads of *narG* amplicons is higher for diversity analyses than reverse reads (Table 4, Figure S5). Results obtained from reverse reads show similar overall trends to those from forward reads (Figures 3, S5-6). Thus, 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. 16 of the 18 OTUs including OTU 1 were only distantly related to *narG* of cultured organisms or environmental sequences (i.e., sequence dissimilarities of OTU representatives were 20-35%), indicating phylogenetic new *narG* in cryoturbated and unturbated peat soil (Figure 3). 95 and 76% of *narG* from cryoturbated and unturbated peat soil, respectively, affiliated with *Actinobacterial narG*. OTUs 2 and 3 were more abundant in cryoturbated than in unturbated peat soil amplicon libraries (Figure 4 A). 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 soil overlapped. Sørensen and Bray-Curtis indices for the β-diversity of *narG* were high (i.e. 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* were 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 4-5). OTUs 2 and 3 both had a relative abundance of approximately 44% and dominated *nirK* in unturbated soil amplicon libraries (Figures 4-5). 99 and 94% of *nirK* from cryoturbated and unturbated peat

soil, respectively, affiliated with *Alphaproteobacterial nirK* (Figures 4-5). Major OTUs of both soils were related to environmental *nirK* from upland soil. 10 of the 22 *nirK* OTUs were only distantly related (i.e., 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 while OTUs 1 and 2 dominated *nirS* in unturbated soil (Figures 4, 6). 99 and 12% of *nirS* from cryoturbated and unturbated peat soil, respectively, affiliated with *Alphaproteobacterial nirS*. One and 82% of *nirS* from cryoturbated and unturbated peat soil, 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). 8 of the 19 *nirS* OTUs were only distantly related (i.e., 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, Figure S5). 95% 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 case of *nirK* (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 soil. UniFrac analysis confirmed significant differences in *nirK* and *nirS* community compositions of cryoturbated and unturbated peat soil ($P<0.002$ 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 soil (Figures 4, 7). Most of the *nosZ* from both soils were affiliated with *Alphaproteobacterial nosZ*. 7 of the 11 *nosZ* OTUs

from cryoturbated and unturbated peat soil were only distantly related (i.e., 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 7). *nosZ* reverse reads yielded similar results (Table 4, Figure S7). Diversity measures of *nosZ* were higher in cryoturbated than in unturbated peat soil (Table 4, 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 (Figure S5). β-diversity as indicated by Sørensen and Bray-Curtis diversity indices of *nosZ* tended to be lower than 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 Material and Methods). 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 were capable of dissimilatory nitrate reduction (Table 5). *narG* copy numbers in unturbated peat soil were significantly lower (about 100 times, P=0.02) than those in cryoturbated peat soil (Table 5). The data is in agreement with the high and low capacities of cryoturbated and unturbated peat soil, respectively, to sustain nitrate dependent denitirification (Figure 2).

Copy numbers of *nirK* were 5 and 35 per ng DNA in cryoturbated and unturbated peat

soil, 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 (i.e., 5 to 7 x 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; Bremner, 1997; Conrad, 1996; 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 approximately 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 1 A). Organisms catalyzing DNR produce N_2O by an unspecific reaction of nitrate reductase with accumulated 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 1 B, 2). Extrapolation of N_2O production from soil endogenous nitrate in microcosms to the field level largely exceed the N_2O emissions measured *in situ*. Thus, current findings demonstrate that cryoturbated peat soil denitrifiers (i) are prone to react to anoxia, (ii) are active under acidic conditions, and (iii) have the potential to account for the *in situ* N_2O emissions of cryoturbated peat circles

(Marushchak *et al*. 2011; Repo *et al*., 2009).

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 1-2, S2, Table 2). Such data provided ecophysiological evidence that denitrifier communitities of cryoturbated and unturbated peat soils were dissimilar.

Pyrosequencing and qPCR of denitrification associated genes substantiated the previous conclusion (Figure 4, Tables 4-5). *narG* copy numbers of cryoturbated peat soil were higher than or in the same range as in agricultural soils or glacier forelands (Bru *et al*., 2007; Deiglmayr *et al*., 2006; Kandeler *et al*., 2006), 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; (Table 4-5, 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 the 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 the acidic peat soils. Indeed, *nirS* abundance in spruce forest soil was positively correlated to decreasing pH from 6.1 to 3.7, while *nirK* abundance was negatively correlated (Barta *et al*., 2010). Such data suggests that low pH and high moisture contents might favor *nirS*-type rather than *nirK*-type denitrifiers in acidic peat soils and highlights 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 nitrous oxide reductase was rather low, as suggested by the low *nosZ nirS*$^{-1}$ ratios (Table 5). The relative abundance of nitrous oxide 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 nitrous oxide 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 were supplied (Figure S2). Diversity measures of detected *nosZ* consistently suggested that denitrifiers capable of N_2O reduction (i.e., harboring the *nosZ* gene) were more diverse in cryoturbated than in unturbated peat soil (Table 4-5, 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 (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 soil by nitrite was more pronounced than by nitrate (Figures 1-2), suggesting that denitrifiers lacking nitrate reductases might contribute to N_2O production and/or nitrate reduction is rate limiting (Vangnai & Klein, 1974; Mahne & Tiedje, 1995; Zumft, 1997).

Denitrifiers in cryoturbated peat thrive at a low pH of 4 (Figures 1-2, 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 at pH 4 were < 10 µM 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 & Tiedje, 1981; Strong & 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 suggests 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 soil (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 1 B, 2, and S2), lending further support to the hypothesis that denitrifier community composition impacts regulation and thus prediction of N_2O fluxes (Ma *et al*., 20111; Philippot *et al*., 2009, 2011; Holtan-Hartwig *et al*., 2000).

Cryoturbated peat soil consumed N_2O that was initially produced from internal-N sources (Figure 1 A), 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 & Kowalchuk, 2008; Yergeau *et al*., 2007; Palmer *et al*., 2010). Ratios of N_2O to total N gases were below 40% at low nitrate and nitrite concentrations and approximately 100% at 500 µM (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 a variety of soils (Blackmer & 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 & Bremner, 1978; Schalk-Otte *et al*., 2000; Simek & 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 (Repo *et al*., 2009; Bockheim, 2007; 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 the acidic peat soils contained hitherto unknown sequences, and the major ones clustered with *Actinobacterial narG* (e.g., OTUs 1 and 3; Figures 3-4). 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, 6). *nirS*-based phylogenies are more congruent with the 16S rRNA-based phylogenies of their hosts than *nirK*-based phylogenies, thus the data suggests 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 utilized 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 the 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 & Kowalchuck, 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 soil. However, the molecular data is largely dependent on the choice of primers. Even though 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 utilized (e.g., Enwall *et* al., 2010; Palmer *et al*., 2010; Green *et al*., 2010; Throbäck *et al*., 2004). Considering the rather high threshold distances utilized in this study for calling OTUs, the diversity analyses of cryo- and unturbated peat soil denitrifiers might be regarded as a minimal estimate of the 'real' denitrifier diversity.

Within these limitations, the present study nonetheless provides evidence that (i) the exceptionally high N_2O emissions from cryoturbated peat circles are associated with a specific diverse, and acid-tolerant denitrifier community, (ii) contrasting denitrifier community compositions are associated with high and low N_2O emission patterns in acidic permafrost affected peat soil, and (iii) 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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Titles and legends to figures

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 soil, respectively. Closed and open symbols represent microcosms with and without acetylene, respectively. Timepoints 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 soil, 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 standard errors of three replicate microcosms are shown in panels A and B.

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 soil, respectively. Closed and open symbols represent microcosms supplemented with nitrite and nitrate, respectively. Mean values and standard errors 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.

Figure 3. Phylogenetic tree of representative *narG* sequences retrieved from unturbated and cryoturbated peat soil. 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 soil. In total, 1 825 and 2 526 sequences from forward reads were obtained from unturbated and cryoturbated peat soil, respectively. Grey 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 *narG* (A), *nirK* (B), *nirS* (C), and *nosZ* (D) derived

OTUs retrieved from unturbated and cryoturbated peat soil. 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 5. Phylogenetic tree of representative *nirK* sequences retrieved from unturbated and cryoturbated peat soil. 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 soil. 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 soil, respectively. Grey 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).

Figure 6. Phylogenetic tree of representative *nirS* sequences retrieved from unturbated and cryoturbated peat soil. 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 soil. Codes preceeding sequence names represent sequence accession numbers in public databases. In total, 2 942 and 285 sequences were obtained from unturbated and cryoturbated peat soil, respectively. Grey 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).

Figure 7. Phylogenetic tree of representative *nosZ* sequences retrieved from unturbated and cryoturbated peat soil. 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 soil. Codes preceeding sequence names represent sequence accession numbers in public databases. In total, 2 097 and 3 709 sequences from forward reads were obtained from unturbated and cryoturbated peat soil, respectively. Grey 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).

Figure 1

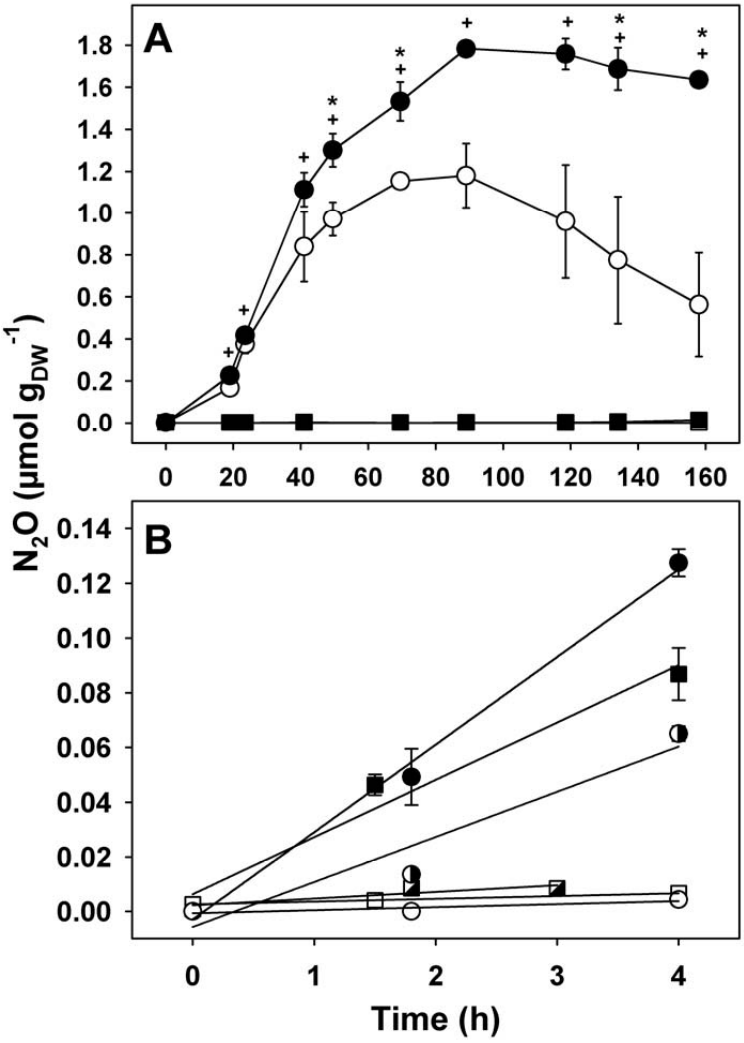
A
B
N_2O (µmol g_{DW}^{-1})
Time (h)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0
20
40
60
80
100
120
140
160
0
1
2
3
4

Figure 2

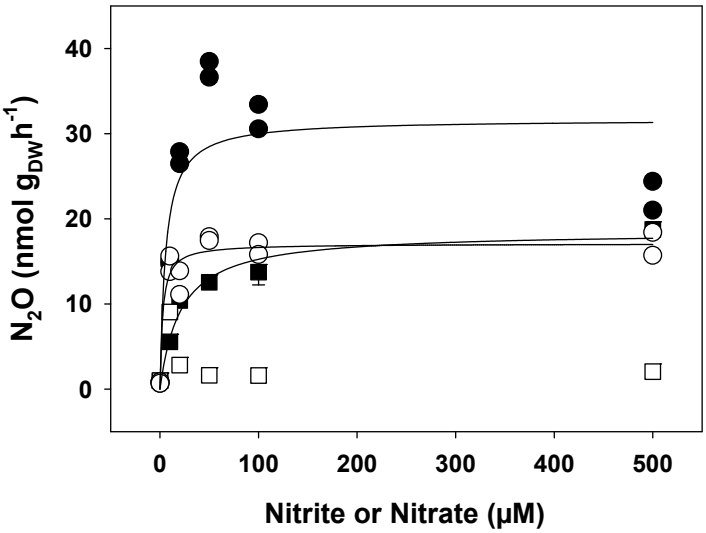
40
30
20
10
0
N_2O (nmol $g_{DW}h^{-1}$)
0
100
200
300
400
500
Nitrite or Nitrate (µM)

Figure 3

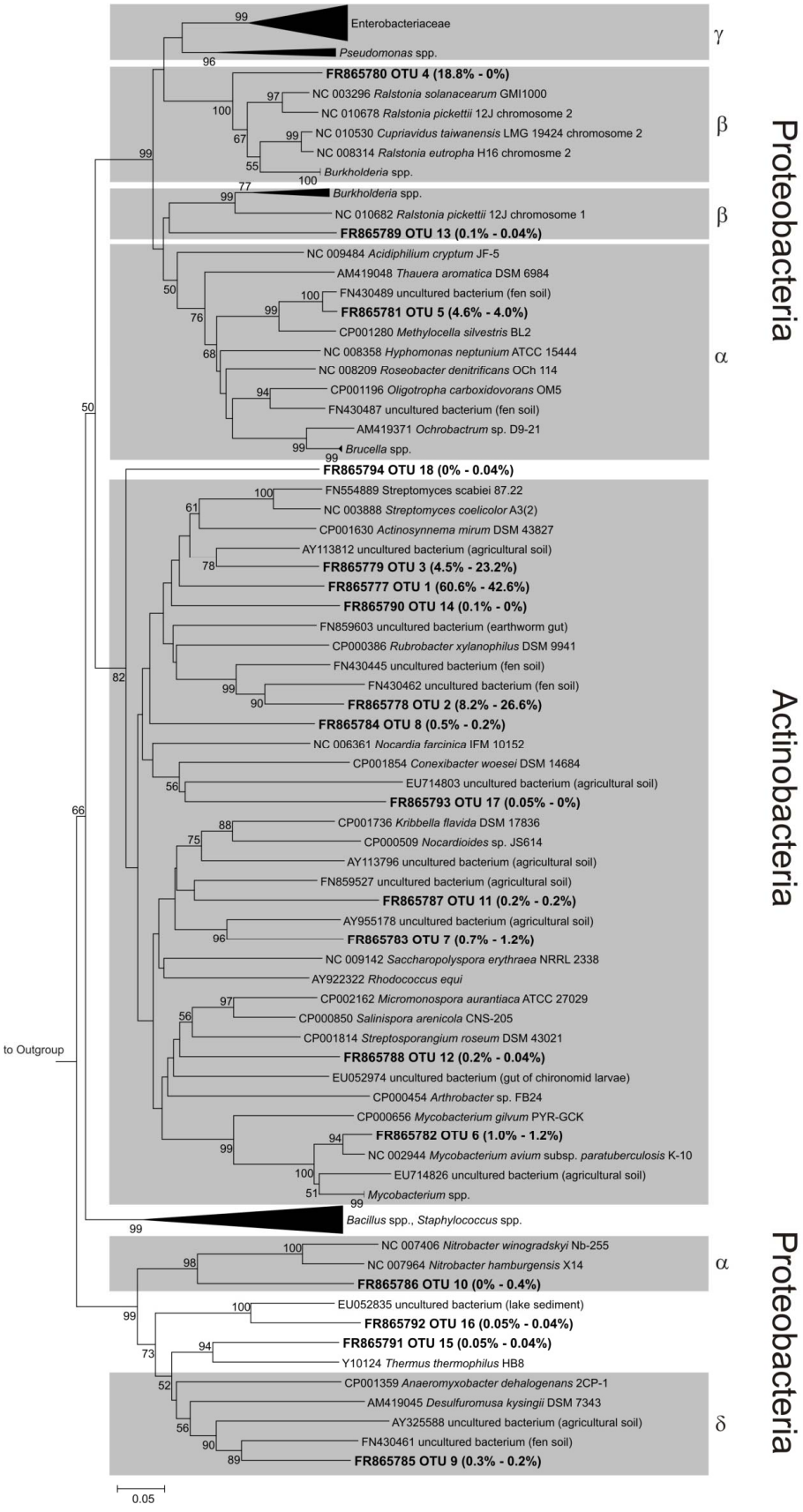
Enterobacteriaceae
Pseudomonas spp.
γ
FR865780 OTU 4 (18.8% - 0%)
NC 003296 Ralstonia solanacearum GMI1000
NC 010678 Ralstonia pickettii 12J chromosome 2
NC 010530 Cupriavidus taiwanensis LMG 19424 chromosome 2
NC 008314 Ralstonia eutropha H16 chromosme 2
Burkholderia spp.
β
Burkholderia spp.
NC 010682 Ralstonia pickettii 12J chromosome 1
FR865789 OTU 13 (0.1% - 0.04%)
β
NC 009484 Acidiphilium cryptum JF-5
AM419048 Thauera aromatica DSM 6984
FN430489 uncultured bacterium (fen soil)
FR865781 OTU 5 (4.6% - 4.0%)
CP001280 Methylocella silvestris BL2
NC 008358 Hyphomonas neptunium ATCC 15444
NC 008209 Roseobacter denitrificans OCh 114
CP001196 Oligotropha carboxidovorans OM5
FN430487 uncultured bacterium (fen soil)
AM419371 Ochrobactrum sp. D9-21
Brucella spp.
α
Proteobacteria
FR865794 OTU 18 (0% - 0.04%)
FN554889 Streptomyces scabiei 87.22
NC 003888 Streptomyces coelicolor A3(2)
CP001630 Actinosynnema mirum DSM 43827
AY113812 uncultured bacterium (agricultural soil)
FR865779 OTU 3 (4.5% - 23.2%)
FR865777 OTU 1 (60.6% - 42.6%)
FR865790 OTU 14 (0.1% - 0%)
FN859603 uncultured bacterium (earthworm gut)
CP000386 Rubrobacter xylanophilus DSM 9941
FN430445 uncultured bacterium (fen soil)
FN430462 uncultured bacterium (fen soil)
FR865778 OTU 2 (8.2% - 26.6%)
FR865784 OTU 8 (0.5% - 0.2%)
NC 006361 Nocardia farcinica IFM 10152
CP001854 Conexibacter woesei DSM 14684
EU714803 uncultured bacterium (agricultural soil)
FR865793 OTU 17 (0.05% - 0%)
CP001736 Kribbella flavida DSM 17836
CP000509 Nocardioides sp. JS614
AY113796 uncultured bacterium (agricultural soil)
FN859527 uncultured bacterium (agricultural soil)
FR865787 OTU 11 (0.2% - 0.2%)
AY955178 uncultured bacterium (agricultural soil)
FR865783 OTU 7 (0.7% - 1.2%)
NC 009142 Saccharopolyspora erythraea NRRL 2338
AY922322 Rhodococcus equi
CP002162 Micromonospora aurantiaca ATCC 27029
CP000850 Salinispora arenicola CNS-205
CP001814 Streptosporangium roseum DSM 43021
FR865788 OTU 12 (0.2% - 0.04%)
EU052974 uncultured bacterium (gut of chironomid larvae)
CP000454 Arthrobacter sp. FB24
CP000656 Mycobacterium gilvum PYR-GCK
FR865782 OTU 6 (1.0% - 1.2%)
NC 002944 Mycobacterium avium subsp. paratuberculosis K-10
EU714826 uncultured bacterium (agricultural soil)
Mycobacterium spp.
Actinobacteria
to Outgroup
Bacillus spp., Staphylococcus spp.
NC 007406 Nitrobacter winogradskyi Nb-255
NC 007964 Nitrobacter hamburgensis X14
FR865786 OTU 10 (0% - 0.4%)
α
EU052835 uncultured bacterium (lake sediment)
FR865792 OTU 16 (0.05% - 0.04%)
FR865791 OTU 15 (0.05% - 0.04%)
Y10124 Thermus thermophilus HB8
CP001359 Anaeromyxobacter dehalogenans 2CP-1
AM419045 Desulfuromusa kysingii DSM 7343
AY325588 uncultured bacterium (agricultural soil)
FN430461 uncultured bacterium (fen soil)
FR865785 OTU 9 (0.3% - 0.2%)
δ
Proteobacteria
0.05

Figure 4

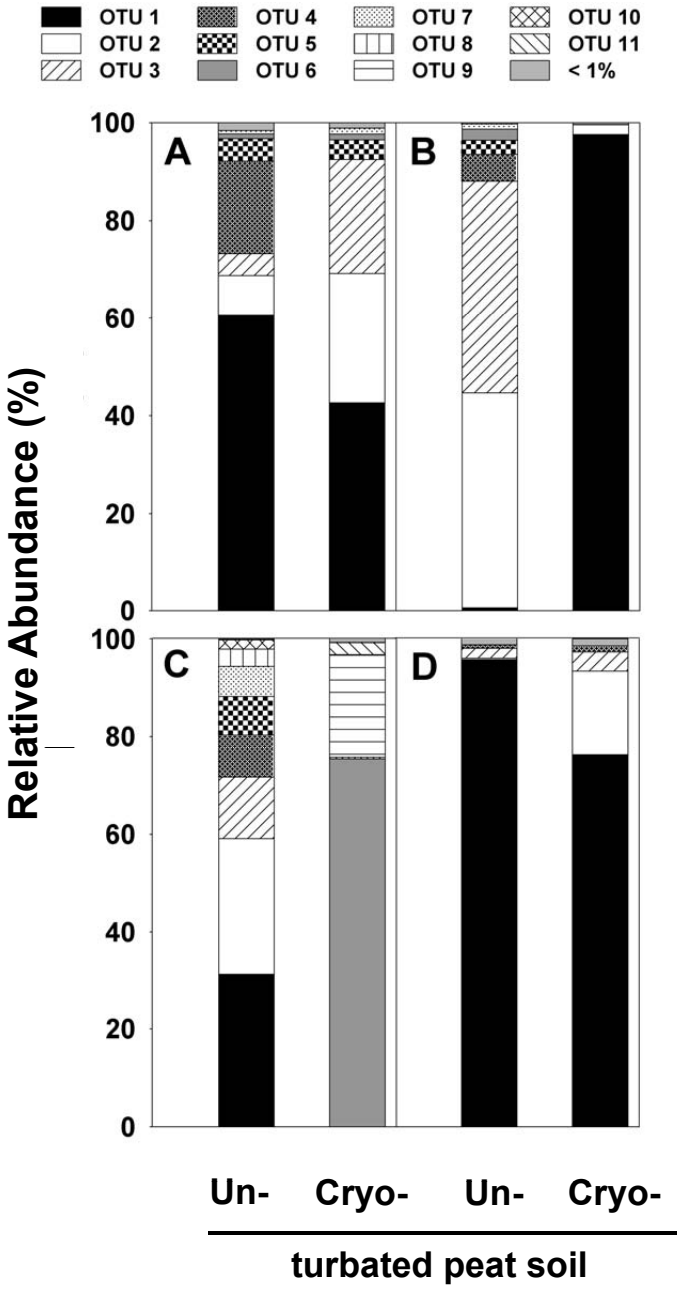
OTU 1
OTU 2
OTU 3
OTU 4
OTU 5
OTU 6
OTU 7
OTU 8
OTU 9
OTU 10
OTU 11
< 1%
A
B
C
D
Relative Abundance (%)
100
80
60
40
20
0
Un-
Cryo-
Un-
Cryo-
turbated peat soil

Figure 5

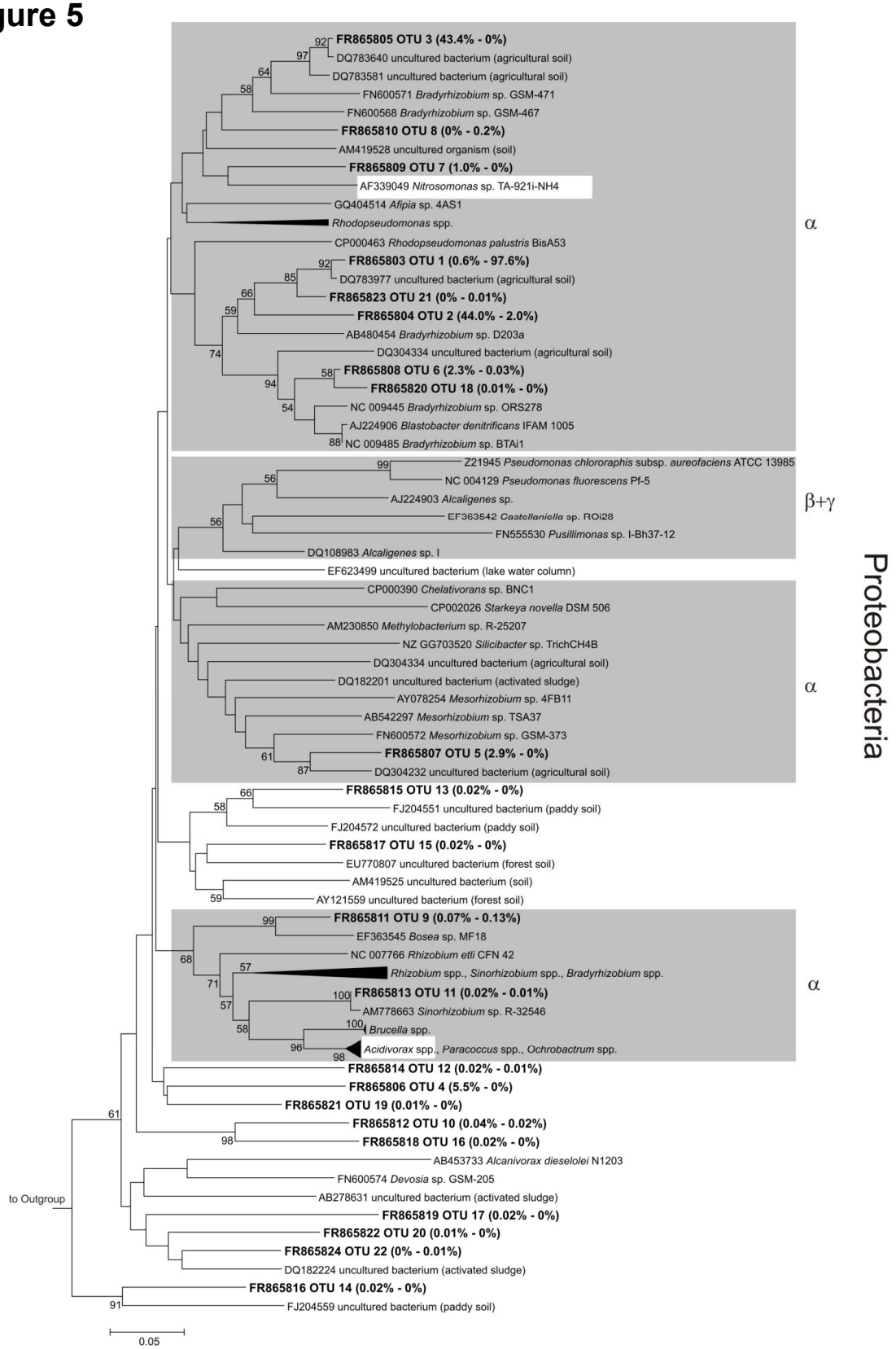
FR865805 OTU 3 (43.4% - 0%)
DQ783640 uncultured bacterium (agricultural soil)
DQ783581 uncultured bacterium (agricultural soil)
FN600571 Bradyrhizobium sp. GSM-471
FN600568 Bradyrhizobium sp. GSM-467
FR865810 OTU 8 (0% - 0.2%)
AM419528 uncultured organism (soil)
FR865809 OTU 7 (1.0% - 0%)
AF339049 Nitrosomonas sp. TA-921i-NH4
GQ404514 Afipia sp. 4AS1
Rhodopseudomonas spp.
CP000463 Rhodopseudomonas palustris BisA53
FR865803 OTU 1 (0.6% - 97.6%)
DQ783977 uncultured bacterium (agricultural soil)
FR865823 OTU 21 (0% - 0.01%)
FR865804 OTU 2 (44.0% - 2.0%)
AB480454 Bradyrhizobium sp. D203a
DQ304334 uncultured bacterium (agricultural soil)
FR865808 OTU 6 (2.3% - 0.03%)
FR865820 OTU 18 (0.01% - 0%)
NC 009445 Bradyrhizobium sp. ORS278
AJ224906 Blastobacter denitrificans IFAM 1005
NC 009485 Bradyrhizobium sp. BTAi1
α
Z21945 Pseudomonas chlororaphis subsp. aureofaciens ATCC 13985
NC 004129 Pseudomonas fluorescens Pf-5
AJ224903 Alcaligenes sp.
EF363542 Castellaniella sp. ROi28
FN555530 Pusillimonas sp. I-Bh37-12
DQ108983 Alcaligenes sp. I
β+γ
EF623499 uncultured bacterium (lake water column)
CP000390 Chelativorans sp. BNC1
CP002026 Starkeya novella DSM 506
AM230850 Methylobacterium sp. R-25207
NZ GG703520 Silicibacter sp. TrichCH4B
DQ304334 uncultured bacterium (agricultural soil)
DQ182201 uncultured bacterium (activated sludge)
AY078254 Mesorhizobium sp. 4FB11
AB542297 Mesorhizobium sp. TSA37
FN600572 Mesorhizobium sp. GSM-373
FR865807 OTU 5 (2.9% - 0%)
DQ304232 uncultured bacterium (agricultural soil)
α
FR865815 OTU 13 (0.02% - 0%)
FJ204551 uncultured bacterium (paddy soil)
FJ204572 uncultured bacterium (paddy soil)
FR865817 OTU 15 (0.02% - 0%)
EU770807 uncultured bacterium (forest soil)
AM419525 uncultured bacterium (soil)
AY121559 uncultured bacterium (forest soil)
FR865811 OTU 9 (0.07% - 0.13%)
EF363545 Bosea sp. MF18
NC 007766 Rhizobium etli CFN 42
Rhizobium spp., Sinorhizobium spp., Bradyrhizobium spp.
FR865813 OTU 11 (0.02% - 0.01%)
AM778663 Sinorhizobium sp. R-32546
Brucella spp.
Acidivorax spp., Paracoccus spp., Ochrobactrum spp.
α
FR865814 OTU 12 (0.02% - 0.01%)
FR865806 OTU 4 (5.5% - 0%)
FR865821 OTU 19 (0.01% - 0%)
FR865812 OTU 10 (0.04% - 0.02%)
FR865818 OTU 16 (0.02% - 0%)
AB453733 Alcanivorax dieselolei N1203
FN600574 Devosia sp. GSM-205
AB278631 uncultured bacterium (activated sludge)
FR865819 OTU 17 (0.02% - 0%)
FR865822 OTU 20 (0.01% - 0%)
FR865824 OTU 22 (0% - 0.01%)
DQ182224 uncultured bacterium (activated sludge)
FR865816 OTU 14 (0.02% - 0%)
FJ204559 uncultured bacterium (paddy soil)
to Outgroup
0.05
Proteobacteria

Figure 6

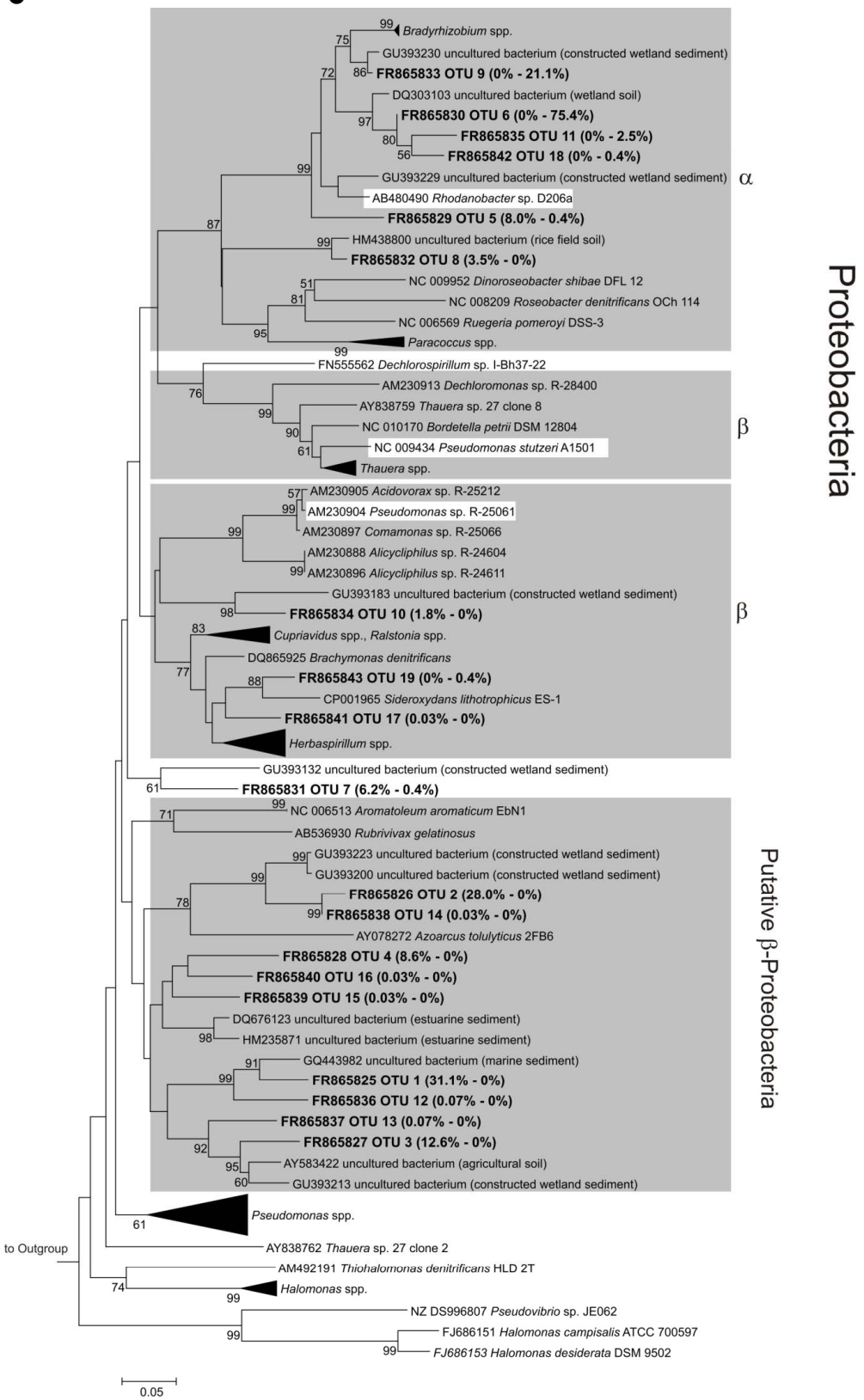
Bradyrhizobium spp.
GU393230 uncultured bacterium (constructed wetland sediment)
FR865833 OTU 9 (0% - 21.1%)
DQ303103 uncultured bacterium (wetland soil)
FR865830 OTU 6 (0% - 75.4%)
FR865835 OTU 11 (0% - 2.5%)
FR865842 OTU 18 (0% - 0.4%)
GU393229 uncultured bacterium (constructed wetland sediment)
AB480490 Rhodanobacter sp. D206a
FR865829 OTU 5 (8.0% - 0.4%)
HM438800 uncultured bacterium (rice field soil)
FR865832 OTU 8 (3.5% - 0%)
NC 009952 Dinoroseobacter shibae DFL 12
NC 008209 Roseobacter denitrificans OCh 114
NC 006569 Ruegeria pomeroyi DSS-3
Paracoccus spp.
α
FN555562 Dechlorospirillum sp. I-Bh37-22
AM230913 Dechloromonas sp. R-28400
AY838759 Thauera sp. 27 clone 8
NC 010170 Bordetella petrii DSM 12804
NC 009434 Pseudomonas stutzeri A1501
Thauera spp.
β
AM230905 Acidovorax sp. R-25212
AM230904 Pseudomonas sp. R-25061
AM230897 Comamonas sp. R-25066
AM230888 Alicycliphilus sp. R-24604
AM230896 Alicycliphilus sp. R-24611
GU393183 uncultured bacterium (constructed wetland sediment)
FR865834 OTU 10 (1.8% - 0%)
Cupriavidus spp., Ralstonia spp.
DQ865925 Brachymonas denitrificans
FR865843 OTU 19 (0% - 0.4%)
CP001965 Sideroxydans lithotrophicus ES-1
FR865841 OTU 17 (0.03% - 0%)
Herbaspirillum spp.
β
Proteobacteria
GU393132 uncultured bacterium (constructed wetland sediment)
FR865831 OTU 7 (6.2% - 0.4%)
NC 006513 Aromatoleum aromaticum EbN1
AB536930 Rubrivivax gelatinosus
GU393223 uncultured bacterium (constructed wetland sediment)
GU393200 uncultured bacterium (constructed wetland sediment)
FR865826 OTU 2 (28.0% - 0%)
FR865838 OTU 14 (0.03% - 0%)
AY078272 Azoarcus tolulyticus 2FB6
FR865828 OTU 4 (8.6% - 0%)
FR865840 OTU 16 (0.03% - 0%)
FR865839 OTU 15 (0.03% - 0%)
DQ676123 uncultured bacterium (estuarine sediment)
HM235871 uncultured bacterium (estuarine sediment)
GQ443982 uncultured bacterium (marine sediment)
FR865825 OTU 1 (31.1% - 0%)
FR865836 OTU 12 (0.07% - 0%)
FR865837 OTU 13 (0.07% - 0%)
FR865827 OTU 3 (12.6% - 0%)
AY583422 uncultured bacterium (agricultural soil)
GU393213 uncultured bacterium (constructed wetland sediment)
Putative β-Proteobacteria
Pseudomonas spp.
to Outgroup
AY838762 Thauera sp. 27 clone 2
AM492191 Thiohalomonas denitrificans HLD 2T
Halomonas spp.
NZ DS996807 Pseudovibrio sp. JE062
FJ686151 Halomonas campisalis ATCC 700597
FJ686153 Halomonas desiderata DSM 9502
0.05

Figure 7

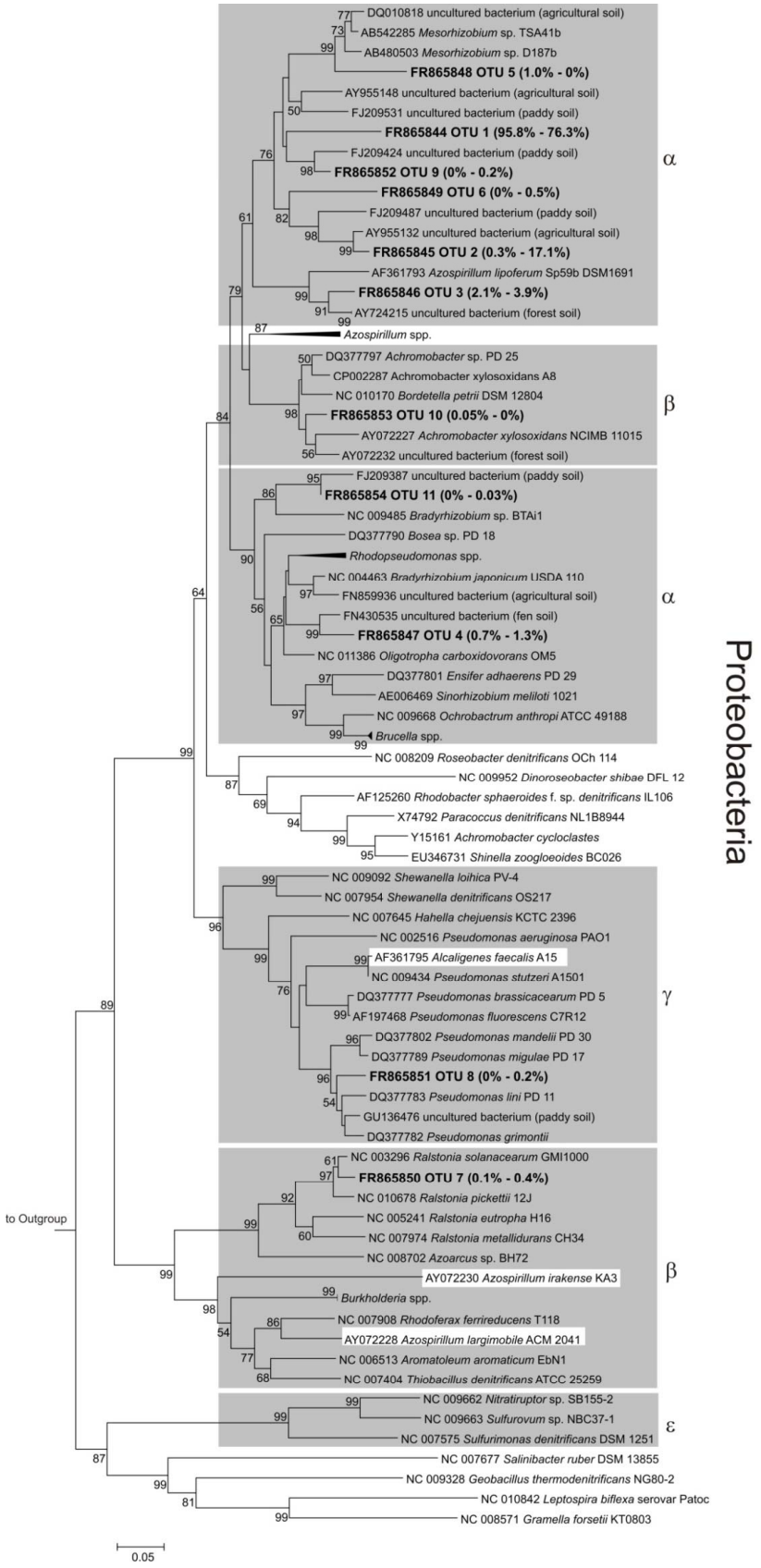
DQ010818 uncultured bacterium (agricultural soil)
AB542285 Mesorhizobium sp. TSA41b
AB480503 Mesorhizobium sp. D187b
FR865848 OTU 5 (1.0% - 0%)
AY955148 uncultured bacterium (agricultural soil)
FJ209531 uncultured bacterium (paddy soil)
FR865844 OTU 1 (95.8% - 76.3%)
FJ209424 uncultured bacterium (paddy soil)
FR865852 OTU 9 (0% - 0.2%)
FR865849 OTU 6 (0% - 0.5%)
FJ209487 uncultured bacterium (paddy soil)
AY955132 uncultured bacterium (agricultural soil)
FR865845 OTU 2 (0.3% - 17.1%)
AF361793 Azospirillum lipoferum Sp59b DSM1691
FR865846 OTU 3 (2.1% - 3.9%)
AY724215 uncultured bacterium (forest soil)
Azospirillum spp.
α
DQ377797 Achromobacter sp. PD 25
CP002287 Achromobacter xylosoxidans A8
NC 010170 Bordetella petrii DSM 12804
FR865853 OTU 10 (0.05% - 0%)
AY072227 Achromobacter xylosoxidans NCIMB 11015
AY072232 uncultured bacterium (forest soil)
β
FJ209387 uncultured bacterium (paddy soil)
FR865854 OTU 11 (0% - 0.03%)
NC 009485 Bradyrhizobium sp. BTAi1
DQ377790 Bosea sp. PD 18
Rhodopseudomonas spp.
NC 004463 Bradyrhizobium japonicum USDA 110
FN859936 uncultured bacterium (agricultural soil)
FN430535 uncultured bacterium (fen soil)
FR865847 OTU 4 (0.7% - 1.3%)
NC 011386 Oligotropha carboxidovorans OM5
DQ377801 Ensifer adhaerens PD 29
AE006469 Sinorhizobium meliloti 1021
NC 009668 Ochrobactrum anthropi ATCC 49188
Brucella spp.
α
NC 008209 Roseobacter denitrificans OCh 114
NC 009952 Dinoroseobacter shibae DFL 12
AF125260 Rhodobacter sphaeroides f. sp. denitrificans IL106
X74792 Paracoccus denitrificans NL1B8944
Y15161 Achromobacter cycloclastes
EU346731 Shinella zoogloeoides BC026
NC 009092 Shewanella loihica PV-4
NC 007954 Shewanella denitrificans OS217
NC 007645 Hahella chejuensis KCTC 2396
NC 002516 Pseudomonas aeruginosa PAO1
AF361795 Alcaligenes faecalis A15
NC 009434 Pseudomonas stutzeri A1501
DQ377777 Pseudomonas brassicacearum PD 5
AF197468 Pseudomonas fluorescens C7R12
DQ377802 Pseudomonas mandelii PD 30
DQ377789 Pseudomonas migulae PD 17
FR865851 OTU 8 (0% - 0.2%)
DQ377783 Pseudomonas lini PD 11
GU136476 uncultured bacterium (paddy soil)
DQ377782 Pseudomonas grimontii
γ
NC 003296 Ralstonia solanacearum GMI1000
FR865850 OTU 7 (0.1% - 0.4%)
NC 010678 Ralstonia pickettii 12J
NC 005241 Ralstonia eutropha H16
NC 007974 Ralstonia metallidurans CH34
NC 008702 Azoarcus sp. BH72
AY072230 Azospirillum irakense KA3
Burkholderia spp.
NC 007908 Rhodoferax ferrireducens T118
AY072228 Azospirillum largimobile ACM 2041
NC 006513 Aromatoleum aromaticum EbN1
NC 007404 Thiobacillus denitrificans ATCC 25259
β
NC 009662 Nitratiruptor sp. SB155-2
NC 009663 Sulfurovum sp. NBC37-1
NC 007575 Sulfurimonas denitrificans DSM 1251
ε
NC 007677 Salinibacter ruber DSM 13855
NC 009328 Geobacillus thermodenitrificans NG80-2
NC 010842 Leptospira biflexa serovar Patoc
NC 008571 Gramella forsetii KT0803
to Outgroup
0.05
Proteobacteria

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

	Temperature (°C)/Time (min)				
Primer set	narG1960f/ narG2650r [1]	F1aCu/ R3Cu [2]	cd3aF/ R3cd [2]	nosZF/ nosZR [3]	Eub341F/ Eub534R [4]
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) [5]	(72) [5]
No. of cycles	40	50	40	35	35
Final elongation	72/5	72/5	72/5	72/5	72/5

[1] Philippot *et al*., 2002
[2] Throbäck *et al*., 2004
[3] Rich *et al*., 2003
[4] Muyzer *et al*., 1993
[5] Fluorescence recorded during elongation step

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.

	Nitrate amended		Nitrite amended	
Soil	v_{max} [1] ($nmol \cdot g_{DW}^{-1} h^{-1}$)	K_M [1] (μM)	v_{max} [1] ($nmol \cdot g_{DW}^{-1} h^{-1}$)	K_M [1] (μM)
unturbated	n.a. [2]	n.a. [2]	18 ± 1	21 ± 5
cryoturbated	17 ± 1	3 ± 1	32 ± 3	6 ± 4

[1] Kinetic parameters (calculated from Figure 2) $\pm$ standard error
[2] not applicable

Table 3: Effect of supplemented electron donors on N_2O production in nitrite-amended anoxic peat soil microcosms. Percentage of N_2O production in each treatment as compared to microcosms supplemented with nitrite only (i.e. control).

	First pulse [1]		Second pulse [2]	
Treatment	Unturbated	Cryoturbated	Unturbated	Cryoturbated
Control [3]	100 [4] (98-102) [5]	100 [6] (79-121)	100 [7] (96-104)	100 [8] (77-123)
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)

[1] Nitrite and electron donors added after 9 days of preincubation

[2] Nitrite and electron donors resupplied 47 days after the first pulse

[3] Microcosms supplemented with nitrite only

[4] N_2O production = 23.1 $nmol \cdot g_{DW}^{-1} h^{-1}$

[5] Ratios of the duplicates and the mean of the control microcosms (%)

[6] N_2O production = 54.9 $nmol \cdot g_{DW}^{-1} h^{-1}$

[7] N_2O production = 16.7 $nmol \cdot g_{DW}^{-1} h^{-1}$

[8] N_2O production = 22.6 $nmol \cdot g_{DW}^{-1} h^{-1}$

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

	Unturbated						Cryoturbated						β Diversity	
Gene marker	No. of sequences	Library coverage (%) [1]	No. of OTUs observed	No. of OTUs estimated [2]	H [3]	E [4]	No. of sequences	Library coverage (%) [1]	No. of OTUs observed	No. of OTUs estimated [2]	H [3]	E [4]	S_s [5]	BC_s [6]
narG forward	1 825	99.8	16	17 (16-27)	1.28 (1.23-1.34)	0.46 (0.44-0.48)	2 526	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)	3 806	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)	3 709	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)	3 664	99.9	11	14 (12-22)	0.75 (0.72-0.79)	0.31 (0.30-0.33)	0.67	0.68

[1] Percent library coverage. $C = (1 - \frac{n_s}{n_t}) \cdot 100$ (n_s=OTUs that occur only once, n_t=total number of sequences)
[2] Chao1 richness estimate with upper and lower 95% confidence intervals given in parentheses.
[3] Shannon-Weaver diversity index with upper and lower 95% confidence intervals given in parentheses.
[4] Species Evenness with upper and lower 95% confidence intervals given in parentheses.
[5] Sørensen similarity index.
[6] Bray Curtis similarity index.

Table 5: Abundance of denitrification-associated genes in peat soil.

	Unturbated		Cryoturbated	
Gene marker	Copy No. per 16S rRNA gene (%) [1,2]	Copy No. per ng DNA [1]	Copy No. per 16S rRNA gene (%) [1,3]	Copy No. per ng DNA [1]
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	$(1.0 \pm 0.6) \times 10^{-4}$	$(2.7 \pm 1.2) \times 10^{0}$	$(1.7 \pm 0.4) \times 10^{-3}$	$(1.2 \pm 0.2) \times 10^{1}$

[1] Mean of 3 (sites) x 6 (technical) replicates $\pm$ standard error (see Material and Methods)

[2] 16S rRNA gene copy numbers were $(1.9 \pm 0.2) \times 10^{6}$ per ng DNA

[3] 16S rRNA gene copy numbers were $(8.0 \pm 1.7) \times 10^{5}$ per ng DNA

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Actinobacterial Nitrate Reducers and Proteobacterial Denitrifiers Are Abundant in N_2O-Metabolizing Palsa Peat

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Palsa peats are characterized by elevated, circular frost heaves (peat soil on top of a permanently frozen ice lens) and are strong to moderate sources or even temporary sinks for the greenhouse gas nitrous oxide (N_2O). Palsa peats are predicted to react sensitively to global warming. The acidic palsa peat Skalluvaara (approximate pH 4.4) is located in the discontinuous permafrost zone in northwestern Finnish Lapland. *In situ* N_2O fluxes were spatially variable, ranging from 0.01 to -0.02 μmol of N_2O m^{-2} h^{-1}. Fertilization with nitrate stimulated *in situ* N_2O emissions and N_2O production in anoxic microcosms without apparent delay. N_2O was subsequently consumed in microcosms. Maximal reaction velocities (v_{max}) of nitrate-dependent denitrification approximated 3 and 1 nmol of N_2O per h per gram (dry weight [g_{DW}]) in soil from 0 to 20 cm and below 20 cm of depth, respectively. v_{max} values of nitrite-dependent denitrification were 2- to 5-fold higher than the v_{max} nitrate-dependent denitrification, and v_{max} of N_2O consumption was 1- to 6-fold higher than that of nitrite-dependent denitrification, highlighting a high N_2O consumption potential. Up to 12 species-level operational taxonomic units (OTUs) of *narG*, *nirK* and *nirS*, and *nosZ* were retrieved. Detected OTUs suggested the presence of diverse uncultured soil denitrifiers and dissimilatory nitrate reducers, hitherto undetected species, as well as *Actino-*, *Alpha-*, and *Betaproteobacteria*. Copy numbers of *nirS* always outnumbered those of *nirK* by 2 orders of magnitude. Copy numbers of *nirS* tended to be higher, while copy numbers of *narG* and *nosZ* tended to be lower in 0- to 20-cm soil than in soil below 20 cm. The collective data suggest that (i) the source and sink functions of palsa peat soils for N_2O are associated with denitrification, (ii) actinobacterial nitrate reducers and *nirS*-type and *nosZ*-harboring proteobacterial denitrifiers are important players, and (iii) acidic soils like palsa peats represent reservoirs of diverse acid-tolerant denitrifiers associated with N_2O fluxes.

Permafrost systems in the Northern Hemisphere cover about 16% of the global soil surface area, store substantial amounts of carbon and nitrogen, and are therefore important players in the global carbon and nitrogen cycles (54, 67). Palsas are elevations of peat soil above the ground level due to uplifting of peat layers by a frozen ice lens and are mainly encountered in the discontinuous permafrost zone (63). Palsa peatlands are widely distributed in the Arctic, including Canada, Norway, Sweden, Iceland, Russia (Siberia), the United States (Alaska), and Finland (62, 75). Palsa development is affected by various environmental factors, such as wind erosion, vegetation cover, snow cover, and ground water table depth (63). High-latitude peatlands have been intensively studied with respect to their capacity to emit methane due to the large amount of stored carbon in peat soils, but nitrous oxide (N_2O) emissions from permafrost regions were generally considered to be insignificant (12, 58, 66). However, recent studies document significant but variable N_2O emissions from permafrost systems including palsas (17, 45, 58). N_2O is the major ozone-depleting substance in the atmosphere (57), and such N_2O emissions might well impact climate change since the global warming potential of N_2O is approximately 300 times that of CO_2 (20). Palsa peats are predicted to be strongly affected by global warming (2, 21, 63). Increasing temperatures are generally anticipated to reduce the water table in northern peatlands and to increase the amount of CO_2, CH_4, and N_2O released from peatland soils (2, 44, 45).

N_2O is produced during nitrification, denitrification, or chemical processes in soils (8, 13). N_2O is an intermediate during denitrification, and denitrification is considered to be the main source of and hypothesized to represent a sink for N_2O in water-saturated soils including peatlands and is an essential part of the nitrogen cycle (11, 13, 48). Nitrate or nitrite is sequentially reduced via nitric oxide (NO) and N_2O to dinitrogen (N_2) during denitrification (76). Such 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* or *norZ*), and N_2O reductases (encoded by *nosZ*) (76, 77). Nitrate reductases likewise catalyze nitrate reduction by nondenitrifying dissimilatory nitrate reducers (52). N_2O and N_2 can be released into the atmosphere, and the ratio of N_2O to N_2 is determined by *in situ* parameters such as pH, temperature, and oxygen content, as well as nitrate/nitrite and electron donor availability (69). pH is one of the main factors impacting denitrification; low pH decreases overall denitrification rates and increases the product ratio of N_2O to N_2 (64). Although pristine northern peatlands including palsas are characterized by low pH and although evidence is accumulating that northern peatlands emit N_2O, palsas might represent temporary sinks for N_2O; however, microbial communities involved in N_2O turnover and N-cycling in northern peatlands are largely unknown to date (24, 45, 47, 48).

There is a particularly high deficit of information on N_2O fluxes from palsa peats, the underlying processes, their regulation, and the associated acid-tolerant microbial communities. Thus, the main objectives of this study were to (i) assess *in situ* N_2O

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TABLE 1 Soil parameters of Skalluvaara palsa peat[a]

Soil layer or source	pH	Moisture content (%)	NO_3^- concn		NO_2^- concn		NH_4^+ concn		Total C ($g\ kg_{DW}^{-1}$)	DOC[b] ($mg\ liter^{-1}$)	Total N ($g\ kg_{DW}^{-1}$)	C/N[c]
			μM	μmol g_{DW}^{-1}	μM	μmol g_{DW}^{-1}	μM	μmol g_{DW}^{-1}				
0–20 cm	4.6	73 ± 0.2	<26	<0.07	<60	<0.16	125.0	6.0	505	217	18	29
Below 20 cm	4.2	76 ± 0.2	<25	<0.08	<57	<0.18	106.0	6.0	501	156	19	26
Stream water	6.7	NA[d]	<4.7	NA	<11	NA	<1.4	NA	NA	7.2	NA	NA

[a] All values for 0- to 20-cm soil and below-20-cm soil, with the exception of moisture content, were determined from pooled soil samples of four replicate palsas.
[b] DOC, dissolved organic carbon.
[c] Carbon-to-nitrogen ratio.
[d] NA, not applicable.

fluxes and *in situ* N_2O emission capacities of palsa peat soil, (ii) determine N_2O production and consumption potentials of palsa peat denitrifiers in microcosms, (iii) derive apparent Michaelis-Menten-like kinetic parameters, and (iv) characterize the denitrifier community composition and abundance in palsa peat soil by bar-coded amplicon pyrosequencing coupled to quantitative PCR of multiple denitrification or nitrate reduction associated genes.

MATERIALS AND METHODS

Sampling site. The palsa peat Skalluvaara is located in northwestern Finnish Lapland (69°49′13″N, 27°9′47″E) at an elevation of 280 m above sea level, 6 km northwest of Stuorra Skállovárri (Great Skalluvaara, 408 m above sea level). The mean annual air temperature is −1.6 ± 1.2°C, the mean air temperature in July is 12.9°C (range, 2.9 to 26.9°C), and the mean monthly precipitation approximates 34.6 ± 6.7 mm (average of years 1962 to 2008, measured at Kevo research station). Palsas are elevated about 20 to 100 cm above the peat surface. Plant cover of palsas and surrounding peat soil consists mainly of *Rubus chamaemorus*, *Vaccinium myrtillus*, *Vaccinium vitis-idea*, *Vaccinium uliginosum*, *Empetrum nigrum*, *Rhododendron tomentosum*, *Cornus suecica*, *Sphagnum fallax*, *Sphagnum riparium*, *Betula pubescens* subsp. *czerepanovii*, and *Betula nana*. Soil temperature was 12°C in surface soil (16 July 2010), and palsas were frozen at 30 to 35 cm below palsa surface. Soil samples from layers at 0 to 20 cm and from 20 cm to the top of the frozen core (i.e., below 20 cm) were collected from four different vegetated palsas in July 2010. Samples were transported on ice to the laboratory and stored at 4°C for microcosm analyses or at −80°C for nucleic acid extractions within 1 h after sampling. Microcosm experiments were conducted within 2 weeks after sampling.

Assessment of *in situ* gas emissions. *In situ* gas emissions of unfertilized palsa peat and palsas fertilized with either nitrate or ammonium were determined in closed Plexiglas chambers. Two liters of water from a nearby stream that was supplemented with 20 mM sodium nitrate or 20 mM ammonium chloride was distributed homogeneously on top of approximately 0.25 m^2 of the soil in four replicates. Soil that received unsupplemented stream water served as a control. Stream water nitrate, nitrate, ammonium, and dissolved organic carbon (DOC) concentrations were negligible (Table 1). Plexiglas chambers were placed on metal collars, which had been inserted into the soil for a few centimeters (31). The transition between Plexiglas chamber and metal collar was sealed with a rubber band to avoid exchange of gases from the chamber with the surrounding air. Gas samples (5 ml per sampling time point) were taken from gas outlets and injected into gas-tight Exetainers (Labco Limited, High Wycombe, United Kingdom) immediately after fertilization and after 0.5, 1, and 3 h.

Assessment of denitrification in soil microcosms. Soil was homogenized and mixed prior to microcosm experiments. Soil slurries at an *in situ* pH of ~4.4 were prepared with 10 g of soil and 3 volumes of deionized water in 125-ml infusion flasks, sealed with gas-tight rubber stoppers and 100% argon in the gas phase. Experiments were done in triplicate. Microcosms were always incubated at 20°C in the dark, which is in the range of temperatures occurring *in situ* (air temperatures of up to 27°C in the month of sampling). Acetylene inhibits the reduction of N_2O to N_2 by blocking nitrous oxide reductases (72). Parallel microcosms with and without acetylene (15% [vol/vol] in headspace) were used to differentiate between total denitrification and N_2O production potentials (72). Experiments without supplemental nitrate or nitrite were conducted to assess denitrification potentials from internal nitrate or nitrite with and without acetylene. For apparent Michaelis-Menten kinetics, soil was preincubated under anoxic conditions to reduce the amount of internal nitrate and nitrite. Plateauing N_2O production indicated that internal nitrate and nitrite were consumed within 24 and 48 h in 0- to 20-cm and below 20-cm palsa peat soils, respectively (see Fig. 2). Preincubated soil was supplemented with 0 to 1,000 μM $NaNO_2$ or $NaNO_3$ or with 0 to 4 μM N_2O. Headspace concentrations of N_2O were determined four or five times via gas chromatography within 8 to 14 h of incubation, and N_2O production and consumption rates were calculated in the linear phase by linear regression of three or four data points (R^2 values were always greater than 0.80). Michaelis-Menten constants (K_m) and the maximum reaction velocities (v_{max}) of denitrification and N_2O consumption were based on the production of N_2O in microcosms supplemented with either nitrate or nitrite and acetylene or on the consumption of N_2O in microcosms supplemented with N_2O. Apparent Michaelis-Menten kinetics were fitted to the data points using the program SigmaPlot, version 10.0 (Systat Software GmbH, Erkrath, Germany) for calculation of K_m and v_{max} according to the following equation (61): $v = (v_{max} \times [S])/(K_m + [S])$ where $[S]$ is the concentration of nitrate or nitrite.

Analytical procedures. Nitrate, nitrite, and ammonium concentrations as well as soil pH and dissolved organic carbon (DOC) were determined in water extracts (10 g of soil in 30 ml of double-distilled H_2O [ddH_2O]; extraction was performed for 24 h at 4°C). Nitrate and nitrite were measured by flow injection analysis using a Dionex DX-500 ion chromatograph equipped with an IonPac AS4A-SC ion exchange column and an ED40 electrochemical detector (Sunnyvale, CA). Ammonium was quantified by flow injection analysis (FIA-LAB; MLE Dresden, Dresden, Germany). Total C and N contents were determined from oven-dried soil with an elemental analyzer (Thermo Quest Flash EA 1112; CE Instruments, Wigan, United Kingdom). DOC was determined after filtration (pore size, 0.45 μm) with a total organic carbon (TOC) analyzer (Multi N/C 2100; Analytik Jena, Jena, Germany). pH was determined with a pH electrode (pH Meter CG 832; Schott Geräte GmbH, Mainz, Germany). Moisture content was obtained by weighing soil samples before and after drying at 60°C for 3 days. N_2O was quantified with a Hewlett-Packard 5980 series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with an electron capture detector, according to published protocols (48).

Extraction of nucleic acids and PCR amplification of *narG*, *nirK*, *nirS*, and *nosZ*. Nucleic acids were extracted from homogenized soil of each sampled soil layer. Extractions were conducted as described previously with a bead-beating protocol (47). One to two micrograms of DNA was obtained per gram of soil (fresh weight) and had a low humic acid content, as indicated by A_{260}/A_{230} ratios approximating 0.94 to 1.16. *narG*, *nirK*, *nirS*, and *nosZ* were amplified using the primer pairs

narG1960f (TAY GTS GGS CAR GAR AA) and narG2650r (TTY TCR TAC CAB GTB GC) (53), F1aCu (ATC ATG GTS CTG CCG CG) and R3Cu (GCC TCG ATC AGR TTG TGG TT) (68), cd3aF (GTS AAC GTS AAG GAR ACS GG) and R3cd (GAS TTC GGR TGS GTC TTG A) (68), and nosZF (CGC TGT TCI TCG ACA GYC AG) and nosZR (ATG TGC AKI GCR TGG CAG AA) (59), respectively. Each primer was preceded by a 6-base-long bar code (ACACAC for 0- to 20-cm soil; ACGTAC for soil below 20 cm) to separate sequences after pyrosequencing. The application of primers fused to A and B adaptors required for pyrosequencing (which is routinely applied for amplicon pyrosequencing) was avoided to minimize primer bias during PCR (5). PCRs and thermal cycling conditions were as described previously (47).

Bar-coded amplicon pyrosequencing of structural genes. Bar-coded PCR products were gel purified with 1% agarose gels using a Montage Gel Extraction Kit (Millipore Corporation, Bedford, MA), according to the manufacturer's instructions, and purified via isopropanol precipitation to remove residual running buffer. Concentrations of the gel-purified PCR products were determined using Quant-iT PicoGreen (Invitrogen, Carlsbad, CA) and an FLx800 Fluorescence Microplate Reader (BioTek, Bad Friedrichshall, Germany). PCR products (i.e., amplicons) of similar lengths of both soil layers were combined in equal amounts (i.e., *narG* and *nosZ* 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 via isopropanol precipitation. A and B sequencing adaptors (GCC TCC CTC GCG CCA TCA G and GCC TTG CCA GCC CGC TCA G, respectively) that are required for pyrosequencing were ligated to the amplicons and sequenced from 5′ (forward) and 3′ (reverse) ends of amplicons. Amplicons were mixed with other samples and subjected to two pyrosequencing analyses at the Göttingen Genomics Laboratory employing a Roche GS-FLX 454 pyrosequencer and GS FLX titanium series reagents (Roche, Mannheim, Germany), according to the manufacturer's instructions. Pyrosequencing runs including *narG* and *nosZ* amplicons yielded 120,775 sequences with a mean sequence length of 372 bp; runs including *nirK* and *nirS* amplicons yielded 173,163 sequences with a mean sequence lengths of 388 bp.

Sequence filtering and analysis. Pyrosequencing and PCR amplification errors can artificially inflate detected sequence diversity (41, 56). Thus, correction or removal of so-called noisy reads is essential to correctly estimate the number of operational taxonomic units (OTUs) in an environmental sample. The AmpliconNoise pipeline achieves reduction of pyrosequencing and PCR amplification errors by flowgram and sequence preclustering, respectively (utilizing the PyroNoise and SeqNoise algorithms, respectively) (56). However, studies have shown that the effect of OTU overestimation becomes less severe at greater clustering distances (33, 41). To assess the effect of pyrosequencing errors on detected diversity of denitrification-related structural gene markers, pyrosequencing reads were clustered (i.e., assigned to OTUs) with and without prior denoising of the data. Undenoised sequences were analyzed using the JAguc2 pipeline (http://wwwagak.informatik.uni-kl.de/research/JAguc/) as described previously (46, 47), while denoising of pyrosequencing reads and subsequent analysis of the denoised sequences were conducted using the AmpliconNoise and Qiime pipelines (10, 56). Sequences with ambiguities and errors in primer or bar code sequences were discarded in both types of analysis. For JAguc2 clustering, *narG* as well as *nosZ* sequences shorter than 350 bp and *nirK* as well as *nirS* sequences shorter than 300 bp were excluded from further analysis. More than 130 bases of forward and reverse reads of nitrite reductase genes (i.e., *nirK* or *nirS*) overlapped. Thus, reverse complements of reverse reads were analyzed together with forward reads. DNA sequences were sorted according to their bar codes and primers, and each subset of sequences was clustered by average linkage clustering after pairwise sequence alignment as this clustering method is rather insensitive to pyrosequencing errors (41). Denoised sequences were aligned using the Needleman-Wunsch algorithm and likewise clustered by an average linkage algorithm. Clustering was redone at different threshold similarities to test the stability of the clusters for the original and the denoised sequence sets.

Denoised data sets were used for subsequent analysis of the denitrification-related structural gene markers. Sequences were assigned to OTUs at species-level threshold distances of 33% (*narG* [49]), 17% (*nirK* [P. Depkat-Jakob et al., unpublished data]), 18% (*nirS* [P. Depkat-Jakob et al., unpublished]), or 20% (*nosZ* [49]) and at 3% sequence difference for each gene marker to allow for comparison of higher-resolved diversity in high-quality data sets. The closest relatives of cluster representatives were determined using BLAST (1). Cluster representatives were edited, translated *in silico*, and aligned with reference sequences using the ClustalW algorithm implemented in MEGA, version 5.0 (40). 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, version 5.0. A total of 10,000 bootstrap replicates were calculated to test the stability of tree topologies (60). OTU tables were rarified; i.e., subsampled OTU tables were created by random sampling at sampling depths of 1,000, 3,000, 80, and 1,000 for *narG*, *nirK*, *nirS*, and *nosZ*, respectively (100 iterations), to remove heterogeneity in sequence numbers obtained from the two soil layers and to estimate the uncertainty in diversity measures. Alpha- and beta-diversity measures and statistical tests were calculated with Qiime from such rarified OTU tables at species-level and at 3% threshold distance (i.e., 97% similarity) for each gene.

Quantification of *narG*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes in soil. Quantitative kinetic real-time PCRs (qPCRs) were performed as described previously in six technical replicates (47). Gene copy numbers were corrected for inhibition (73). Inhibition factors ranged from 0.5 to 1.0, 0.2 to 0.4, 0.2 to 1.0, 0.8 to 1.0, and 0.5 to 0.6 for qPCR analyses of *narG*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes, respectively.

Nucleotide sequence accession numbers. The OTU representatives of *narG*, *nirK*, *nirS*, and *nosZ* gene sequences derived from bar-coded amplicon pyrosequencing were deposited at EMBL under accession numbers HE616587 to HE616628. Complete amplicon sequence libraries were deposited in the European Nucleotide Archive (ENA) Sequence Read Archive under study number ERP001096 (http://www.ebi.ac.uk/ena/data/view/ERP001096).

RESULTS

Soil parameters. Soil moisture content of vegetated palsa peat soil was 74% ± 0.3% (Table 1). Soil pH was 4.6 and 4.2 in water extracts from 0- to 20-cm and below-20-cm palsa peat soils, respectively (Table 1). Nitrate and nitrite were below the detection limit (i.e., <0.08 μmol per gram, dry weight [g_{DW}^{-1}], and 0.17 μmol g_{DW}^{-1}, respectively). Ammonium was 125 μM and 106 μM in 0- to 20-cm and below-20-cm palsa peat soils, respectively (Table 1). Dissolved organic carbon was higher in upper than in lower soil layers; total carbon and nitrogen contents were similar in both soil layers, and C/N ratios were 29 and 26 in 0- to 20-cm and below-20-cm soil, respectively (Table 1).

***In situ* gas emissions of vegetated palsa peat soil.** Mean N_2O emissions from unfertilized palsa peat soil were rather small (Fig. 1). However, N_2O fluxes varied from 0.01 to −0.02 μmol of N_2O m^{-2} h^{-1}, indicating that individual palsas can be either sources or sinks for N_2O. Ammonium did not significantly increase N_2O emission from palsa peat soil ($P = 0.6$) (Fig. 1). Initial N_2O emission from nitrate-fertilized palsa peat soil was significantly higher than from unfertilized or ammonium-fertilized palsa peat soil ($P < 0.05$ and $P < 0.01$, respectively), and 1.6 μmol of N_2O m^{-2} accumulated in gas chambers within 3 h following nitrate fertilization (Fig. 1).

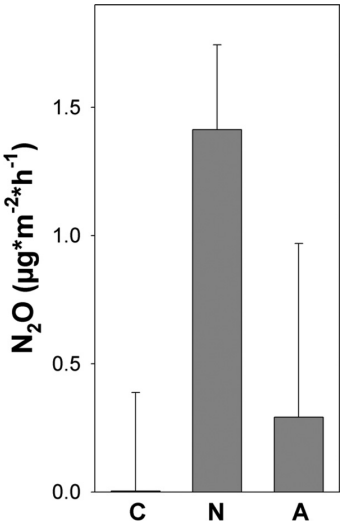

FIG 1 Effect of nitrate and ammonium fertilization on *in situ* fluxes of N_2O from palsa peat soil within 3 h following fertilization (see Materials and Methods for details). Mean values and standard errors of four replicates are shown for unfertilized controls (C) and for treatments fertilized with a solution of 20 mM $NaNO_3$ (N) or 20 mM NH_4Cl (A).

Denitrification activities in palsa peat soil microcosms. N_2O was produced in anoxic microcosms at an *in situ* pH of 4.5 with unsupplemented palsa peat soil from 0 to 20 cm and below 20 cm of depth in the absence of acetylene, and 0.4 and 1 nmol of N_2O g_{DW}^{-1} accumulated during the first 52 h (t_{52}) of incubation, respectively (Fig. 2). N_2O was subsequently consumed in microcosms without acetylene within the next 115 h (t_{167}) of incubation in microcosms with palsa peat soil from below 20 cm, whereas only minor N_2O consumption occurred in microcosms with 0- to 20-cm palsa peat soil ($P = 0.001$ and $P = 0.07$, respectively, for comparison of t_{52} and t_{167}). Below-20-cm palsa peat soil consumed N_2O in atmospheric and subatmospheric concentrations and reduced the concentration from 343 ppb (52 h) to 57 ppb (167 h) (Fig. 2).

Acetylene stimulated the accumulation of N_2O in such microcosms ($P = 0.02$) (Fig. 2); 0.5 and 2 nmol of N_2O g_{DW}^{-1} accumulated in the presence of acetylene within the first 96 h of incubation in microcosms with 0- to 20-cm and below-20-cm palsa peat soils, respectively. N_2O concentrations remained constant after 96 h (t_{96}) of incubation ($P = 0.2$ and $P = 0.3$ for comparison of t_{96} and t_{167}), indicating that all endogenous nitrate or nitrite had been consumed. The amount of N_2O that accumulated in the presence of acetylene indicates that the initial concentration of nitrate/nitrite was at least 0.07 and 0.24 µM (1.1 and 4.0 nmol g_{DW}^{-1}) in 0- to 20-cm and below-20-cm palsa peat soils, respectively. The collective microcosm data suggest that N_2 accounted for approximately 50 to 90% of the gaseous denitrification products at *in situ* pH and nitrate/nitrite concentrations.

Effect of supplemental N oxides on denitrification and N_2O consumption. Nitrate stimulated the production of N_2O in microcosms with palsa peat soil from 0 to 20 cm and below 20 cm of depth ($P \leq 0.03$ for all nitrate concentrations). N_2O production in nitrate-supplemented microcosms was less than 25% of the N_2O production in nitrite-supplemented microcosms (see Fig. S1 in the supplemental material). N_2O production rates in acetylene-amended microcosms with palsa peat soil from both depths increased with increasing nitrate concentrations in the range of 0 to

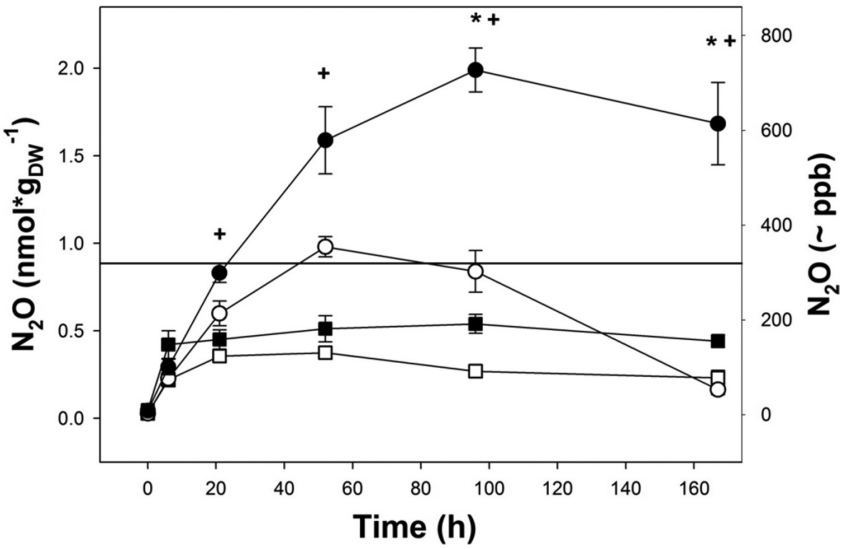

FIG 2 Production and consumption of N_2O by unsupplemented palsa peat soil. Squares and circles represent 0- to 20-cm and below-20-cm palsa peat soils, respectively. Closed and open symbols represent microcosms with and without acetylene, respectively. Mean values and standard errors of three replicates are shown. Time points at which N_2O concentrations in below-20-cm soil with acetylene differed significantly ($P < 0.05$) from N_2O concentrations in below-20-cm soil without acetylene (*) or in 0- to 20-cm palsa peat soil with acetylene (+) are indicated. The horizontal line indicates the atmospheric N_2O concentration (319 ppb).

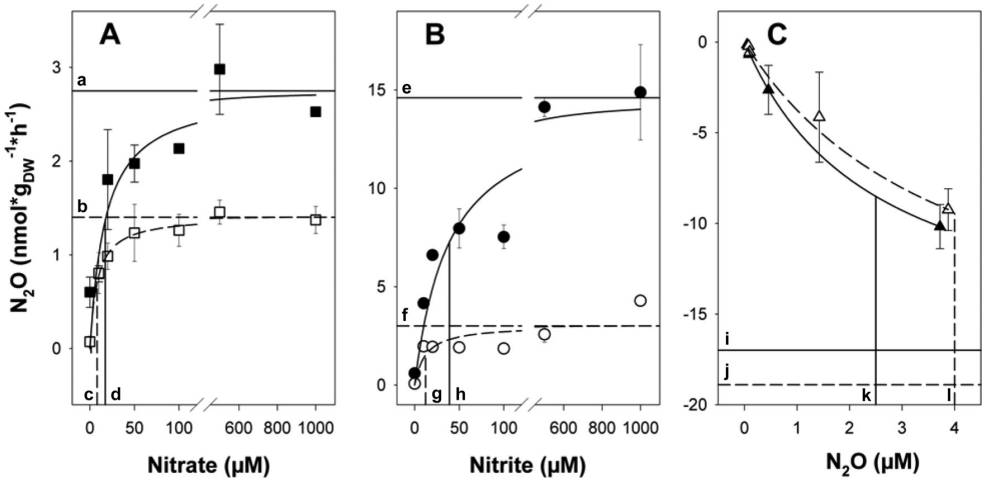

FIG 3 Apparent Michaelis-Menten kinetics of nitrate-dependent (A) and nitrite-dependent (B) N_2O production in the presence of acetylene and N_2O consumption (C) in anoxic palsa peat soil microcosms. Squares, circles, and triangles represent microcosms supplemented with nitrate, nitrite, and N_2O, respectively. Closed and open symbols represent 0- to 20-cm and below-20-cm palsa peat soils, respectively. The *x* axis displays the amount of supplemented (i.e., additional) nitrate, nitrite, or N_2O. Mean values and standard errors of three replicate microcosms are shown. Solid and dashed lines are indicative of 0- to 20-cm and below-20-cm palsa peat soils, respectively. Curves represent Michaelis-Menten fits of the data. Horizontal and vertical lines represent v_{max} and K_m values, respectively. Letters indicate the following values: (i) nitrate-dependent v_{max} (a, 2.75; b, 1.4) and K_m (c, 8.1; d, 17.3), (ii) nitrite-dependent v_{max} (e, 14.6; f, 3.0) and K_m (g, 12.3; h, 39.1), and (iii) N_2O-dependent v_{max} (i, −17.0; j, −18.9) and K_m (k, 2.5; l, 4.0).

50 μM supplemental nitrate and essentially leveled off for nitrate concentrations greater than 50 μM, indicating that denitrifiers in palsa peat are saturated with 50 μM nitrate (Fig. 3A). N_2O production in microcosms with 0- to 20-cm palsa peat soil was about twice as high as in microcosms with below-20-cm palsa peat soil, indicating a greater denitrification potential in the upper soil layer. The ratio of N_2O to combined N_2 and N_2O approximated 50% and 60% for all supplied nitrate concentrations in microcosms with 0- to 20-cm and below-20-cm palsa peat soils, respectively (see Fig. S2), indicating that a substantial portion of N_2O was reduced to N_2 by denitrification.

Supplemental nitrite likewise stimulated the production of N_2O without apparent delay in anoxic microcosms with palsa peat soil ($P \leq 0.02$ for all nitrite concentrations) (see Fig. S1 in the supplemental material). N_2O production rates increased with increasing nitrite concentrations in the range of 0 to 500 and 0 to 20 μM supplemental nitrite for soil from 0 to 20 cm and below 20 cm, respectively (Fig. 3B). N_2O production was higher in 0- to 20-cm soil microcosms than in below-20-cm soil microcosms, underlining the higher denitrification potential of the upper soil layer. The ratio of N_2O to combined N_2 and N_2O varied from 70% to 90% in microcosms with palsa peat soil from both soil layers for all nitrite concentrations (see Fig. S2), indicating that N_2O consumption capacities were in similar ranges when nitrate or nitrite was supplied as an electron acceptor.

Supplemental N_2O was consumed in microcosms with palsa peat soil from both layers (Fig. 3C). Increasing N_2O concentrations stimulated consumption. N_2O consumption rates were similar in microcosms with palsa peat soil from 0 to 20 cm and below 20 cm. Palsa peat soil showed the potential to reduce supplied N_2O to subatmospheric concentrations in such experiments (i.e., approximately 1,000 ppb of N_2O was reduced to 280 ppb within 10 h [data not shown]). Initial N_2O production rates of palsa peat soil microcosms amended with nitrate or nitrite in the presence of acetylene or N_2O consumption rates in the absence of acetylene followed apparent Michaelis-Menten kinetics in both soil layers (Fig. 3). Apparent maximal reaction velocities (v_{max}) were highest for N_2O consumption and higher for nitrite than for nitrate-dependent N_2O production (Fig. 3).

Effect of pyrosequencing and PCR errors on detected diversity of structural gene markers. Denoising of the pyrosequencing reads prior to OTU assignment reduced the number of detected OTUs for all tested gene markers (see Fig. S3 in the supplemental material). The reduction of the OTU number was especially pronounced at small clustering distances (see Fig. S3). At 0% clustering distance, OTU numbers of the original sequence sets were 9 to 30 times higher than OTU numbers of denoised sequence sets. The number of detected OTUs declined rapidly with increasing clustering distance, and similar OTU numbers were obtained for original and denoised sequence sets at clustering distances of 5 to 10% (see Fig. S3).

Phylogenetic analysis of denitrifiers. In total, 28,570 denoised, quality-filtered sequences of the structural gene markers *narG*, *nirK*, *nirS*, and *nosZ* (3,571 ± 751 sequences per gene marker and soil layer on average) were obtained. Forward and reverse reads for *nirK* and *nirS* (amplicon lengths of approximately 470 and 410 bp, respectively) overlapped almost completely and were therefore pooled per gene for further analyses. As overlaps of forward and reverse reads of *narG* and *nosZ* amplicons (amplicon lengths of approximately 670 and 700 bp, respectively) were not sufficient, they were analyzed separately. More than 99% of analyzed sequences generated from amplicons of a certain gene-specific (i.e., *narG*, *nirK*, *nirS*, and *nosZ*) primer set were affiliated with the target gene. Coverage levels of all amplicon libraries were greater than 99% when sequence dissimilarity thresholds of 33%, 17%, 18%, and 20% for *narG*, *nirK*, *nirS*, and *nosZ*, respectively,

TABLE 2 Analysis of *narG*, *nirK*, *nirS*, and *nosZ* derived from palsa peat soil based on two distinct threshold similarities utilized for calling OTUs

Gene marker (read)	Threshold similarity (%)	Soil layer (cm)	No. of sequences	Good's coverage (%)[a]	No. of OTUs observed	No. of OTUs estimated[b]	H^c	E^d
narG (forward)	67	0 to 20	2,956	100.0	4	4 (4–4)	0.56	0.28
		>20	1,925	100.0	3	3 (3–3)	0.81	0.51
	97	0 to 20	2,956	92.1	165	284 (232–375)	3.39	0.46
		>20	1,925	94.7	67	157 (103–293)	2.21	0.36
narG (reverse)	67	0 to 20	3,541	100.0	2	2 (2–2)	0.01	0.01
		>20	1,774	100.0	1	1 (1–1)	0.00	n.a.
	97	0 to 20	3,541	97.0	195	417 (325–576)	3.22	0.42
		>20	1,774	97.9	73	117 (92–172)	2.47	0.40
nirK	83	0 to 20	8,313	100.0	4	4 (4–4)	1.49	0.75
		>20	3,299	99.9	8	9 (8–16)	0.71	0.24
	97	0 to 20	8,313	99.9	34	40 (35–61)	2.33	0.46
		>20	3,299	99.4	48	67(54–105)	1.72	0.31
nirS	82	0 to 20	85	100.0	4	4 (4–4)	0.78	0.25
		>20	833	99.6	12	15 (12–37)	2.39	0.75
	97	0 to 20	85	97.5	7	8 (7–15)	1.09	0.39
		>20	833	97.4	46	85 (59–159)	3.36	0.61
nosZ (forward)	80	0 to 20	1,563	99.9	5	5 (5–5)	1.30	0.56
		>20	1,044	99.9	6	6 (6–6)	1.47	0.58
	97	0 to 20	1,563	99.5	32	38 (33–59)	2.24	0.45
		>20	1,044	99.3	25	29 (26–44)	2.37	0.51
nosZ (reverse)	80	0 to 20	1,856	100.0	4	4 (4–4)	1.27	0.63
		>20	1,381	99.9	6	6 (6–6)	1.45	0.56
	97	0 to 20	1,856	99.6	29	33 (30–51)	2.03	0.42
		>20	1,381	99.5	27	32 (28–55)	2.32	0.49

[a] Percent library coverage (Good's coverage): $C = (1 - n_s/n_t) \times 100$, where n_s is the number of OTUs that occur only once and n_t is the total number of sequences.
[b] Chao1 richness estimate with upper and lower 95% confidence intervals given in parentheses.
[c] Shannon diversity index.
[d] Species evenness.

were utilized for calling OTUs (Table 2). The mean coverage was 98% (ranging from 92 to 100%) when OTUs were called at a 3% sequence dissimilarity threshold, indicating that the number of quality-filtered sequences generated was sufficient for subsequent analyses.

Forward reads of *narG* amplicons yielded more OTUs than reverse reads although similar numbers of sequences were obtained, indicating that the utility of the 3′ ends of *narG* amplicons is higher for diversity analysis than 5′ ends (Table 2; see also Table S1 in the supplemental material). However, results obtained from reverse reads show similar overall trends (Table 2; see also Table S1 and Fig. S4 and S5 in the supplemental material). Thus, information presented below refers to forward reads only. In total, *narG* sequences were assigned to four species-level OTUs (Table 2). Four and three OTUs were detected in palsa peat soil from 0 to 20 cm and below 20 cm, respectively. OTU representatives were only distantly related to publicly available *narG* sequences (sequence dissimilarities ranged from 16 to 33%), indicating phylogenetic novelty. OTU 1 affiliated with actinobacterial *narG*, while OTU 2 affiliated with alphaproteobacterial *narG* (Table 3; see also Fig. S4). OTUs of *narG* from palsa peat soil were related to *narG* genes of uncultured bacteria from upland and fen soil as well as from sediments and to *narG* genes of *Methylocella sylvestris*, *Actinosynnema mirum*, *Rubrobacter xylanophilus*, *Oligotropha carboxidovorans*, and *Desulfurispirillum indicum* (Table 3; see also Fig. S4). OTU 1 dominated *narG* in both layers and accounted for 87% and 76% of the sequences in 0- to 20-cm and below-20-cm soil, respectively, while OTU 2 accounted for 13% and 24% of the sequences in 0- to 20-cm and below-20-cm soil, respectively (Table 3; see also Fig. S4). Rarefaction analysis (i.e., random subsampling of each amplicon library) was applied to minimize the potential bias introduced by varying the numbers of sequences per amplicon library. OTUs called at species-level threshold distances of 33% were split into several OTUs at a 3% threshold distance in original and rarified data sets (Fig. 4 and Table 2; see also Table S1 in the supplemental material). In rarified data sets, *narG* sequences were likewise assigned to four OTUs at a 33% species-level threshold distance, which were split into 111 3%-OTUs (at a 3% threshold distance); 85, 22, 2, and 1 3%-OTUs were derived from sequences of 33%-OTUs 1, 2, 3, and 4, respectively. *narG* community composition differed between soil layers at a 3% threshold distance; 3%-OTU 1.1 dominated *narG* in 0- to 20-cm palsa peat soil, while 3%-OTU 1.2 dominated *narG* in below-20-cm palsa peat soil (Fig. 4). Differences in observed and estimated OTU numbers were rather insignificant at the 33% species-level threshold distance when rarified data sets were utilized (see Table S1). Shannon diversity indices, species evenness, and Chao1 richness estimates of *narG* from 0- to 20-cm and below-20-cm palsa peat soils were significantly different at a 3% threshold difference; alpha-diversity of *narG* tended to be higher in soil

TABLE 3 Amino acid identities of *in silico* translated OTU representatives of denitrification-associated genes retrieved from acidic palsa peat soil to closely related sequences

Gene (read)[b]	OTU no. (accession no.)	Closest relative: Name (accession no.)	Closest relative: Identity (%)[a]	Closest cultured relative: Name (accession no.)	Closest cultured relative: Identity (%)[a]	Relative abundance of OTUs in amplicon libraries (%): 0 to 20 cm	Relative abundance of OTUs in amplicon libraries (%): Below 20 cm
narG (forward)	1 (HE616587)	Uncultured bacterium (AY955190)	82	*Streptomyces coelicolor* A3(2) (NC 003888)	78	87.3	75.8
	2 (HE616588)	*Oligotropha carboxidovorans* OM 5 (CP001196)	84	*Oligotropha carboxidovorans* OM 5 (CP001196)	84	12.6	24.1
	3 (HE616589)	Uncultured bacterium (FJ556609)	68	*Oceanithermus profundus* DSM 14977 (CP002361)	64	0.03	0.1
	4 (HE616590)	*Rubrobacter xylanophilus* DSM 9941 (CP000386)	72	*Rubrobacter xylanophilus* DSM 9941 (CP000386)	72	0.07	0
narG (reverse)	1 (HE616591)	Uncultured bacterium (AY955190)	86	*Actinosynnema mirum* DSM 43827 (CP001630)	75	99.9	100
	2 (HE616592)	*Nitrobacter hamburgensis* X14 (NC 007964)	71	*Nitrobacter hamburgensis* X14 (NC 007964)	71	0.1	0
nirK[c]	1 (HE616593)	Uncultured bacterium (DQ783977)	97	*Bradyrhizobium* sp. strain ORS278 (NC 009445)	84	57.6	0.33
	2 (HE616594)	Uncultured bacterium (EF623499)	78	*Rhodopseudomonas palustris* BisA53 (CP000463)	76	12.8	82.9
	3 (HE616595)	Uncultured bacterium (DQ304355)	96	*Bradyrhizobium* sp. strain GSM-467 (FN600568)	85	26.6	0.09
	4 (HE616596)	Uncultured bacterium (EU790857)	80	*Alcaligenes* sp. strain I (DQ108983)	79	0	16.4
	5 (HE616597)	Uncultured bacterium (EU790858)	92	*Bosea* sp. MF18 (EF363545)	92	2.9	0
	6 (HE616598)	Uncultured bacterium (DQ783907)	82	*Bradyrhizobium* sp. strain D203a (AB480454)	76	0	0.12
	7 (HE616599)	Uncultured bacterium (DQ783888)	85	*Bradyrhizobium* sp. strain BTAi1 (NC 009485)	81	0	0.06
	8 (HE616600)	Uncultured bacterium (EF623499)	77	*Methylobacterium* sp. strain R-25207 (AM230850)	75	0	0.03
	9 (HE616601)	Uncultured bacterium (EU790858)	79	*Rhizobium etli* CFN 42 (NC 007766)	78	0	0.03
nirS[d]	1 (HE616602)	Uncultured bacterium (GQ443982)	92	*Rubrivivax gelatinosus* (AB536930)	80	0	36.2
	2 (HE616603)	Uncultured bacterium (GU393200)	86	*Pseudomonas* sp. strain I-Bh25-14 (FN555560)	75	0	31.6
	3 (HE616604)	Uncultured bacterium (GU393229)	87	*Bradyrhizobium* sp. strain TSA26 (AB542313)	84	86.3	8.5
	4 (HE616605)	Uncultured bacterium (GU393213)	88	*Thiobacillus denitrificans* ATCC 25259 (CP000116)	76	0	9.0
	5 (HE616606)	Uncultured bacterium (HM438800)	95	*Dechlorospirillum* sp. strain I-Bh37-22 (FN555562)	80	0	5.3
	6 (HE616607)	Uncultured bacterium (DQ676123)	85	*Thiobacillus denitrificans* ATCC 25259 (CP000116)	80	0	4.6
	7 (HE616608)	Uncultured bacterium (DQ676123)	84	*Aromatoleum aromaticum* EbN1 (NC 006513)	78	0	3.1
	8 (HE616609)	Uncultured bacterium (GU393183)	78	*Cupriavidus* sp. strain TSA25 (AB542312)	74	0	0.84
	9 (HE616610)	Uncultured bacterium (GU393076)	92	*Bradyrhizobium* sp. strain TSA1 (AB542304)	86	6.3	0
	10 (HE616611)	Uncultured bacterium (GU393229)	87	*Rhodanobacter* sp. strain D206a (AB480490)	85	5.0	0.12
	11 (HE616612)	Uncultured bacterium (AY583422)	89	*Cupriavidus metallidurans* CH34 (CP000352)	77	0	0.48
	12 (HE616613)	Uncultured bacterium (GU393076)	94	*Bradyrhizobium* sp. strain TSA1 (AB542304)	87	2.5	0
	13 (HE616614)	Uncultured bacterium (DQ303103)	84	*Rhodanobacter* sp. strain D206a (AB480490)	82	0	0.12
	14 (HE616615)	Uncultured bacterium (DQ676123)	82	*Cupriavidus* sp. strain N24 (AB480486)	78	0	0.12
nosZ (forward)	1 (HE616616)	Uncultured bacterium (FN430557)	96	*Oligotropha carboxidovorans* OM 5 (CP001196)	85	51.1	51.9
	2 (HE616617)	Uncultured bacterium (FN430533)	91	*Burkholderia pseudomallei* K96243 (BX571965)	90	43.4	37.6
	3 (HE616618)	Uncultured bacterium (FN430550)	87	*Pseudomonas brassicacearum* PD 5 (DQ377777)	82	3.8	8.1
	4 (HE616619)	*Alcaligenes faecalis* A15 (AF361795)	95	*Alcaligenes faecalis* A15 (AF361795)	95	1.5	1.0
	5 (HE616620)	*Azospirillum* sp. TSO41-3 (AB545691)	83	*Azospirillum* sp. strain TSO41-3 (AB545691)	83	0	1.2
	6 (HE616621)	Uncultured bacterium (FN430537)	90	*Azospirillum brasilense* SpT60 DSM 2298 (AF361792)	90	0.06	0
	7 (HE616622)	Uncultured bacterium (FN430542)	89	*Burkholderia pseudomallei* K96243 (BX571965)	87	0	0.1
nosZ (reverse)	1 (HE616623)	Uncultured bacterium (FN430557)	95	*Rhodopseudomonas palustris* HaA2 (CP000250)	81	52.3	52.1
	2 (HE616624)	Uncultured bacterium (FN430533)	91	*Azospirillum lipoferum* (AF361793)	85	42.4	36.5
	3 (HE616625)	Uncultured bacterium (FN430533)	87	*Mesorhizobium* sp. strain TSA41b (AB542285)	79	4.7	10.0
	4 (HE616626)	*Ralstonia solanacearum* GMI1000 (AL646053)	85	*Ralstonia solanacearum* GMI1000 (AL646053)	85	0.59	0.36
	5 (HE616627)	Uncultured bacterium (FJ209438)	83	*Mesorhizobium* sp. strain D246 (AB480521)	76	0	0.94
	6 (HE616628)	*Bradyrhizobium japonicum* USDA 110 (BA000040)	88	*Bradyrhizobium japonicum* USDA 110 (BA000040)	88	0	0.07

[a] Determined after alignment in MEGA, version 5.0.
[b] Where indicated, analyses were based on forward or reverse reads.
[c] Analyses included forward and reverse complements of reverse reads; minimal sequence overlaps of forward and reverse reads were 130 bases.
[d] Analyses included forward and reverse complements of reverse reads; minimal sequence overlaps of forward and reverse reads were 190 bases.

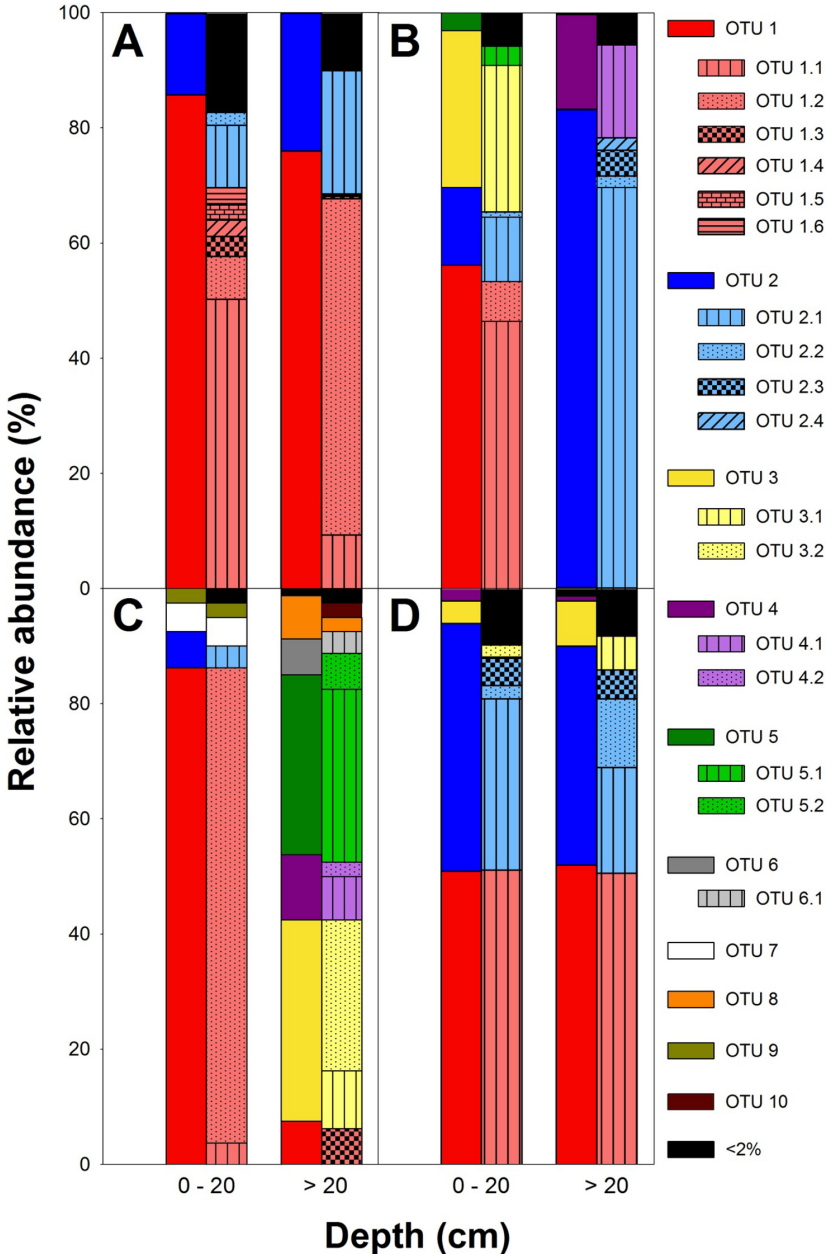

FIG 4 Relative abundances of OTUs derived from *narG* forward reads (A), *nirK* (B), *nirS* (C), and *nosZ* forward reads (D) retrieved from palsa peat soil at species level (left bars) and at 3% (right bars) threshold distances from rarified data sets. OTUs were rarified at sampling depths of 1,000, 3,000, 80, and 1,000 for *narG*, *nirK*, *nirS*, and *nosZ*, respectively. OTUs called at species-level threshold distances were enumerated 1 through 10; subclusters of those OTUs called at 3% threshold distance were enumerated 1.1 through 6.1. All OTUs that had relative abundances below 2% in both layers were grouped. Please note that the same color coding for different structural genes does not indicate whether such genes were derived from the same organisms.

from 0- to 20-cm soil than in soil from below 20 cm (see Table S1). Beta-diversity measures showed larger differences in community composition at 3% than at the 33% threshold distance (see Table S1).

nirK genes were assigned to nine species-level OTUs. Four and eight OTUs were detected in palsa peat soils from 0 to 20 cm and from below 20 cm, respectively (Table 2). OTUs 1 and 2 dominated *nirK* in palsa peat soils from 0 to 20 cm and below 20 cm,

respectively (about 58% and 83%, respectively). OTU 3 accounted for 27% of *nirK* in palsa peat soil from 0 to 20 cm but for only 0.1% of *nirK* from below-20-cm palsa peat soil, while OTU 4 accounted for 16% of *nirK* from below-20-cm palsa peat soil but was not detected in 0- to 20-cm palsa peat soil (see Fig. S6 in the supplemental material). Sequence dissimilarities of OTU representatives to publicly available *nirK* sequences ranged from 3 to 25%, indicating the presence of new and known denitrifiers in palsa peat soil (Table 3). All *nirK* detected in palsa peat soil affiliated with alphaproteobacterial *nirK*. OTUs 2, 4, and 8 were related to *nirK* of uncultured bacteria and more distantly related to *nirK* of *Methylobacterium* sp., while OTUs 1 and 3 were related to *nirK* of *Bradyrhizobium* sp. and *Rhodopseudomonas* sp. (Table 3; see also Fig. S6). In rarified data sets, *nirK* sequences were likewise assigned to 9 OTUs at a 17% species-level threshold distance which were split into 67 3%-OTUs; 20, 30, 2, 4, and 3 OTUs were derived from sequences of 17%-OTUs 1, 2, 3, 4, and 5, respectively. Alpha-diversity measures (i.e., Shannon diversity index, species evenness, and Chao1) differed significantly between the two soil layers and suggested a higher diversity of *nirK* in soil from 0 to 20 cm than from soil below 20 cm when rarified data sets were utilized (see Table S1). Beta-diversity measures indicated little overlap of the two communities, confirming the observed OTU patterns (Fig. 4; see also Table S1,).

Fourteen species-level OTUs of *nirS* were detected in the original data set (Table 2). Four and 12 OTUs were detected in 0- to 20-cm and below-20-cm palsa peat soils, respectively (Table 2). *nirS* of palsa peat soil from 0 to 20 cm was dominated by OTUs affiliated with alphaproteobacterial *nirS*, while *nirS* genes from soil below 20 cm were dominated by *nirS* affiliated with betaproteobacterial *nirS* (Table 3; see also Fig. S7 in the supplemental material). Many OTUs were related to *nirS* of uncultured wetland or marine sediment bacteria and distantly related to *nirS* of *Bradyrhizobium* sp. (0 to 20 cm) and other *Alphaproteobacteria* and to *Azoarcus tolulyticus* (below 20 cm) (Table 3; see also Fig. S7). Sequence dissimilarities of OTU representatives to publicly available *nirS* sequences ranged from 5 to 22%, indicating the presence of phylogenetically new denitrifiers in palsa peat soil. In rarified data sets, *nirS* sequences were assigned to 10 OTUs (rather than 14 OTUs for the original data set) at an 18% species-level threshold distance (see Table S1) since the low number of sequences obtained for 0- to 20-cm peat soil limits the number of sequences analyzed from soil below 20 cm of depth. Such 18%-OTUs were split into 19 3%-OTUs; 4, 3, 2, 2, 2, and 2 OTUs were derived from sequences of 18%-OTUs 1, 2, 3, 4, 5, and 6, respectively. The *nirS* community composition differed between soil layers at 18% and 3% threshold distances, and *nirS* genes from OTU 1 were assigned to different 3%-OTUs in the upper and lower soil layers (Fig. 4). Alpha-diversity measures were significantly higher in below-20-cm soil than in 0- to 20-cm soil at both threshold distances; beta-diversity measures indicated little overlap of the two communities when rarified data sets were utilized, as was observed for *nirK* (Fig. 4; see also Table S1).

nosZ forward reads were assigned to seven species-level OTUs. Five and six OTUs were detected in palsa peat soil from 0 to 20 cm and from below 20 cm, respectively (Table 2). OTUs 1 and 2 dominated *nosZ* amplicon libraries of palsa peat soil from both soil layers (see Fig. S8 in the supplemental material). Sequence dissimilarities of OTU representatives to publicly available *nirS* sequences ranged from 4 to 18%, indicating the presence of phylogenetically new denitrifiers capable of N_2O consumption. Essentially all *nosZ* genes were affiliated with alpha- and betaproteobacterial *nosZ* and clustered with *nosZ* of wetland and upland soils, as well as *Bradyrhizobium japonicum* and *Azospirillum lipoferum* (Table 3; see also Fig. S8). *nosZ* reverse reads yielded similar results (Table 2; see also Fig. S9 in the supplemental material). In rarified data sets, *nosZ* sequences formed 6 OTUs at a 20% species-level threshold distance which were split into 39 3%-OTUs; 24, 7, and 5 3%-OTUs were derived from OTUs 1, 2, and 3, respectively. *nosZ* community composition was similar in both soil layers at 20% and 3% threshold distances. However, minor differences in the 3%-OTU composition of 20%-OTUs 2 and 3 were detected (Fig. 4). Alpha-diversity was in general marginally higher in below-20-cm palsa peat soil; beta-diversity measures indicated a higher proportion of shared OTUs and a more common phylogeny than observed for the other gene markers when rarified data sets were utilized (see Table S1).

TABLE 4 Copy numbers of denitrification-associated genes in palsa peat soil

Gene	Copy no. by soil layer			
	From 0–20 cm		Below 20 cm	
	Per 16S rRNA gene (%)[a,g]	Per ng of DNA	Per 16S rRNA gene (%)[b,g]	Per ng of DNA
narG[c]	$(8.9 \pm 0.5) \times 10^{-1}$	$(1.5 \pm 0.1) \times 10^{4}$	$(4.9 \pm 0.4) \times 10^{0}$	$(5.1 \pm 0.2) \times 10^{4}$
nirK[d]	$(1.2 \pm 0.3) \times 10^{-3}$	$(1.4 \pm 0.3) \times 10^{0}$	$(3.3 \pm 0.9) \times 10^{-4}$	$(8.6 \pm 2.3) \times 10^{-1}$
nirS[e]	$(1.6 \pm 0.2) \times 10^{-1}$	$(2.5 \pm 0.3) \times 10^{2}$	$(1.4 \pm 0.3) \times 10^{-2}$	$(3.7 \pm 0.7) \times 10^{1}$
nosZ[f]	$(2.7 \pm 0.5) \times 10^{-3}$	$(4.3 \pm 0.8) \times 10^{1}$	$(8.4 \pm 1.0) \times 10^{-3}$	$(8.8 \pm 0.8) \times 10^{1}$

[a] 16S rRNA gene copy numbers were $(1.6 \pm 0.1) \times 10^{6}$ per ng DNA.
[b] 16S rRNA gene copy numbers were $(1.0 \pm 0.1) \times 10^{6}$ per ng DNA.
[c] Assay validation for *narG:* detection limit, 10^{1} copies; PCR efficiency, 108.2%; standard curve r^2, 0.993.
[d] Assay validation for *nirK:* detection limit, 10^{0} copies; PCR efficiency, 106.0%; standard curve r^2, 0.993.
[e] Assay validation for *nirS:* detection limit, 10^{1} copies; PCR efficiency, 97.5%; standard curve r^2, 0.993.
[f] Assay validation for *nosZ:* detection limit, 10^{1} copies; PCR efficiency, 101.5%; standard curve r^2, 0.989.
[g] Assay validation for 16S rRNA: detection limit, 10^{1} copies; PCR efficiency, 95.9%; standard curve r^2, 0.994.

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 Materials and Methods). Copy numbers of *narG* accounted for 1 and 5% of bacterial 16S rRNA gene copy numbers in 0- to 20-cm and below-20-cm palsa peat soils, respectively (Table 4). Copy numbers of *narG*, *nirK*, *nirS*, and *nosZ* were essentially in the same order of magnitude in both soil layers although gene ratios of copy numbers from 0- to 20-cm soil to below-20-cm soil were 0.3, 1.6, 6.8, and 0.5 for *narG*, *nirK*, *nirS*, and *nosZ*, respectively (Table 4), suggesting a tendency for nitrite reductase genes to be more abundant in upper than lower soil layers. *nirS* copy numbers were approximately 100 times higher than *nirK* copy numbers (Table 4). *nosZ* copy numbers were in the same magnitude as *nirS* copy numbers in below-20-cm palsa peat soil while the *nirS* copy numbers were about 10 times higher than *nosZ* copy numbers in 0- to 20-cm palsa peat soil (Table 4).

DISCUSSION

Denitrifiers in palsa peat soil as a source and sink for N_2O. N_2O emission was stimulated *in situ* with nitrate rather than ammo-

nium, and new as well as diverse denitrification-associated genes were abundant, highlighting the importance of denitrifiers for N_2O fluxes in acidic palsa peats (Fig. 1 and Table 2; see also Table S1 and Fig. S4 to S9 in the supplemental material). Although the N_2O flux measurements were performed only once, N_2O emission rates were in the range of those observed for previously analyzed vegetated palsas that were characterized by similar C/N ratios (Table 1) (45). However, unvegetated palsas with C/N ratios similar to those of the vegetated palsa peat emit large amounts of N_2O; such emissions are in the same range as determined for agricultural and tropical soils (45, 58, 71). Thus, although the C/N ratio is recognized as a strong determinant for N_2O emission, it is not the sole determinant for N_2O emission capabilities of peatlands (38). Unvegetated palsas are characterized by a low ratio of ammonium to nitrate, indicating a good nitrification-derived nitrate supply to denitrifiers (45). In contrast, high ratios of ammonium to nitrate in vegetated tundra peatlands as observed also in Skalluvaara palsa peat (Table 1) indicate restricted nitrification activity and thus low nitrate availability (45). Copy numbers of *narG* and of *nirK* and *nirS* were at the lower end or in the same range of those detected in soil or other permafrost-affected acidic peatlands, respectively (9, 14, and 47), demonstrating that nitrate reducers and denitrifiers were abundant. Nitrate and nitrite stimulated denitrification in palsa peat soil without apparent delay at ratios of 60 to 90% of N_2O to total N gas highlighting N-oxide limitation of denitrifiers (Fig. 3; see Fig. S1 and S2 in the supplemental material), indicating that denitrifiers in palsa might become a significant source of N_2O when nitrate is available. Such data (i) suggest that denitrification rather than nitrification is the major source of N_2O in Skalluvaara palsa peat soil, (ii) indicate that denitrifiers are prone to react to nitrate supply, and (iii) are in line with the hypothesis that N_2O emissions from vegetated permafrost-affected peatlands are low due to nitrification-limited nitrate supply to denitrifiers (45).

Many nitrate-limited denitrifiers hosting N_2O reductases utilize N_2O as a terminal electron acceptor under anoxic conditions (34, 76, 78). Acidic wetlands including vegetated palsa peats represent temporary sinks of atmospheric N_2O, and N_2O consumption of atmospheric levels was detected *in situ* at the Skalluvaara palsa peat site and in microcosms (Fig. 1 and 2) (11, 24, 39, 45, 48). The acetylene inhibition technique indicated that N_2 was a denitrification end product in unsupplemented palsa peat soil microcosms (Fig. 2). Nitrous oxide reductase gene copy numbers were present and low compared to levels in less acidic soils and in the same range as those of acidic tundra soil (9, 14, and 47). The ratio of detected nitrite reductase to N_2O reductase genes approximated 10 and 1 in upper and lower soil layers, respectively (Table 4), suggesting that detected denitrifiers capable of N_2O production outnumbered those capable of N_2O consumption in the upper layer of palsa peat. The ratio of nitrite reductase genes to N_2O reductase genes is highly variable in soils, and often nitrite reductase copy numbers largely exceed N_2O reductase copy numbers (14, 28, 47). Such findings are in agreement with the fact that only approximately two-thirds of the genomes of cultured denitrifiers harbor *nosZ* (35). However, *nosZ* copy numbers in the lower soil layer were similar to those of the upper soil layer, indicating similar genetic potentials to consume N_2O. Such genetic potentials were essentially reflected by the maximal reaction velocities, likewise suggesting (i) higher N_2O production in upper than lower soil layers and (ii) similar N_2O consumption capacities in upper and lower soil layers (Fig. 3). Interestingly, maximal reaction velocities for N_2O consumption exceeded those for N_2O production, and ratios of maximal reaction velocities for N_2O production to N_2O consumption ranged from 0.1 to 0.9 (Fig. 3). Ratios in the same range were observed for acidic to pH-neutral soils in Finland, Sweden, and Germany and demonstrate a high relative capacity of the acidic Skalluvaara peat soil to consume N_2O (30). Given equilibrium concentrations of dissolved N_2O in the range of 10 nM N_2O when 380 ppb of N_2O in the atmosphere is assumed and dissolved N_2O concentrations of up to 8 nM in pore water of a German fen (23), the data indicate that N_2O will be used as a terminal electron acceptor by acid-tolerant palsa peat denitrifiers, especially when the electron acceptors nitrate and nitrite are depleted, allowing palsa peats to act as temporary N_2O sinks.

New denitrifier communities in palsa peat soil differ with depth. Denitrifier diversity was analyzed by bar-coded amplicon pyrosequencing of multiple denitrification-associated structural genes (Table 2; see also Table S1 in the supplemental material). Variability among technical replicate bar-coded amplicon pyrosequencing analyses of 16S rRNA genes might be rather high when OTUs are called at a 97% sequence similarity threshold, and only 30 to 65% of estimated total OTUs are detected, suggesting that bar-coded amplicon pyrosequencing underestimates shared taxa when coverage is low (74). Such variabilities are attributed to primer biases originating from the utilization of primers with different bar codes and might be minimized by applying two-step PCR approaches (5). However, average coverage levels of 99% as obtained for denitrification-associated structural genes (Table 2), utilization of two sequence distances for calling OTUs as well as forward and reverse reads, and rarifying data sets for determining beta-diversity (see Table S1) suggest that artifacts in the denitrification-associated gene-based estimates of denitrifier beta-diversity were minimal. Within the limitations of target gene detectability by the utilized primer sets, tagged amplicon pyrosequencing-based approaches are well suited for capturing the alpha-diversity of detectable microbial communities when rigorous sequence correction algorithms are applied (see Materials and Methods) even if coverage levels are well below 99% (74).

Most detected *narG* sequences from Skalluvaara palsa peat clustered within the *Actinobacteria* and were related to *narG* of *Actinosynnema* sp. (Table 3; see also Fig. S4 in the supplemental material). *Actinosynnema* sp.-related actinobacterial *narG* genes were the dominant group in unturbated peat plateau tundra and cryoturbated peat circles of Russian permafrost-affected peat soil, indicating that this group is common in the nitrate-reducer community of permafrost-affected peatland soils (47). Actinobacterial *narG* genes are predominant in Canadian permafrost soil, and *Actinobacteria* is the dominant taxon in the active layer in Canadian arctic permafrost soil, thus highlighting the importance of *Actinobacteria* in the nitrate reducer community of palsa peats and other permafrost systems (47, 65).

Proteobacteria are abundant in permafrost soils, which is in line with the affiliation of detected *nirK*, *nirS*, and *nosZ* from palsa peat with *Proteobacteria* (see Fig. S6 to 8 in the supplemental material) (65). Since the phylogenies of *nirS* and *nosZ* are basically congruent with 16S rRNA-based phylogenies (29, 35, 49), *Proteobacteria* are suggested as important denitrifiers in permafrost environments. Pronounced differences were detected in the diversity of the nitrite reductase genes between soil layers for *nirK* and *nirS*. Indeed, nitrite reductase communities reflect differences in environmental conditions more strongly than nitrate or N_2O reduc-

tase communities (6, 9, 14, 47). Copy numbers of *nirS* genes (i.e., the predominant detected nitrite reductase genes) were significantly higher in the upper soil layer than in the lower soil layer (Table 4). Such a trend was reflected in the nitrite-dependent kinetic parameters, likewise suggesting dissimilar denitrifier communities in upper and lower soils (Fig. 3). Such findings are supported by recent studies demonstrating a positive correlation of *nirS* and 16S rRNA genes with denitrification potentials (14, 51). Although current PCR-based analyses of nitrate reducer communities including denitrifiers underestimate the real diversity in the environment due to the selectivity of the primers utilized, the collective data represent a minimal estimate of denitrifier diversity and abundance and indicate abundant, diverse, acid-tolerant, and uncultured nitrate reducers including denitrifiers in palsa peat soil (25, 29, 36). The composition of *nirK* and *nirS* OTUs of Skalluvaara palsa peat soil was similar to the community composition in unturbated permafrost-affected tundra peat soil, while especially *nirS* community composition of cryoturbated peat circle soil differed strongly from both unturbated tundra and palsa peat soil (47), indicating site-specific differences in permafrost-nitrite reducer communities that might relate to observed differences in N_2O emissions.

The community composition of detected N_2O-consuming denitrifiers, as indicated by *nosZ* sequence analysis, clearly differed between Finnish palsa peat and Russian tundra peat soil, as *nosZ* genes from Finnish palsa peat soil were dominated by sequences related to *Bradyrhizobium* and *Azospirillum*, while *nosZ* genes from Russian tundra peat soil are dominated by *Mesorhizobium*-related *nosZ* genes (47). N_2O emissions from Russian cryoturbated peat circles are much higher than from palsa peat soil, and ratios of N_2O to N_2 are likewise higher in Russian peat soil (47), indicating that *Mesorhizobium*-related denitrifiers in Russian peat soil, which are absent in Skalluvaara peat soil, might be candidates for key denitrifiers impacting the N_2O consumption capability of permafrost-affected soils.

Predominance of detected *nirS*-type rather than *nirK*-type denitrifiers. Detected genes indicative of cytochrome cd_1-dependent nitrite reductases (i.e., *nirS*) outnumbered genes indicative of copper-dependent nitrite reductases (i.e., *nirK*) by approximately 2 orders of magnitude (Table 4), suggesting that *nirS*-type denitrifiers are important for denitrification in palsa peat. Substantially higher copy numbers of *nirS* than *nirK* are detected in certain (semi-)aquatic habitats and acidic soils by a range of different primers, suggesting that *nirS*-type denitrifiers are well adapted to low pH and high water content (3, 22, 32, 47). However, the primer systems for *nirK* and *nirS* used in this study and many others target mainly diverse *Proteobacteria* excluding recently described *nirK*-hosting acid-tolerant *Rhodanobacter* species (25, 26, 68). Thus, conclusions are limited to detectable proteobacterial *nirK*-type and *nirS*-type denitrifiers. Niche differentiation rather than competitive exclusion is hypothesized as a mechanism for the occurrence of *nirS*-type and *nirK*-type denitrifiers detected with currently available primers (9, 18, 37). Abundance of such *nirS*-type denitrifiers is impacted by pH, nitrate, soil moisture, and manganese content, and a positive correlation of *nirS* abundance and soil moisture was revealed (9, 14, 15, 18, 27), suggesting that moisture content and the low pH might favor putative proteobacterial *nirS*-type denitrifiers in palsa peat soils.

Denitrifier community composition and regulation of N_2O fluxes. The community composition of soil denitrifiers impacts on denitrification capacities, on the response of denitrification to soil pH, and thus on the release of N_2O into the atmosphere (7, 16, 30). Acidic pH impairs the activity of N_2O reductase, most likely by posttranscriptional effects (4, 43). Cells of the neutrophilic model denitrifier *Paracoccus denitrificans* grown under pH-neutral conditions that were subjected to N_2O reduction assays display only 50% of N_2O reduction rates at pH 6.1 compared to a pH of 7.5. Transcription of *nosZ* in cells grown at pH 6.1 is essentially not affected compared to those at pH 7.5, while N_2O reduction capabilities are severely impaired by the low pH (4). Soil denitrifiers likewise show lower N_2O reduction activities at pH 6.1 than at pH 8.0 but higher transcriptional activities of *nosZ* at the lower pH, which might be viewed as an attempt to partially compensate for N_2O reduction activities (43). Thus, current knowledge suggests that the posttranscriptional effects at low pH impairing N_2O reduction activities include negative effects on (i) the activity of the mature N_2O reductase enzyme and (ii) the export/and or assembly of premature N_2O reductases (76).

Indeed, the relative proportion of N_2O in total N gases is higher in acidic than in pH-neutral soil (64). However, denitrifiers adapted to a low pH of their environment occur in certain habitats (16, 48, 50). Some acid-tolerant strains of *Rhodanobacter* sp. (e.g., *Rhodanobacter denitrificans* and *Rhodanobacter thiooxydans*) are capable of complete denitrification to N_2 at pH 4 (i.e., N_2O reduction) and are associated with denitrification in acidic subsurface environments (25, 26, 55, 70). Such findings, half-lives of mature model N_2O reductases during N_2O turnover of approximately 5 min (as reviewed in reference 76), and high N_2O reduction capabilities in palsa peat soil microcosms at pH 4 for greater than 8 h under anoxic conditions (Fig. 3C) argue in favor of a robust acid-tolerant N_2O reducing capability of palsa peat organisms rather than measuring the activity of N_2O reductases that had been expressed in pH-neutral microsites.

Soil pH exerts strong effects on the community composition of soil bacteria, and phylotypic diversity is lower in acidic than in pH-neutral soils (19, 42). Such pH effects are in line with putative new denitrifiers that were detected in palsa peats and other acidic wetlands (47, 48) (see also Fig. S4 to S9 in the supplemental material) and with the predominance of certain OTUs in amplicon libraries (Fig. 4). Although soil pH is an important environmental parameter impairing activity and community structure of microorganisms, adaption of denitrifier communities to low pH is a common feature of acidic peat denitrifier communities, which are not well represented in denitrifiers of culture collections to date.

Permafrost environments and N_2O fluxes. Even though evidence is accumulating that permafrost-affected systems may play an important role for N-cycling and global N_2O fluxes, many details are still unresolved (45, 47, 58). Further in-depth studies on denitrification and denitrifier communities in permafrost-affected systems are thus needed to reach a higher level of understanding of the possible source and sink functions for N_2O in permafrost peatlands. DNA-based denitrifier community analyses reflect long-term impact of environmental conditions and do not allow for conclusions on the active fraction of the community at a given time point (i.e., resting and dormant cells are included in such analyses). Detection of new organisms via PCR-based structural gene analyses is particularly limited by the selectivity of primers utilized, further complicating matters (25, 35, 68). Thus, transcriptomic and gene expression-based approaches in the future are needed to identify links between active members of the

denitrifier community and observed differences in N_2O fluxes from permafrost peatlands in future studies.

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4.2 Manuscripts in preparation

Denitrification activity of a new and diverse denitrifier community in a pH neutral fen soil in Finnish Lapland is nitrate limited

Manuscript in preparation

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Wetlands are sources of the greenhouse gas nitrous oxide (N_2O). Peatlands cover about 25% of the Finnish land area and might significantly impact on N_2O fluxes. Denitrifiers release N_2O as an intermediate. The denitrifier community in a pH-neutral fen (pH app. 6.9) in Finnish Lapland was investigated. N_2O emission was not observed *in situ* from unsupplemented fen soil during gas chamber measurements, but nitrate and ammonium addition significantly increased in situ N_2O emissions. Stimulation with nitrate was stronger than with ammonium, indicating denitrification rather than nitrification as a potential source of N_2O *in situ*. N_2O was produced and subsequently consumed in gas chambers, indicating complete denitrifcation to N_2. In unsupplemented anoxic microcosms, fen soil produced N_2O only when acetylene was added to block nitrous oxide reductase, likewise indicating 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 $N_2O \cdot h^{-1} \cdot g_{DW}^{-1}$, respectively. N_2O was below 30% of total produced N gases in fen soil when concentrations of nitrate and nitrite were <500 μM, indicating essentially complete denitrification. 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 approximately 28,000 quality filtered sequences indicated up to 26 species-level operational taxonomic units (OTUs), and up to 363 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 N_2O consumption potential, and (ii) a highly diverse, nitrate limted denitrifier community associated with potential N_2O fluxes in a pH-neutral fen soil.

1 Introduction

Northern peatlands store more than 30% of soil carbon and nitrogen [12], even though they cover only about 3% of the terrestrial surface. They are therefore important players in the global carbon and nitrogen cycles. Northern peatlands are very diverse ecosystems, including many types of pristine and managed soils. High latitude peatlands have been intensively studied with respect to their capacity to emit methane due to the large amount of stored carbon in peat soils . However, the emission of nitrous oxide (N_2O) has also been investigated [20, 23, 24]. N_2O is one of the major greenhouse gases, ranging in importance after carbon dioxide and methane [10]. The global warming potential of N_2O is about 300 times higher than that of CO_2, and its atmospheric concentration has been rising from 270 ppb to 319 ppb from 1750 to 2005, thereby largely contributing to the increase in radiative forcing [10]. Furthermore, N_2O is now the major ozone-depleting substance [33]. N_2O in soils is generally produced during nitrification, denitrification, or chemical processes [3, 7]. Denitrification is considered to be the main source of N_2O in water-saturated soils including peatlands [7, 29]. During denitrification, nitrate or nitrite are sequentially reduced via nitric oxide (NO) and N_2O to dinitrogen (N_2) [44]. 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 (endoded by *norBC*, and N_2O reductases (encoded by *nosZ*) [44]. N_2O or N_2 can be released into the atmosphere, the ratio of N_2O to N_2 is determined by *in situ* parameters such as pH, temperature, as well as nitrate/nitrite and electron donor availability [6]. pH is one of the main factors impacting denitrification, as low pH decreases overall denitrification rates and increases the product ratio of N_2O to N_2 [38]. Even though N_2O emissions from high latitude peatlands have been studied, many studies have focused on N_2O emissions from managed peatlands [20, 22, 23]. Pristine northern peatlands can be net sources of N_2O even though emission rates are generally low [11, 20, 24], on the other hand peatlands can act as temporary sinks for N_2O [11, 28]. Moreover, the denitrifier communities involved in N_2O turnover in northern peatlands are largely unknown.

High latitude peatlands are likely to be strongly affected by increasing temperatures due to climate change [19]. Global warming might reduce the water table in northern peatlands and influence the amount of N_2O released from the soil [39]. A constantly lowered water table increases the N_2O fluxes from nutrient rich peat, whereas fluxes from nutrient-poor peat remain largely unaffected [23]. The aim of this study was to

assess denitrification in a pH neutral pristine fen. The main objectives were to (i) assess *in situ* N_2O emissions of fen soil, (ii) determine the N_2O production and consumption capacities of fen soil, (iii) link differences in the denitrifier community composition to physiological differences of the denitrification in the peat soil, and (iv) identify possible key factors controlling N2O fluxes from high latitude peat soils.

2 Material and Methods

2.1 Sampling site

Samples were taken from a fen and a palsa peat in Finnish Lapland. The fen Puukkosuo is located in southeastern Finnish Lapland (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. 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.

2.2 Soil parameters

Nitrate, nitrite and ammonium concentrations as well as soil pH and dissolved organic carbon (DOC) were determined in watery extracts (10 g of soil in 30 ml ddH_2O; extraction for 24 h at 4°C). Nitrate and nitrite were measured by flow injection analysis using a Dionex DX-500 ion chromatograph equipped with an IonPac AS4A-SC ion-exchange column and an ED40 electrochemical detector (Sunnyvale, CA, USA). Ammonium was measured by Flow Injection Analysis (FIA-LAB, MLE Dresden, Germany). Total C and N contents were determined from oven-dried soil with an elemental analyzer (Thermo Quest Flash EA 1112, CE Instruments, Wigan, UK). DOC was determined after filtration (pore size 0.45 μM) with a TOC-Analyzer (multi N/C 2100; Analytik Jena, Jena, Germany). pH was determined with a pH electrode (pH Meter CG 832, Schott Geräte GmbH, Mainz, Germany). Moisture content was determined by weighing the soil before and after drying at 60°C for 3 days.

2.3 Assessment of in situ gas emissions

In situ gas emissions of unsupplemented soil and soil supplemented with either nitrate or ammonium were determined in closed plexiglas 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, soil was supplied with 2 l water from a nearby stream with 20 mM of added nitrate or ammonium in 4 replicate treatments each and incubated for 3 hours. Unsupplemented controls received pure stream water. Gas samples (5 ml per sampling timepoint) were taken from gas outlets and injected into gas tight exetainers (Labco Limited, High Wycombe, UK). Concentrations of N_2O were gaschromatographically determined in the laboratory. Gas samples were taken at the start of the experiment and after 0.5, 1 and 3 hours. N2O was quantified with a Hewlett-Packard 5980 series II gas chromatograph (Hewlett-Packard Paolo Alto, CA) equipped with an electron capture detector [28].

2.4 Assessment of denitrification in soil microcosms

Soil of of each sampling site and sampled soil layer was homogenized and mixed prior to the microcosm experiments. Soil slurries at *in situ* pH were prepared with 10 g of soil and 3 volumes of deionized water in 125-ml infusion flasks. The flasks were sealed with gas-tight rubber stoppers. The gasphase was 100% argon. Experiments were done in triplicate. Microcosms were always incubated at 20 °C in the dark. Acetylene blocks nitrous oxide reductases and thus the reduction of N_2O to N_2 [42]. Parallel microcosms with and without acetylene (15% [vol/vol] in headspace) were used to differentiate between total denitrification and N_2O-production potentials [42]. Experiments without supplemental nitrate or nitrite were conducted to assess denitrification potentials from internal nitrate or nitrite with and without acetylene. For apparent Michaelis-Menten kinetics, soil was pre-incubated for 24 hours under anoxic conditions to reduce the concentrations of internal nitrate and nitrite. Such preincubated soil was supplemented with 0 to 1000 μM of $NaNO_2$ or $NaNO_3$ or 0 to 61 μM N_2O. Michaelis-Menten constants (K_M) and the maximum reaction velocities (v_{max}) were based on the production of N_2O in microcosms supplemented with either nitrate or nitrite and acetylene and consumption of N_2O in microcosms supplemented with N_2O. Headspace concentrations of N_2O were

determined four or five times via gas chromatography within 8 to 14 h of incubation, and N_2O production rates were calculated in the linear phase by linear regression of three or four data points (R^2 values were always greater than 0.89). Nitrite consumption was tested exemplarily in nitrite supplemented microcosms with fen soil from 0 to 20 cm by colorimetric determination of nitrite concentrations at each sampling timepoint [13]. Apparent Michaelis-Menten kinetics were fitted to the data points using the program SigmaPlot 10.0 (Systat Software GmbH, Erkrath, Germany) for calculation of K_M and v_{max} according to the following equation [37]: $v = \frac{v_{max} \cdot [S]}{K_M + [S]}$, where [S] is the concentration of nitrate or nitrite.

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

Nucleic acids were extracted from homogenized soil of each site and sampled soil layer. A bead-beating protocol tailored for the efficient removal of PCR-inhibiting humic acids by aluminum sulfate precipitation prior to cell lysis was applied [30]. In brief, the minimal amount of aluminium sulfate for efficient humic acid removal depends on the humic acid content of a soil sample, and was experimentally determined to be 200 μl of a 0.2 M aluminumsulfate solution per 0.5 g of soil samples from Puukkosuo fen soil. Following bead beating, nucleic acids were purified by two organic solvent extractions and isopropanol precipitation to obtain high quality DNA. DNA yields were 4 to 12 μg DNA per gram freshweight of soil. A260/A230 values approximating 0.94 to 1.56 indicated DNA with moderate to low humic acid content.

narG, *nirK*, *nirS*, and *nosZ* were amplified using the primer pairs narG1960f (TAY GTS GGS CAR GAR AA)/narG2650r (TTY TCR TAC CAB GTB GC) [31], F1aCu (ATC ATG GTS CTG CCG CG)/R3Cu (GCC TCG ATC AGR TTG TGG TT) [41], cd3aF (GTS AAC GTS AAG GAR ACS GG)/R3cd (GAS TTC GGR TGS GTC TTG A) [41], and nosZF (CGC TGT TCI TCG ACA GYC AG)/nosZR (ATG TGC AKI GCR TGG CAG AA) [34], respectively. Each primer was preceeded by a 6 basepair-long barcode (ACTGCG for 0 to 20 cm fen soil, AGTATG for 20 to 40 cm fen soil) to separate sequences after pyrosequencing. PCR reactions and thermal cycling conditions were the same as previously published [26].

2.6 Barcoded amplicon pyrosequencing of structural genes

Barcoded PCR products were gel purified with 1% agarose gels using a Montage Gel Extraction Kit (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions, and purified via isopropanol precipitation to remove residual running buffer. Concentrations of the gel-purified PCR products were determined using Quant-iTTM PicoGreen (Invitrogen, Carlsbad, CA, USA) and a FLx800 Fluorescence Microplate Reader (BioTek, Bad Friedrichshall, Germany). PCR products (i.e., amplicons) of similar lengths of both soil types were combined in equal amounts (i.e., *narG* and *nosZ* 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 via isopropanol precipitation. A and B sequencing adaptors (GCC TCC CTC GCG CCA TCA G and GCC TTG CCA GCC CGC TCA G, respectively) that are required for pyrosequencing were ligated to the amplicons and sequencing from 5' (forward) and 3' (reverse) ends of amplicons was performed at the Göttingen Genomics Laboratory employing the Roche GS-FLX 454 pyrosequencer and GS FLX Titanium series reagents (Roche, Mannheim, Germany) according to the manufacturer's instructions.

2.7 Sequence filtering and analysis

Pyrosequencing and PCR amplification errors can artificially inflate detected sequence diversity [18, 32]. Thus, correction or removal of so-called noisy reads is essential to correctly estimate the number of operational taxonomic units (OTUs) in an environmental sample. The AmpliconNoise pipeline achieves reduction of pyrosequencing and PCR amplification errors by flowgram and sequence preclustering, respectively (utilizing the PyroNoise and SeqNoise algorithms, respectively) [32]. Denoising of pyrosequencing reads and subsequent analysis of the denoised sequences was conducted using the AmpliconNoise and Qiime pipelines [5, 32]. Sequences with ambiguities and errors in primer or barcode sequences were discarded. DNA sequences were sorted according to their barcodes and primers, and each subset of sequences was clustered by average linkage clustering after pairwise sequence alignment, as this clustering method is expected to be rather insensitive to pyrosequencing errors [18]. Denoised sequences were aligned using the Needleman-Wunsch algorithm and clustered [i.e., assigned to operational taxonomic units (OTUs)] by average linkage at species-level threshold similarities of 67% (*narG*,

[27], 83% (*nirK*, Depkat-Jakob et al., unpublished), 82% (*nirS*, Depkat-Jakob et al., unpublished), or 80% (*nosZ*, [27]], as well as at 97% threshold similarity for all genes. Cluster representatives were edited, translated *in silico*, and aligned with reference sequences using the ClustalW algorithm implemented in MEGA 5.0 [17]. 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. Stability of tree topologies was assessed by calculating 10 000 bootstrap replicates [36]. Diversity measures were calculated as described [15].

2.8 Quantification of narG, nirK, nirS, nosZ, and 16S rRNA genes in soil

Quantitative kinetic real-time PCRs (qPCRs) of structural and 16S rRNA genes were conductd 6 technical replicates as described [26]. Negative controls with sterilized water instead of DNA template were included in every PCR setup. Obtained gene copy numbers were inhibtion corrected, inhibtion factors ranged from 0.5-0.6, 0.3-0.4, 0.5-1, 0.9-1.0, and 0.9-1.0 for qPCR analyses of *narG*, *nirK*, *nirS*, *nosZ*, and 16S rRNA genes, respectively. Inhibition corrected structural gene copy numbers were normalized per ng DNA and 16S rRNA gene copy numbers, since such normalized gene copy numbers are less sensitive to varying DNA extraction efficiencies (that is, DNA extraction bias) than copy numbers per gram dry weight soil.

3 Results

3.1 Soil parameters

Soil moisture content of Puukkosuo fen soil was (90±0.1)% (Table 1). Soil pH of Puukkosuo fen was 6.85±0.01. Nitrate was below the detection limit (4 $\mu g \cdot g_{DW}^{-1}$) (Table 1). Carbon and nitrogen contents were slightly higher in 20 to 40 cm than in 0 to 20 cm fen soil, but C/N ratios were similar in both soil layers (15 an 14 in 0 to 20 cm and 20 to 40 cm fen soil, respectively; Table 1).

Table 1: Soil parameters of Puukkosuo fen.

Soil layer (cm)	pH	Moisture content (%)	NO_3^- (μM)	NO_3^- ($\mu mol \cdot g_{DW}^{-1}$)	NO_2^- (μM)	NO_2^- ($\mu mol \cdot g_{DW}^{-1}$)	NH_4^+ (μM)	NH_4^+ ($\mu mol \cdot g_{DW}^{-1}$)	Total C [1] ($g \cdot kg_{DW}^{-1}$)	DOC [2] ($mg \cdot l^{-1}$)	Total N [3] ($g \cdot kg_{DW}^{-1}$)	C/N [4]
0 to 20	6.8	90	14.4	7.7	b.d. [5]	b.d.	77.2	11.9	434	63.2	29	15
below 20	6.9	90	b.d.	b.d.	b.d.	b.d.	37.7	6.3	492	65.1	35	14
Stream water		n.a. [6]	b.d.	n.a.	b.d.	n.a.	< 1.4	n.a.	n.a.	8.8	n.a.	n.a.

[1] Total carbon.
[2] Dissolved organic carbon.
[3] Total nitrogen.
[4] Carbon to nitrogen ratio.
[5] Below detection limit.
[6] Not applicable.

3.2 In situ N_2O emissions of fen soil

Only minor amounts of N_2O were emitted from unsupplemented fen soil during gas chamber measurements (Figure 1). Nitrate-addition increased N_2O emission. However, emission of N_2O was restricted to the first 30 minutes after nitrate-addition, and initially emitted N_2O was subsequently consumed after 30 minutes (Figure 1). Ammonium likewise increased N_2O emission from fen soil (Figure 1). N_2O emission was linear within the first hour after ammonium-addition, and initially emitted N_2O was subsequently consumed after the first hour.

3.3 Denitrification activities in unsupplemented fen soil microcosms

In anoxic microcosms unsupplemented soil from both soil layers produced only small amounts of N_2O without addition of acetylene (Figure 2). However, N_2O production was significantly higher in anoxic microcosms when N2O -reductase was blocked by acetylene (Figure 2). 234 nmol N_2O per g_{DW} accumulated within the first 94 hours in acetylene-amended microcosms with 0 to 20 cm fen soil, and the concentration of N_2O stayed constant after that (Figure 2), indicating that endogenous nitrate had been

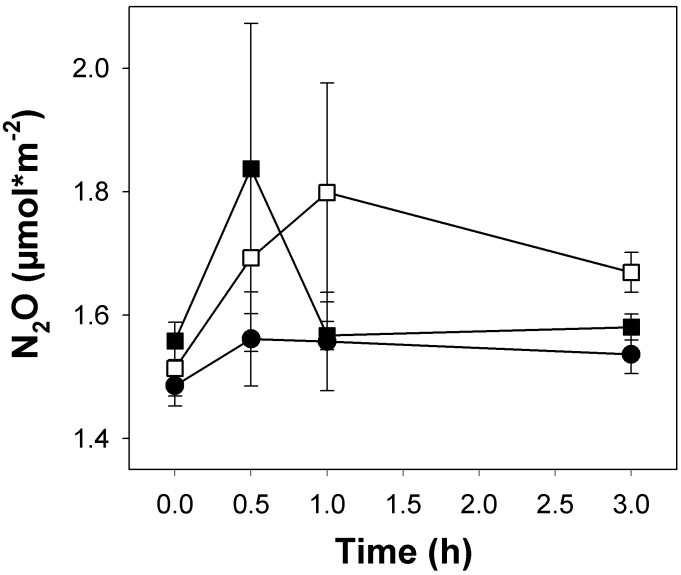

Figure 1: Effect of nitrate and ammonium fertilization on *in situ* N_2O emissions from pH-neutral fen soil. Mean values and standard errors of 4 replicates are displayed. Closed circles represent unfertilized controls, closed and open squares represent soil fertilized with 20 mM $NaNO_3$ and 20 mM NH_4Cl, respectively.

consumed. Only minor amounts of N_2O were detected in microcosms without acetylene after 24 hours of anoxic incubation, and this initially produced N_2O was subsequently consumed (Figure 2).

N_2O production was significantly lower in microcosms with 20 to 40 than in those with 0 to 20 cm fen soil (Figure 2). 11 nmol N_2O per g_{DW} were produced 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, only 0.6 nmol N_2O per g_{DW} accumulated within the first 94 hours. The initially accumulated N_2O was subsequently consumed within the next 74 hours.

3.4 Effect of supplemental nitrate, nitrite, and N_2O on denitrification in fen soil

Supplemental nitrate and nitrite stimulated the production of N_2O without apparent delay in microcosms with fen soil from both soil layers (Figure S1). Stimulation with nitrate was smaller than with nitrite, and N_2O production in nitrate-supplemented microcosms was less than 25% of that N_2O in nitrite-supplemented microcosms (Figure S1). In microcosms with fen soil from 0 to 20 cm, N_2O production in acetylene-amended mi-

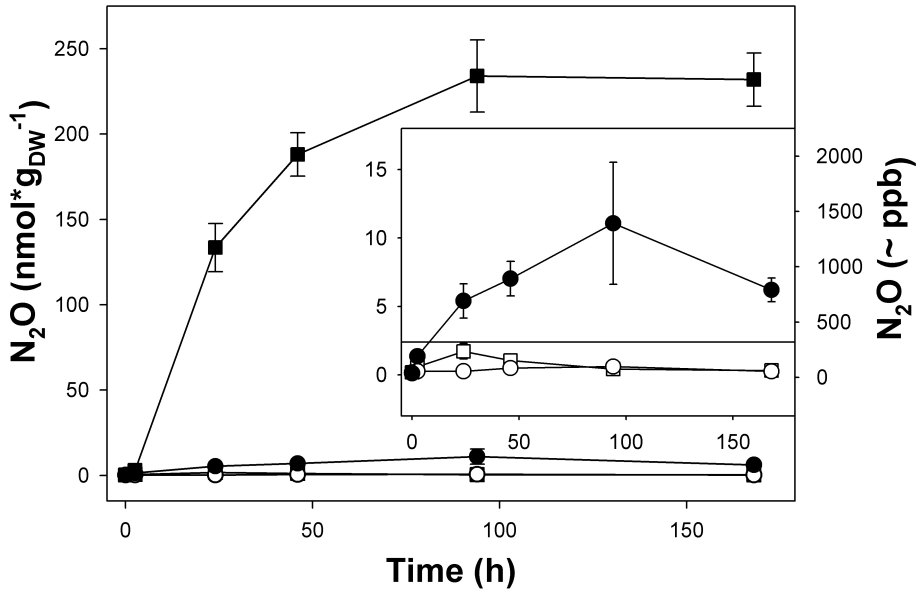

Figure 2: N_2O production and consumption in unsupplemented anoxic fen soil microcosms. Squares and circles represent 0 to 20 cm and 20 to 40 cm fen soil, respectively. Open and closed symbols represent microcosms without and with acetylene addition, respectively. Mean values and standard errors of three replicates are displayed. The inset represents an enlargement of the lower N_2O concentrations to allow better visualization of N_2O production and consumption in 20 to 40 cm fen soil microcosms and microcosms without acetylene addition.

crocosms was in a similar magnitude for all supplemented nitrate concentrations, while 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 larger nitrate concentrations (Figure 3 A). In nitrite-supplemented microcosms with fen soil, N_2O production rates increased with increasing nitrite concentrations in both soil layers (Figure 3) A. N_2O production was higher in 0 to 20 cm fen soil than in 20 to 40 cm fen soil. Significant differences in N_2O production were observed in microcosms with and without acetylene in both soil layers and with both electron acceptors. The ratio of N_2O to (N_2+N_2O) 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 (Figure S2), indicating that more than half of the N_2O produced from nitrate was further reduced to N_2 in fen soil. The ratio of N_2O to (N_2+N_2O) 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 N_2O to (N_2+N_2O) was between 50% and 100% for all supplied nitrite concentrations (Figure S2), indicating that N_2O was the main product of denitrification in that soil layer when nitrite was the electron acceptor provided.

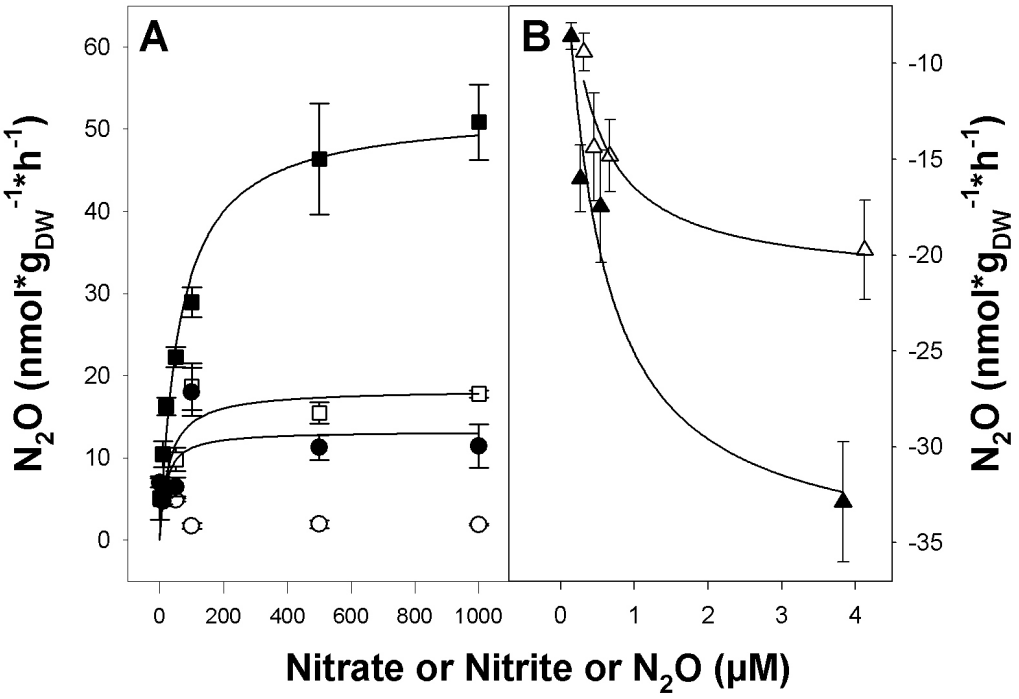

Figure 3: Effect of added nitrate, nitrite (A) and N_2O (B) on N_2O production and consumption in anoxic fen soil microcosms. Closed and open symbols represent 0 to 20 cm and 20 to 40 cm fen soil microcosms, respectively. Circles, squares and triangles represent microcosms supplemented with nitrate, nitrite and N_2O, respectively. Mean values and standard errors of three replicates are displayed.

Supplemental N_2O was consumed in microcosms with fen soil from 0 to 20 cm and 20 to 40 cm (Figure 3 B). Consumption was stimulated by increasing N_2O concentrations (Figure 3 B). Consumption was higher in 0 to 20 cm than in 20 to 40 cm fen soil. Supplied N_2O concentrations were consumed to sub-atmospheric levels (...), demonstrating the potential of fen soil to consume N_2O in atmospheric concentrations. Initial nitrite-dependent N_2O production rates of fen soil microcosms amended with acetylene followed apparent Michaelis-Menten kinetics, as did nitrate-dependent N_2O production rates of fen soil microcosms from 0 to 20 cm depth and N_2O-dependent N_2O consumption rates in both layers (Figure 3 B). Apparent maximal reaction velocities (v_{max}) were highest for

nitrite-dependent N_2O production, followed by N_2O-dependent N_2O consumption rates. v_{max} was lowest for nitrate-dependent N_2O production (Table 2). Apparent Michaelis-Menten constants K_M were much lower for N_2O consumption than for N_2O production, indicating a high affinity of fen denitrifiers to N_2O (Table 2).

Table 2: Soil parameters.

Soil layer (cm)	Nitrate amended		Nitrite amended		N_2O amended	
	K_M [1] (μM)	v_{max} [1] (nmolcoth^{-1}·g$_{DW}^{-1}$)	K_M [1] (μM)	v_{max} [1] (nmolcoth^{-1}·g$_{DW}^{-1}$)	K_M [1] (μM)	v_{max} [1] (nmolcoth^{-1}·g$_{DW}^{-1}$)
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. [2]	n.a. [2]	18.3 ± 21.0	13.3 ± 3.3	0.30 ± 0.09	- 21.4 ± 2.0

[1] Kinetic parameters (calculated from Figure 3) ± standard error.
[2] Not applicable.

3.5 Phylogenetic analysis of denitrifiers in pH-neutral fen soil

In total, aproximately 14 000 denoised sequences of the strucural gene markers *narG*, *nirK*, *nirS*, and *nosZ* were included into 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 combination of forward and revers 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 (amplicons 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 [26, 29]. More than 99% of analyzed 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 DNA sequence dissimilarities of 33%, 17%, 18%, and 20% for *narG*, *nirK*, *nirS*, and *nosZ*, respectively (Table 3), indicating that the number of sequences generated was sufficient. At 3% sequence dissimilarity, libary coverage varied from 80% to 97% (Table 3), indicating that still more diversity might be detectable for some genemarkters.

Table 3: Detected species-level and 97%-similarity-level diversity of all obtained sequences of *narG*, *nirK*, *nirS*, and *nosZ* derived from pH-neutral fen soil..

Gene marker	Threshold similarity (%)	Soil layer (cm)	No. of sequences	Library coverage (%) 1	No. of OTUs observed [1]	No. of OTUs estimated [2]	H [3]	E [4]
narG	67	0 to 20	1 141	99.7	7	10 (7-32)	1.28	0.45
		20 to 40	1 697	99.9	4	4 (4)	1.20	0.60
	97	0 to 20	1 141	79.8	359	856 (699-1084)	7.08	0.83
		20 to 40	1 697	93.7	230	383 (322-486)	5.45	0.69
nirK	83	0 to 20	1 814	99.7	23	27 (24-45)	1.53	0.34
		20 to 40	1 876	99.8	17	18 (17-24)	1.41	0.35
	97	0 to 20	1 814	93.8	174	404 (309-565)	3.59	0.48
		20 to 40	1 876	96.1	109	311 (211-512)	2.06	0.30
nirS	82	0 to 20	3 146	99.9	22	24 (22-34)	2.14	0.48
		20 to 40	3 382	99.9	23	23 (23-29)	2.79	0.62
	97	0 to 20	3 146	93.8	301	741 (592-966)	4.36	0.53
		20 to 40	3 382	96.7	185	407 (316-561)	3.86	0.51
nosZ	80	0 to 20	572	100.0	8	8 (8)	1.79	0.60
		20 to 40	530	99.8	8	8 (8)	0.79	0.26
	97	0 to 20	572	86.4	120	308 (218-479)	5.13	0.74
		20 to 40	530	96.4	40	83 (53-179)	2.93	0.55

[1] Percent library coverage C = (1 - ns/nt) * 100 (ns = OTUs that occur only once, nt = total number of sequences)
[2] Chao1 richness estimate with upper and lower 95% confidence intervals given in parentheses.
[3] Shannon-Weaver diversity index.
[4] Species Evenness.

Table 4: Detected species-level and 97%-similarity-level diversity of rarified *narG*, *nirK*, *nirS*, and *nosZ* derived from pH-neutral fen soil..

Gene marker	Threshold similarity (%)	Soil layer (cm)	α-Diversity								β-Diversity			
			No. of OTUs observed [1]	Comparison observed OTUs	No. of OTUs estimated [2]	Comparison estimated OTUs	H [3]	Comparison H	E [4]	Comparison E	S_S [5]	BC_S [6]	D_{UU} [7]	D_{WU} [8]
narG	67	0 to 20	6.75 ± 0.05	$P < 0.001$	8.80 ± 0.14	$P < 0.001$	1.28 ± 0.001	$P < 0.001$	0.46 ± 0.002	$P < 0.001$	0.31 ± 0.01	0.25 ± 0.001	0.40 ± 0.006	0.20 ± 0.001
		20 to 40	3.66 ± 0.05		3.66 ± 0.05		1.20 ± 0.002		0.65 ± 0.007					
	97	0 to 20	330 ± 0.44	$P < 0.001$	814 ± 5.3	$P < 0.001$	7.03 ± 0.003	$P < 0.001$	0.84 ± 0.001	$P < 0.001$	0.78 ± 0.001	0.85 ± 0.001	0.71 ± 0.001	0.56 ± 0.001
		20 to 40	178 ± 0.53		312 ± 3.3		5.35 ± 0.006		0.72 ± 0.001					
nirK	83	0 to 20	21.8 ± 0.10	$P < 0.001$	26.0 ± 0.34	$P < 0.001$	1.53 ± 0.002	$P < 0.001$	0.34 ± 0.001	$P < 0.001$	0.22 ± 0.003	0.64 ± 0.001	0.41 ± 0.004	0.30 ± 0.001
		20 to 40	16.2 ± 0.07		18.5 ± 0.39		1.41 ± 0.002		0.35 ± 0.001					
	97	0 to 20	154 ± 0.34	$P < 0.001$	381 ± 4.59	$P < 0.001$	3.57 ± 0.003	$P < 0.001$	0.49 ± 0.001	$P < 0.001$	0.75 ± 0.002	0.93 ± 0.001	0.67 ± 0.002	0.40 ± 0.001
		20 to 40	93.4 ± 0.38		256 ± 5.17		2.04 ± 0.004		0.31 ± 0.001					
nirS	82	0 to 20	21.0 ± 0.09	$P < 0.001$	24.1 ± 0.34	$P = 0.12$	2.14 ± 0.002	$P < 0.001$	0.49 ± 0.001	$P < 0.001$	0.13 ± 0.003	0.78 ± 0.001	0.19 ± 0.005	0.32 ± 0.001
		20 to 40	22.3 ± 0.07		23.5 ± 0.20		2.78 ± 0.001		0.62 ± 0.001					
	97	0 to 20	260 ± 0.55	$P < 0.001$	667 ± 7.26	$P < 0.001$	4.34 ± 0.003	$P < 0.001$	0.54 ± 0.001	$P < 0.001$	0.78 ± 0.001	0.81 ± 0.001	0.68 ± 0.001	0.40 ± 0.001
		20 to 40	154 ± 0.45		368 ± 4.38		3.84 ± 0.003		0.53 ± 0.001					
nosZ	80	0 to 20	8.00 ± 0.00	$P = 0.01$	8.00 ± 0.00	$P = 0.16$	1.78 ± 0.003	$P < 0.001$	0.60 ± 0.001	$P < 0.001$	0.25 ± 0.001	0.30 ± 0.001	0.30 ± 0.001	0.18 ± 0.001
		20 to 40	7.94 ± 0.02		7.96 ± 0.028		0.79 ± 0.002		0.26 ± 0.001					
	97	0 to 20	110 ± 0.29	$P = 0.10$	292 ± 3.49	$P < 0.001$	5.10 ± 0.005	$P < 0.001$	0.75 ± 0.001	$P < 0.001$	0.68 ± 0.001	0.75 ± 0.001	0.56 ± 0.001	0.47 ± 0.001
		20 to 40	38.8 ± 0.11		80.4 ± 1.68		2.92 ± 0.002		0.55 ± 0.001					

[1] Number of OTUs observed in rarifies OTU tables ± standard error.
[2] Chao1 richness estimate of rarified OTUs ± standard error.
[3] Shannon diversity index of rarified OTUs ± standard error.
[4] Species Evenness of rarified OTUs ± standard error.
[5] Sørensen similarity index of rarified OTUs ± standard error.
[6] Bray Curtis similarity index of rarified OTUs ± standard error.
[7] Unweighted Unifrac distance of rarified OTUs ± standard error.
[8] Weighted Unifrac distance of rarified OTUs ± standard error.
[9] P-values of T-test for pairwise comparisons of values from upper an lower soil layers are given.

Forward reads of *narG* amplicons yielded more OTUs than reverse reads although similar number of sequences were obtained, indicating that the utility of 3-prime ends of *narG* amplicons is higher for diversity analyses than 5-prime ends (Table 3). However, results obtained from reverse reads show similar overall trends (Table 3). Thus, information presented below refers to forward reads only. In total, narG sequences were assigned to 7 species-level OTUs. 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 (Figure 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), while about 40% of narG belonged to OTUs 2 and 3 (Figure 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; Figure 4 A). Most of the OTUs were only distantly related to *narG* of cultured organisms or environmental sequences (i.e. sequence dissimilarities of OTU representatives were >20%) (Figure 5). Sequences of OTUs 1, 2, and 3 affiliated with *narG* of *Alphaproteobacteria*, *Actinobaceria*, and *Deinococci*, respectively, more specifically they were related to *narG* of uncultured bacteria and to those of *Oligotropho carboxidovorans*, *Salinispora arenicola*, and *Thermus* sp., respectively (Figure 5). Diversity of *narG* as described by the Shannon diversity index, species Evenness, and Chao1 richness estimates was similar in 0 to 20 cm and 20 to 40 cm fen soil at species-level threshold similarities (Table 3). Observed *narG* diversity was higher at 97% threshold similarity than at species-level threshold similarities (Table 3, 4). At 97%, 359 and 230 in 0 to 20 cm and 20 to 40 cm fen soil, respectively (Table 3), and also the Shannon diversity and Species Evenness index indicated higher diversity at this threshold-level.

In total, *nirK* were assigned to 24 species-level OTUs. 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 (Figure 4 B). OTU 2 dominated *nirK* in fen soil from 0 to 20 cm (about 60%), while OTUs 1 dominated *nirK* in fen soil from 20 to 40 cm respectively (about 70%; Figure 4 B). Most OTUs were related to *Alphaproteobacterial nirK*. OTUs 1, 2, and 3 were related to *nirK* of *Bradyrhizobium* sp., *Rhizobium etli*, and *Alcaligenes* sp., respectively (Figure 6). Further OTUs were related to *nirK* of *Pseudomonas* sp., *Afipia* sp., or uncultured bacteria (Figure 6). In total, 25 species-level OTUs of *nirS* were detected. 22 and 23 OTUs were detected in fen soil from 0 to 20 cm and from 20 to 40 cm, respectively (Table 3). Community composition of *nirS* differed more strongly between the soil layers than *nirK* community

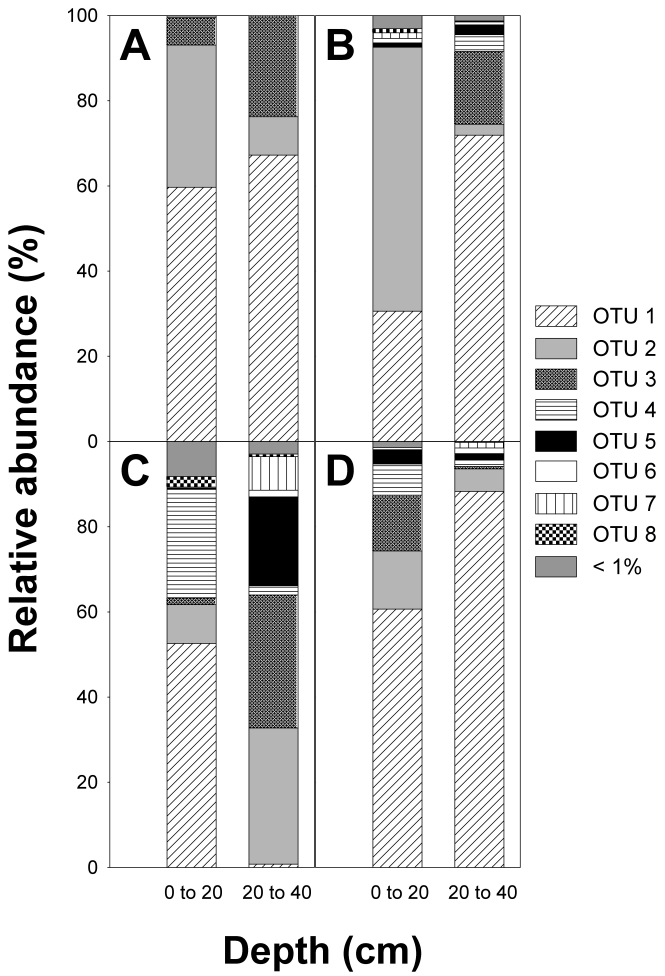

Figure 4: Relative abundances of denitrification-associated genes in pH-neutral fen soil. OTUs of *narG* forward reads (A), *nirK* (B), *nirS* (C), and *nosZ* forward reads were derived at species-level thresholds of 33%, 17%, 18%, and 20%, respectively, from original sequence data sets (i.e., not rarified).

composition (Figure 4 B, C). *nirS* of fen soil was dominated by OTUs affiliated to *Beta-* and *Gammaproteobacterial nirS*, however, about 26% of detected *nirS* from 20 to 40 cm affiliated with *Alphaproteobacterial nirS* (Figure 7). 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., *Acidovorax* sp. and *Bradyrhizobium* sp., and *Azoarcus* sp. (Figure 7). Shannon diversity, species Evenness, and Chao1 richness estimates of *nirK* and *nirS* were similar in both soil layers, *nirK* diversity was slightly higher in 0 to 20 cm fen soil, while *nirS* diversity was slightly higher in 20 to 40 cm fen soil (Table 3). Observed *nirK*

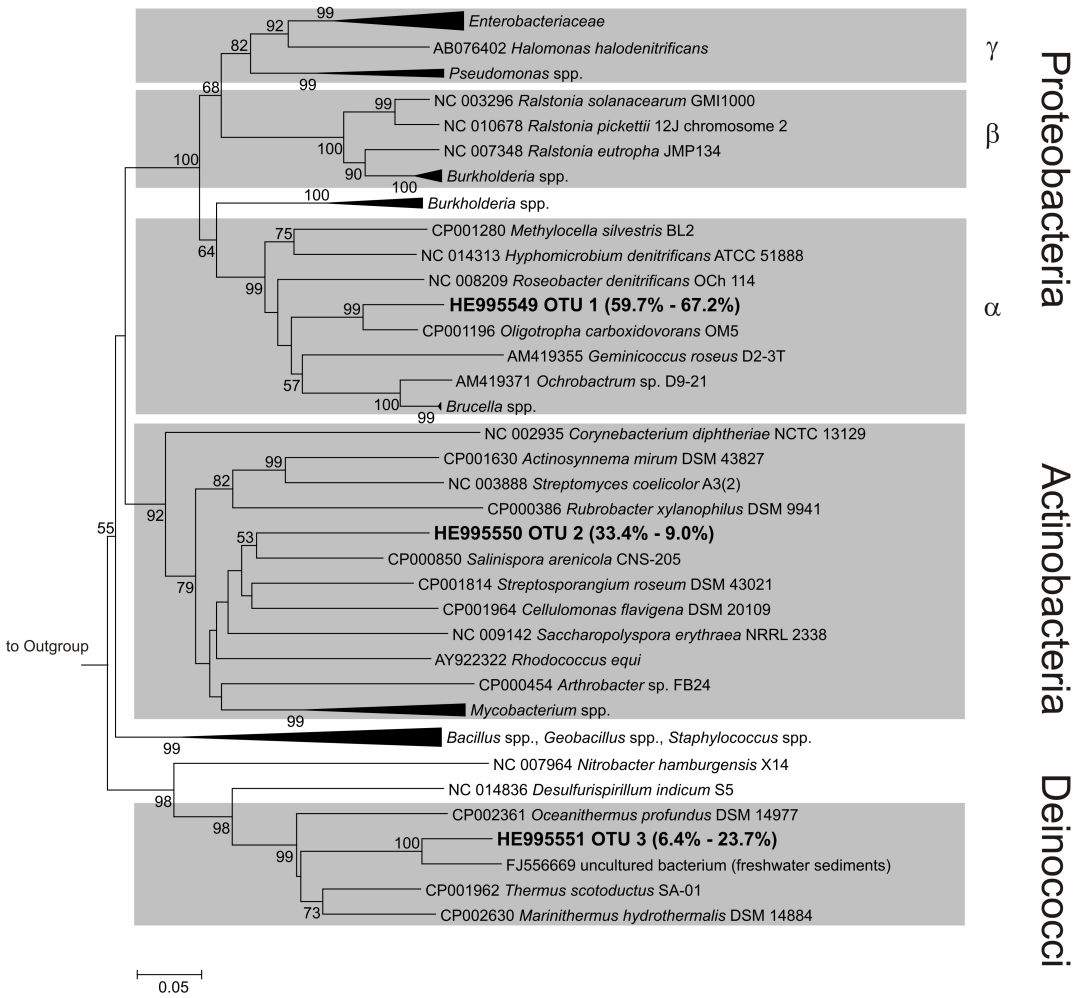

Figure 5: Phylogenetic tree of *narG* OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil. The tree was calculated based on translated amino acid sequences. OTUs were grouped at a species-level threshold dissimilarity of 33%. Numbers preceeding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parantheses 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. *narG* of *Haloarcula marismortui* ATCC 43049 was used as outgroup to root the tree.

and *nirS* diversity was higher at 97% threshold similarity than at species-level threshold similarities, and *nirS* diversity, as indicated by the Shannon diversiyty index and species Evenness, was higher than *nirK* diversity (Table 3, 4). At 97%, detected OTU numbers

of *nirK* and *nirS* were higher in 0 to 20 cm (174 and 301, respectively) than in 20 to 40 cm soil (109 and 185, respectively) (Table 3).

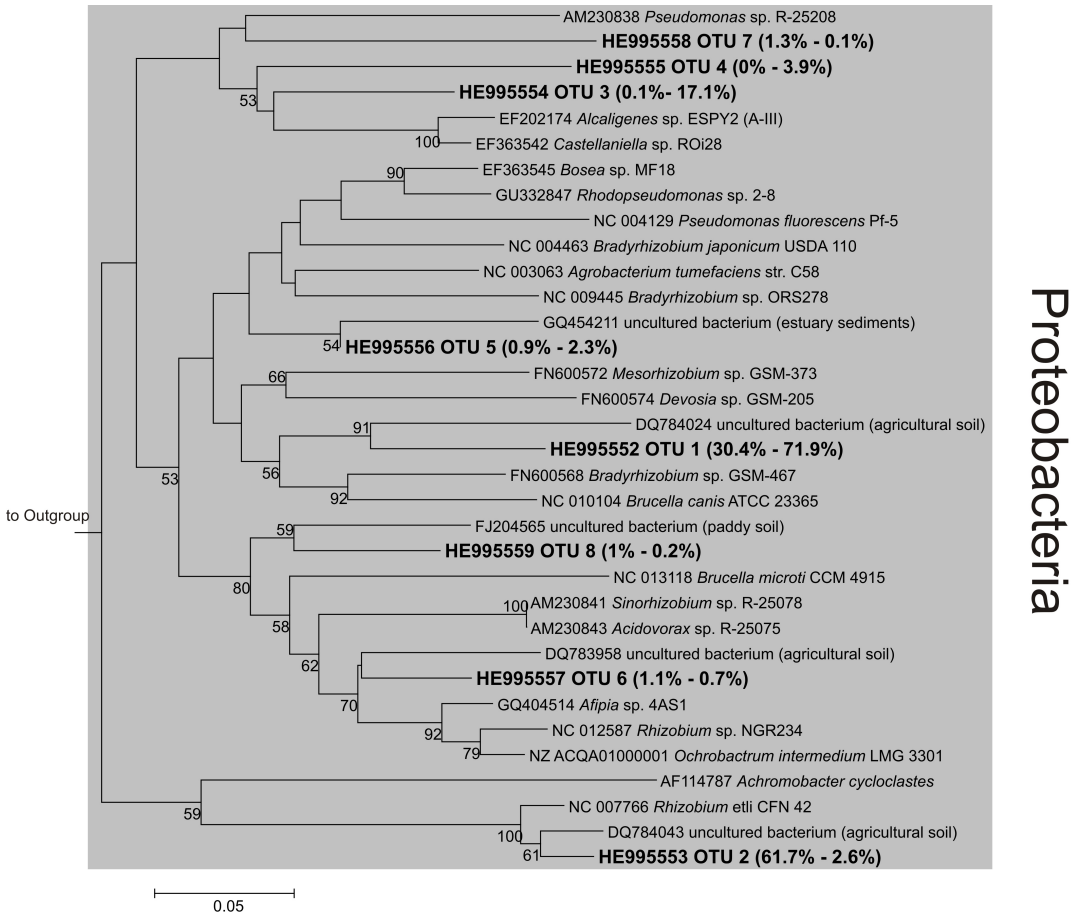

Figure 6: Phylogenetic tree of *nirK* OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil. The tree was calculated based on translated amino acid sequences. OTUs were grouped at a species-level threshold dissimilarity of 17%. Numbers preceeding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parantheses 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.

In total, *nosZ* forward reads were assigned to 10 species-level OTUs. 8 OTUs were detected in each soil layer (Table 3), indicating that the number of denitrifying taxa capable of N_2O reduction is similar in both layers of fen soil. OTUs 1 dominated *nosZ* of fen soil

from both soil layers (Figure 4 D). Essentially all nosZ from both soil layers were affiliated with Alpha- and Betaproteobacterial *nosZ* (Figure 8). Most *nosZ* sequences from fen soil were more distantly related to nosZ of cultured organisms, indicating hitherto uncultured denitrifiers capable of N_2O reduction in fen soil. *nosZ* sequences clustered with *nosZ* of wetland and upland soils, as well as *Bradyrhizobium* sp., *Herbaspirillum* sp., and *Thiobacillus denitrificans* (Figure 8). Shannon diversity indices and species Evennes indicated higher species-level diversity of *nosZ* in 0 to 20 cm than in 20 to 40 cm fen soil, while Chao1 richness estimates were identical for both soil layers (Table 3). Similar trends were reflected by the analysis at 97% threshold similarity (Table 3, 4).

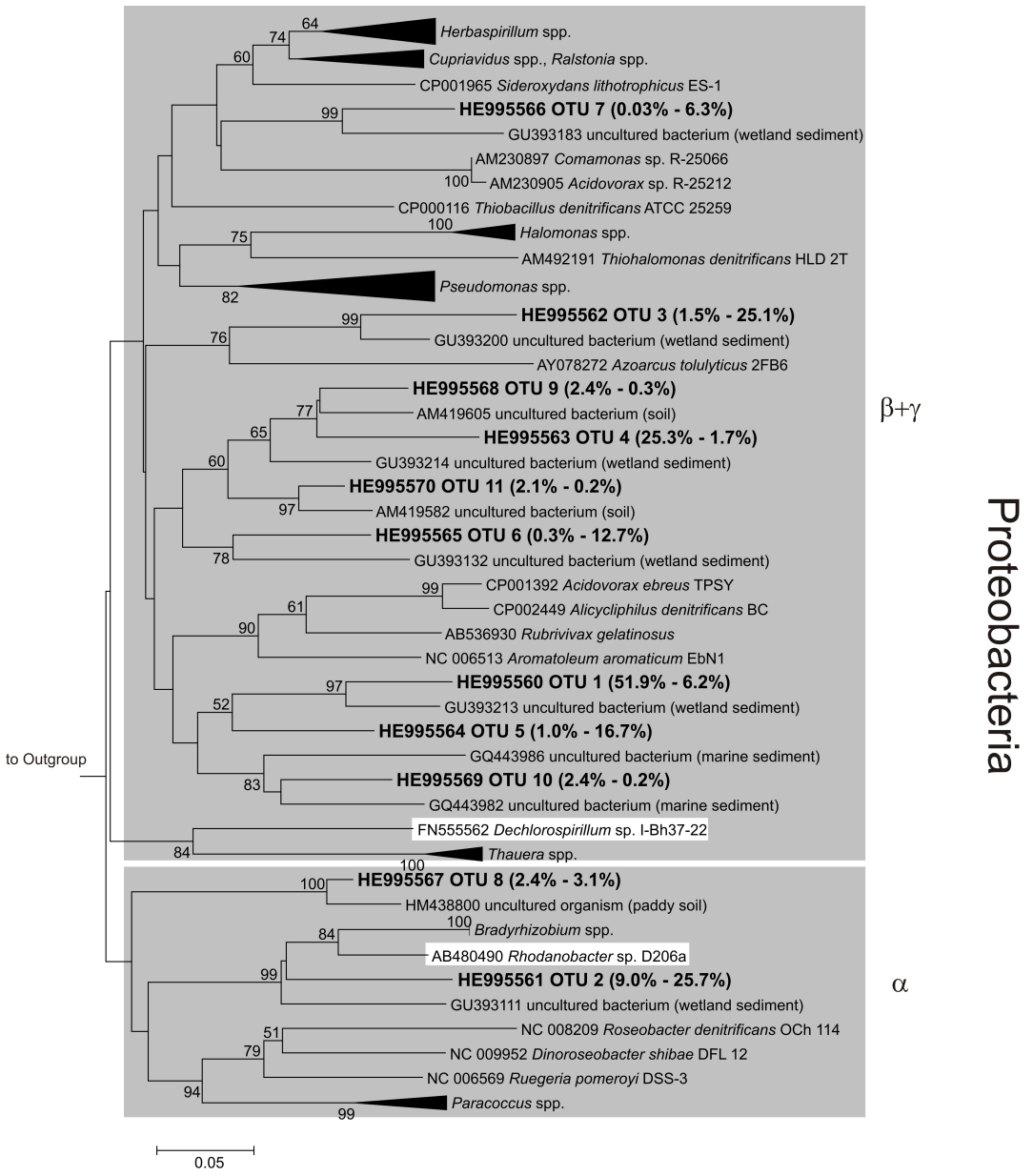

Figure 7: Phylogenetic tree of *nirS* OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil. The tree was calculated based on translated amino acid sequences. OTUs were grouped at a species-level threshold dissimilarity of 18%. Numbers preceeding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parantheses 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.

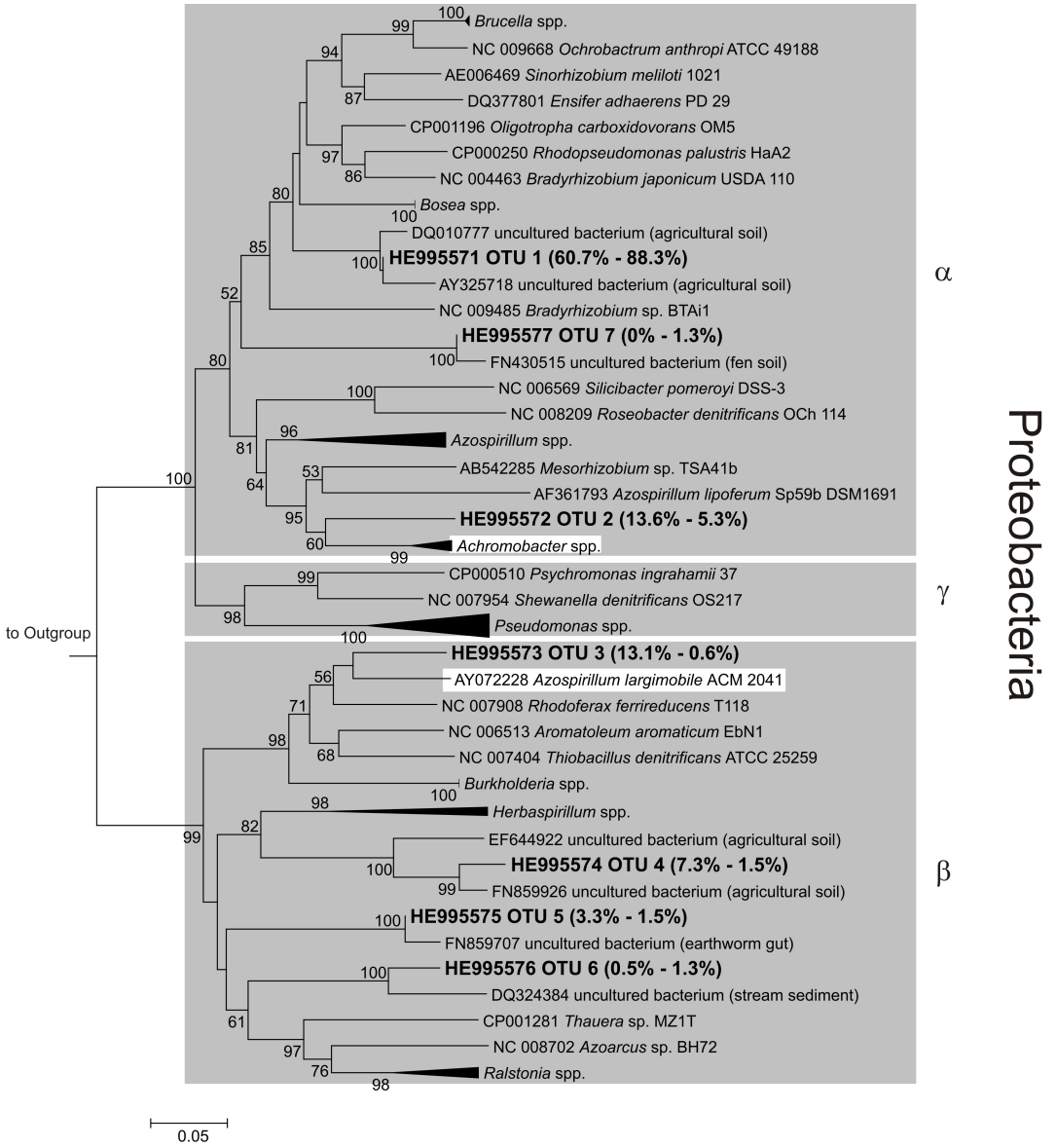

Figure 8: Phylogenetic tree of *nosZ* OTU representatives detected in 0 to 20 cm and 20 to 40 cm fen soil. The tree was calculated based on translated amino acid sequences. OTUs were grouped at a species-level threshold dissimilarity of 20%. Numbers preceeding sequence names refer to sequence accession numbers of reference sequences from public databases. Values given in parantheses 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.

3.6 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). 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 (Table 5), indicating a high relative abundance of dissimilatory nitrate reducers in fen soil. Copy numbers of *nirK*, *nirS*, and *nosZ* were lower than *narG* copy numbers, indicating a higher abundance of nitrate reducers than of denitrifiers (Table 5). Copy numbers of *nirS* were about 100x and 10x higher than copy numbers of *nirK* and *nosZ*, respectively, in both soil layers (Table 5).

Table 5: Abundance of denitrification-associated genes in fen soil.

	0 to 20 cm		20 to 40 cm	
Gene marker	Copy No. per 16S rRNA gene (%) [1]	Copy No. per ng DNA	Copy No. per 16S rRNA gene (%) [2]	Copy No. per ng DNA
narG	$(7.1 \pm 0.6) \cdot 10^{0}$	$(3.7 \pm 0.2) \cdot 10^{4}$	$(2.7 \pm 0.3) \cdot 10^{0}$	$(2.3 \pm 0.3) \cdot 10^{4}$
nirK	$(4.4 \pm 0.4) \cdot 10^{-3}$	$(2.3 \pm 0.2) \cdot 10^{-1}$	$(2.0 \pm 0.7) \cdot 10^{-3}$	$(3.9 \pm 1.2) \cdot 10^{0}$
nirS	$(2.5 \pm 1.1) \cdot 10^{-1}$	$(1.3 \pm 0.6) \cdot 10^{2}$	$(1.4 \pm 0.4) \cdot 10^{-1}$	$(2.6 \pm 0.5) \cdot 10^{2}$
nosZ	$(1.6 \pm 0.2) \cdot 10^{-2}$	$(8.5 \pm 0.7) \cdot 10^{1}$	$(5.7 \pm 0.5) \cdot 10^{-3}$	$(4.9 \pm 0.4) \cdot 10^{1}$

[1] 16S rRNA gene copy numbers were (... ± ...) x 106 per ng DNA.
[2] 16S rRNA gene copy numbers were (... ± ...) x 106 per ng DNA.

4 Discussion

4.1 pH neutral fen soil as N_2O sink and hotspot of denitrifier diversity

Peatlands are important ecosystems in the northern hemisphere and cover more than 30% of Finland [9]. Climate warming has a strong impact on peatlands [19]. No significant release of N_2O from fen soil was observed, indicating that under the present conditions fen soil is a not source of N_2O. N_2O emissions from natural wetlands are highly variable, and many water -saturated soils are also sinks for N_2O [11, 40]. Indeed, fen soil in situ consumed initially produced N_2O from nitrate or ammonium (Figure 1), indicating that Puukkosuo fen soil can be a sink for N_2O. Undrained, pH-neutral fens are sources of N_2, and can be sources or sinks for N_2O depending on environmental conditions [1, 20, 35]. 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 [20]. Nitrate concentrations in Puukkosuo fen soil were likewise low (Table 1). The source- or sink-strength for N_2O is correlated with the amount of atmospheric N-deposition which can resupply nitrate for denitrification [20]. In a nutrient-rich sedge fen, lower below-surface N_2O concentrations than at atmospheric equilibrium are observed, likely because N_2O consumption dominates N_2O production [1, 20]. Thus, mainly complete denitrification to N_2 occurs in pristine pH-neutral fens at in situ nitrate concentrations, [20, 35]. Thus, the absence of *in situ* N_2O emission from Puukkosuo fen soil is likely due to complete denitrification to N_2 as the main or only end product (Figure 1). Nitrate addition does not neccessarily increase the product ratio of N_2O to total N-gases, which might be due to concomitant N_2O reduction or uptake of excess nitrate by plants [35]. Indeed, added nitrate only caused temporary *in situ* emission of N_2O and the initially produced N_2O was subsequently consumed (Figure 1). This indicates that the fen might act as a sink rather than a source of N_2O. This is also supported by microcosm studies with fen soil. Unsupplemented fen soil from both sampled soil layers produced N_2O in acetylene-amended microcosms, demonstrating the denitrification potential of the fen soil. However, nearly no N_2O was produced in the absence of acetylene and initially produced N_2O was subsequently consumed. This indicates complete denitrification to N_2 in the fen.

nosZ copy numbers in 0 to 20 cm fen soil were of a similar magnitude nitrite reductase copy numbers (Table 5), indicating that a high percentage of denitrifiers in that soil possessed a complete denitrification pathway. The ratio of nitrite to nitrous oxide

reductases was larger in 20 to 40 cm fen soil, indicating an increased amount of denitrifiers lacking nitrous oxide reductase in the lower soil layer. The ratio of nitrite reductase genes to N_2O reductase genes is highly variable in soils, and often nitrite reductase copy numbers largely exceed N_2O reductase copy numbers [14, 26]. Approximately 2/3 of the genomes of cultured denitrifiers harbor *nosZ* [16]. Indeed the ratio of N_2O to total N-gases was lower in microcosms with 0 to 20 cm fen soil than in 20 to 40 cm fen soil when nitrate or nitrite were supplied (Figure S2). This was also reflected in consumption potentials of supplied N_2O: even though both soil layers consumed N_2O, consumption was about 2x higher in 0 to 20 cm than in 20 to 40 cm fen soil (Figure 3 B).

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

Supplemental nitrate and nitrite resulted in immediate N_2O production in fen and soil after internal nitrate and nitrite were consumed. Stimulation was greater with nitrite than with nitrate in both soil layers (Figure 3 A). This reflects the fact that all true denitrifiers use nitrite as electron acceptors, while many cultured denitrifiers lack the ability to use nitrate as electron acceptor [21, 44]. Stimulation was also greater in the top soil layer of both soil types (Figure 3 A), reflecting a greater denitrification potential of the top soil. In other wetland soils, denitrification potentials are also highest in the top soil layers [28]. In 20 to 40 cm fen soil, N_2O production decreased with increasing nitrate concentrations. The ratio of N_2O to total N-gases was lower in 0 to 20 cm fen soil than in 20 to 40 cm fen soil, indicating a more efficient N_2O reduction in the upper soil layer. N_2O produced in lower layers of fen soil can diffuse upwards and be further reduced to N_2 by denitrifiers in upper soil layers, and thus emission of N_2O into the atmosphere can be prevented [1, 11, 28]. Fens can also act as a sinks for N_2O [11, 28].

The analysis of denitrification-specific gene markers revealed similarities in the community composition of nitrate and N_2O reducers (as indicated by detected *narG* and *nosZ*, respectively) in 0 to 20 cm and 20 to 40 cm fen soil, while significant differences were observed in the community composition of the nitrite reductase genes *nirK* and *nirS* (Figure 4), indicating that nitrite reductases show a higher variability in fen soil than nitrate and N_2O reductases. Nitrite reductase community composition is highly variable in other types of peatland soils, including frost affected systems, while variations in nitrate and N_2O reductase community composition are much less pronounced [26, 29]. Indeed, the distribution of nitrite reductases is much stronger affected by changes in

environmental conditions than those of nitrate or N_2O reductases [2, 4, 8]. Nitrite reductase genes from fen soil affiliated with *Proteobacterial nirK/S* (Figure 6, Figure 7), indicating that *Proteobacteria* play an important role in the denitrification in pH-neutral fen soil. *Proteobacteria*-affiliated sequences were also detected for *narG* and *nosZ* (Figure 5, Figure 8), underlining the importance of this group. Denitrification-associated genes related to *Proteobacteria* are also found in acidic peatlands, such as acidic fen soils or frost-affected peatlands [28, 26, 29], indicating that *Proteobacteria* might be general peatland denitrifiers. Sequences of *narG* moreover affiliated with *Actinobacterial narG* (10-30%; Figure 5), indicating a that *Actinobacteria* are further important players involved in nitrate reduction and denitrification in pH-neutral fen soil. *Actinobacteria* are common in soils, and are in general considered to be more tolerant to more extreme environmental conditions such as low pH or low temperature [25, 43]. *Actinobacteria* and *Actinobacteria*-affiliated gene markers are moreover frequently detected in a variety of peatlands including acidic fen soils, frost-affected tundra and palsa peat soils [28, 26, 29]. However, in those more extreme environments, *Actinobacteria* often dominate the nitrate reducer communities [26, 29]. The nitrate reducer community in pH-neutral fen soil also contained a substantial portion of *Deinococci*-affiliated *narG* (Figure 5), which are not detected in the above mentioned more extreme habitats such as acidic fens, frost-affected tundra and palsa peat soils [28, 26, 29]. The collective data indicate that (i) a core nitrate reducer/denitrifier community might be common to all kinds of (northern) peatlands, (ii) the community composition is affected by environmental parameters, and (iii) 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 or cold temperatures.

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6 Supplemental Figures

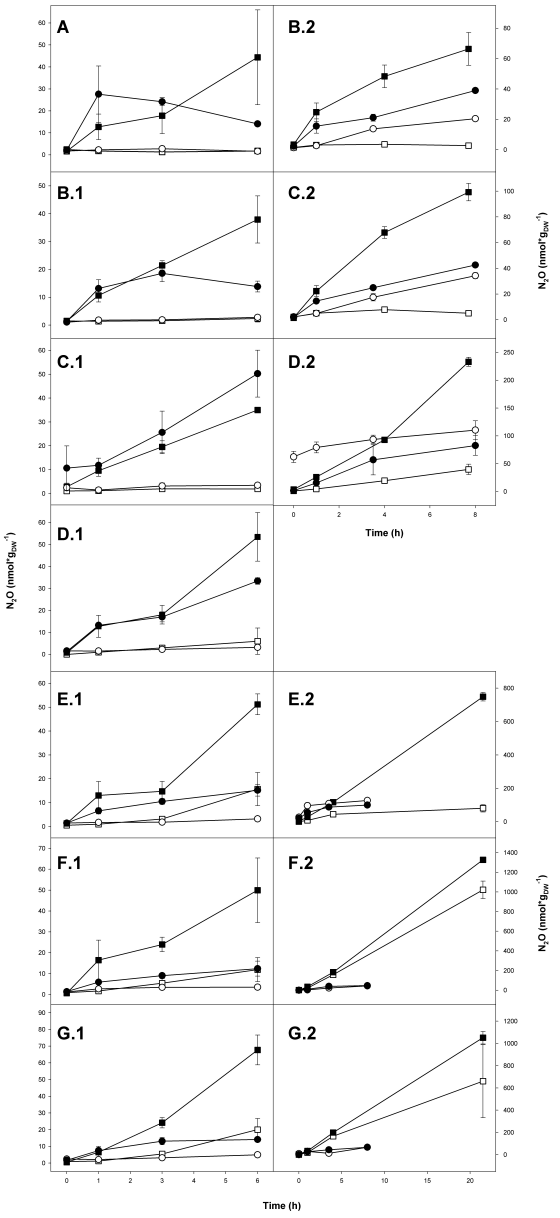

Figure S1: Effect of supplemental nitrate (1) and nitrite (2) on N_2O production and consumption in microcosms with fen soil. Squares and circles represent fen soil from 0 to 20 cm and 20 to 40 cm depth, respectively. Microcosms with and without acetylene are represented by closed and open symbols, respectively. 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.

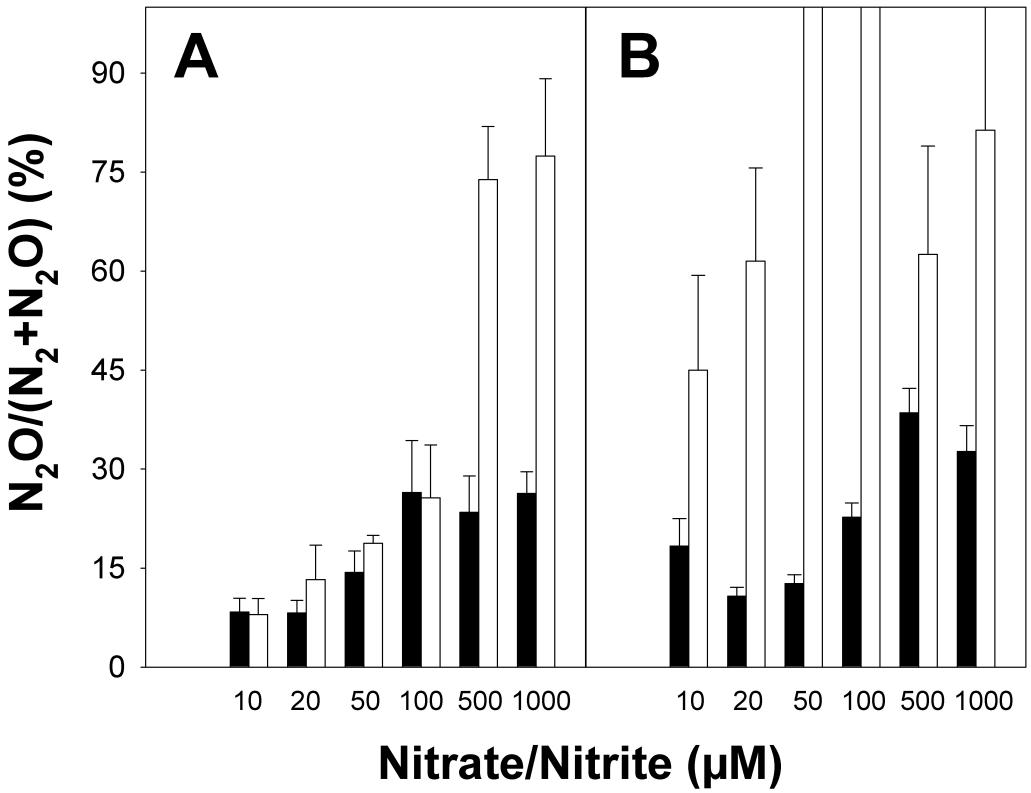

Figure S2: Effect of supplemental nitrate (black) or nitrite (white) on the ratio of N_2O to total N gases in anoxic microcosms with fen soil from 0 to 20 cm (A) and 20 to 40 cm (B) depth. Mean values and standard errors of three replicates are shown.

Stability of Methanogenic Diversity in an Acidic Fen under the Influence of Experimental Drought

Manuscript in preparation

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The response of a methanogenic community of an acidic fen to enhanced drought and subsequent rewetting was investigated. Experimental drought reduced methanogenic capacities of fen soil and prolonged initial lag phases before the onset of methanogenesis. Moreover, oxygen strongly inhibited methanogenic potentials. Based on the Shannon diversity index, experimental drought reduced the detected sequence diversity of the structural gene marker *mcrA* (encoding the alpha-subunit of the methyl coenzyme M reductase), while rewetting restored detected diversity to the original level. qPCR analysis of *mcrA* indicated that methanogens were most abundant in 20 to 30 cm soil, while highest *mcrA* transcript numbers were found in the 0 to 20 cm soil. The relative abundance of *mcrA* genes was reduced by the experimental drought, while rewetting increased the relative abundance of *mcrA*. Experimental drought did not affect transcript to gene ratios of *mcrA*, while a lower transcript to gene ratio was detected after rewetting. Gene-level TRFLP-profiles indicated slight seasonal shifts in the community composition of fen methanogens which were more pronounced than possible drought effects. Detected *mcrA* were affiliated with *mcrA* of *Methanocellaceae*, *Methanomicrobiales*, *Methanobacteriaceae*, and *Methanosarcinaceae*. Transcript-level TRFLP-profiles indicated a shift in the active methanogenic community after the drought, while rewetting restored similar transcript profiles in control and drought plots. The collective data indicate that experimental drought affected the abundance and acitivity but not the community composition of fen methanogens.

1 Introduction

Climate change might lead to a higher frequency of extreme weather events like prolonged drought and extreme precipitation events in future years [61]. These events will likely change conditions in peatland soils, which are sources of the greenhouse gas methane. Methane (CH_4) is an important greenhouse gas with the second-largest radiative forcing after CO_2 (about 20% of the total radiative forcing) [12]. From 1750 to 2000, the CH_4 concentration in the atmosphere increased from 0.7 to 1.8 ppm, but concentrations have remained fairly stable since 1990 [12]. CH_4 is mainly produced biologically via methanogenesis, which is an important part of the anaerobic degradation processes, e.g., in the digestive system of ruminants or termites, in wetland and forest soils as well as in oceans [6]. Wetlands are the most important single source of naturally produced CH_4, and their emission accounts for 25% of the global CH_4 emissions [62]. Wetland soils are usually water-saturated, thus anoxic conditions are known to occur already in near-surface layers [5]. Anoxic processes occur in wetlands, and alternative electron acceptors (NO_3^-, Mn_4^+, Fe_3^+, SO_4^{2-} , and HCO_3^-) are sequentially used for the oxidation of carbon [71].

Peatlands are known to assimilate more CO_2 from the atmosphere than they release into it, and the carbon stored in peatlands accounts for about 30% of all terrestrial soil carbon [17]. Peatlands cover about 3% of the earth surface [4]. In northern peatlands, about 20% of the assimilated CO_2 is released into the atmosphere as CH_4 [53]. The studied minerotrophic fen Schlöppnerbrunnen produces and emits CH_4 *in situ* [32, 33].

CH_4 is produced by obligate methanogenic archaea via hydrogenotrophic, acetoclastic and methylotrophic pathways [21]. CO_2, formate, and alcohols are used as electron acceptors in hydrogenotrophic methanogenesis, which is most widespread as it is found in all methanogenic orders [2]. Acetoclastic and methylotrophic methanogenesis use acetate and methylated compounds (e.g. methanol, methylamine), respectively, as electron acceptors and occcur within the *Methanosarcinales* (methylotrophic also in *Methanosphaera*, *Methanobacteriales*) [2, 21]. Most *Methanosarcinales* produce CH_4 both hydrogenotrophically and acetoclastically, while members of the family *Methanosaetaceae* are obligate acetoclastic methanogens [21]. Methanogenesis in peatlands can be dominated by hydrogenotrophic or acetoclastic methanogenesis [16, 23, 42]. However, CH_4 production in soil from the analysed fen site is dominated by hydrogenotrophic methanogenesis [32, 65]. The methanogenic archaeal community in the fen Schlöppnerbrunnen consists of *Methanocellales*, *Methanomicrobiales*, *Methanobacte-*

riales, and *Methanosarcinales* as revealed by 16S rRNA gene analyses [8, 20, 24, 65].

Methanogenesis in soils is regulated by pH, temperature, groundwater level, the amount of organic material, and the amount of electron acceptors (Segers, 1998). Alternative electron acceptors such as nitrate, sulfate, and Fe(III) usually inhibit methanogenesis as nitrate-, sulfate- and iron-reducers outcompete methanogens for electrons [37]. However, those processes also occur simultaneously *in situ* when H_2 concentrations are sufficiently high [5]. Indeed, nitrate reduction/denitrification, sulfate and iron reduction also occur in the fen Schlöppnerbrunnen [48, 49, 52, 56]. Highest CH_4 production rates are found at or below the water table in both oligo- and ombrotrophic peatlands, and lowering of the water table reduces the amount of methane produced in soil [3, 26].

The methyl-coenzyme M reductase catalyses the formation of methane from methyl-coenzyme M and is therefore a key enzyme in methanogenesis [2]. Thus the gene *mcrA*, coding for the α-subunit of the methyl-coenzyme M reductase, has been used as a structural gene marker to characterize the methanogenic community in different habitats [14, 15, 25, 40].

The present study aims to investigate the effects of prolonged experimental drought and subsequent rewetting on methanogenic potentials in an acidic fen. Moreover, the influence of drought and rewetting on the abundance and community structure of methanogens was assessed.

2 Material and Methods

2.1 Study site and experimental setup

The minerotrophic fen Schlöppnerbrunnen is located in the Lehstenbach catchment in the Fichtelgebirge, Bavaria, Germany (50° 07' 53" N, 11° 52' 51" E), at approximately 700 m above sea level. The fen has a mean annual air temperature of 5.3°C and a mean annual precipitation of 1160 mm [11]. The fen soil is a Fibric Histosol on granite bedrock. Vegetation consists mainly of *Molinia caerulea*, *Eriophorum vaginatum*, *Carex canescens*, and *Juncus effusus* [49].

In 2006, 2007 and 2008, the water table was artificially lowered in 3 treatment (i.e., drought) plots (size 7.2 m x 5 m). Roofs were erected to shield the plots from rain water, and additional drainage was accomplished by ditches. The roofs had large open side walls to minimize temperature and wind speed effects. Additionally, 3 untreated plots served as control sites. Roofs and drainage on the drought plots were installed on August 14th 2006, May 10th 2007 and June 10th 2008, and kept in place until September 26th, July 29th and August 7th, respectively. On average, experimental drought lowered the water table to 0.21 m, 0.25 m and below 0.6 m below the surface in 2006, 2007, and 2008, respectively. After the experimental drought period, roofs and drainage were removed and drought plots were rewetted with artificial rainwater. For more detailed description of the experimental setup refer to [45]. The water table in the drought plots was considerably lowered during the course of the experiment in each year, even though a slight water table drawdown was also observed in the control sites [45]. Soil was sampled for microcosm experiments before the start of the experimental drought in 2006 (August 14th) and 5 days after rewetting (October 2nd). Soil samples were taken from each plot from soil depths 0 to 10 cm and 30 to 40 cm with a soil corer, as those depths show differences in methanogenic potential [65]. Samples were stored at 4°C and processed within 2 days. Sampling for molecular analyses was done before the start of the experimental drought in 2008 (June 09th), at the end of the experimental drought period (July 27th), and 4 days after rewetting (August 11th). Soil samples were taken from each plot from soil depths 0 to 40 cm at 10-cm intervals with a soil corer. Soil samples were quick-frozen in liquid nitrogen and stored at -80°C until further processing.

2.2 Microcosms

Methanogenic capacities were assessed in anoxic fen soil microcosms. Soil of each plot and soil layer was homogenized prior to the experiment. 4.5 g of soil were diluted 20-fold with artificial pore water [65] and placed in 125-ml infusion flasks. Flasks were sealed with gas-tight rubber stoppers. The gasphase was 100% N_2. One microcosm was set up from each plot (i.e., control and drought plots 1 to 3) and sampled soil layer (i.e., 0 to 10 cm and 30 to 40 cm). Microcosms were incubated at 15°C in the dark. CH_4 was quantified with a Hewlett-Packard 5980 series II gas chromatograph (Hewlett-Packard Paolo Alto, CA) equipped with a flame ionization detector [65].

The effect of oxygen (O_2) on methanogenic potentials of fen soil was assessed in microcosms supplemented with different O_2 concentrations. Microcosms were supplemented with 100 μM formate to stimulate methanogenesis. 20 g of soil from sites adjecent to the treatment plots (sampled in 2010) were diluted 5-fold with artificial pore water and placed in 500-ml infusion flasks. Flasks were sealed with gas-tight rubber stoppers. A preincubation of 7 days was conducted under anoxic conditions (i.e., with 100% argon in the gasphase) to reduce alternative electron acceptors present in Schlöppnerbrunnen fen soil [8, 65]. After preincubation, 100 μM formate and different amounts of O_2 (resulting in headspace concentrations of 0%, 0.17%, 0.6% and 23%) were supplied. Each O_2 concentration was tested in three replicate microcosms. Difluoromethane is an inhibitor of methanotrophy [44], and was thus added to the microcosms (1.5% [vol/vol]) to inhibit methanotrophy that might otherwise occur in the presence of O_2. The inhibitory effect of difluoromethane on methanotrophy in oxic fen soil microcosms had been tested prior to the experiments (Figure S1). Microcosms were incubated at 20°C in the dark on an overhead-shaker; O_2 concentrations were monitored and readjusted on a daily basis.

2.3 Extraction of nucleic acids

Nucleic acids from all sampled soil layers were extracted using a bead-beating protocol [18] followed by separation of DNA and RNA using the Qiagen RNA/DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

2.4 Amplification of mcrA

mcrA was amplified using the primers ME1 (GCM ATG CAR ATH GGW ATG TC)/ME2 (TCA TKG CRT AGT TDG GRT AGT) [19] from DNA extracts of one treatment and

one control plot. The PCR was preceeded by an initial denaturation (94°C, 5 min). During PCR, denaturation and elongation were conducted at 94°C and 72°C, respectively. The annealing temperature was lowered from 55°C to 50°C in 10 pre-cycles, followed by 25 cycles with an annealing temperature of 50°C. Denaturation, annealing and elongation were for 0.75 min, 0.75 min and 1.5 min, respectively. The PCR was completed by an end-elongation of 10 min at 72°C.

2.5 Cloning, screening, and sequencing

PCR products of *mcrA* were gel-purified using the Montage Gel Extraction Kit (Millipore Corporation, Bedford, MA, USA). Purified PCR products of each plot type and timepoint (i.e., all 4 soil depths) were pooled prior to ligation. The purified PCR products were ligated into the pGEM-T vector (Promega, Mannheim, Germany) and transformed into competent *E. coli* JM109 (Promega) according to the manufacturer's protocol. Clones were screened for insert-positive vectors via amplification with primers $M13_{uni}$/$M13_{rev}$ [41]. Prior to sequencing, M13-PCR products were purified using the Millipore Multiscreen 96-well filtration system (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's protocol. The sequencing was done commercially by Macrogen (Seoul, South Korea).

2.6 Sequence analysis

Sequences from the different clone libraries were in silico tagged with barcodes to allow for identification of the sequences after analysis. Sequences were clustered [i.e., assigned to operational taxonomic units (OTUs)] at species-level threshold distances of 16% (see below) using the JAguc2 pipeline (http://wwwagak.informatik.uni-kl.de/research/JAguc/; [46]). In brief, sequences are pairwisely aligned prior to calculation of a distance matrix and clustered with the average similarity method, which requires a minimum of computing time. Sequence subsets with different barecodes can be combined and clustered together, as JAguc2 allows identification of sequences in the clusters according to their barcodes. The closest relatives of cluster representatives were determined using BLAST [1]. Cluster representatives were edited, translated in silico, and aligned with reference sequences using the ClustalW algorithm implemented in MEGA 5.0 [35]. 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. Stability of tree topologies was assessed by calculating 10 000 bootstrap

replicates [54]. Diversity measures were calculated as described [22, 70].

2.7 Comparative analysis of archaeal 16S rRNA genes and mcrA

Pairwise similarities of *mcrA* and archaeal 16S rRNA genes were compared to evaluate the validity of deducing species-level OTUs from environmental *mcrA* sequences as described earlier [24, 30, 34, 47]. 88 *mcrA* sequences from pure cultures were retrieved from GenBank along with the associated 16S rRNA sequences. Fragments of *mcrA* and 16S which correspond to regions amplified by the primers ME1/ME2 used in this study (*mcrA*) and 4Fa/1492R [36] (16S) were analyzed. Threshold similarities for species-level OTU definition were estimated as previously described [47]. A 16S rRNA gene similarity of ≥97% corresponded to an average *mcrA* sequence similarity of 90% (Figure S2). This is in good agreement with findings from an earlier study with a more limited set of methanogens [58]. 90% of methanogens with a 16S rRNA gene similarity of ≥97% had a *mcrA* sequence similarity of ≥84%. Thus, this value was used as threshold similarity (i.e., any two sequences that are less similar than the threshold similarity with a very high probability belong to different species) in further analyses.

2.8 Reverse transcription

Reverse transcription of extracted RNA into cDNA was conducted using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol.

2.9 Quantitative PCR

Quantitative PCR (qPCR) was performed with an iQTM5 Real-Time qPCR cycler (Bio-Rad, Munich, Germany). All qPCR reactions were run in 3 replicates. Negative controls were included, and standard curves were determined for each qPCR. qPCR reactions were performed in 20 μl reaction mixtures that were composed of SensiMix Plus SYBR Green & Fluorescein (Bioline GmbH, Luckenwalde, Germany), 1.2 μl 50 mM $MgCl_2$ (Bioline GmbH), 150 ng/μl bovine serum albumin, 0.2 - 1.6 pM of each primer (Biomers, Ulm, Germany), 5 μl of template DNA and sterilized deionized water. The primer set ME1/ME2 was used for qPCR of *mcrA*, yielding about 760 bp-sized fragments. The primer set Arc344F/Arc915R [51, 59] was used for quantification of the archaeal 16S rRNA genes, yielding about 570 bp-sized fragments. The *mcrA* qPCR assay contained

the following steps: initial denaturation (95°C, 10 min), 40 cycles of denaturation (95°C, 45 s), primer annealing (61°C, 30 s), and elongation (72°C, 1 min), and a final elongation (72°C, 5 min). The archaeal 16S rRNA qPCR assay contained the following steps: initial denaturation (95°C, 10 min), 40 cycles of denaturation (95°C, 40 s), primer annealing (55.7°C, 40 s), and elongation (72°C, 40 min). In the case of *mcrA*, fluorescence was recorded in an additional step after elongation (80°C, 20s) to avoid interference of primer dimers with the fluorescence measurement. Agarose gel electrophoresis, melting curve analysis, and sequencing of amplicons generated with the same primers indicated that the amplification was specific. The lower limits of quantification were 101 gene copy numbers μl^{-1} of DNA extract. 16S rRNA gene copy numbers were determined concomitantly for all environmental samples in order to quantify mcrA-harboring methanogens in soil relative to the total archaeal population [70].

Inhibition of qPCR was assessed as previously described [70]. Unless otherwise stated, inhibition corrected structural gene copy numbers were normalized per ng DNA and 16S rRNA gene copy numbers, since such normalized gene copy numbers are less sensitive to varying DNA extraction efficiencies (that is, DNA extraction bias) than copy numbers per gram dry weight soil.

2.10 Terminal restriction fragment length polymorphism analysis (TRFLP)

Triplicate qPCR reations of *mcrA* from DNA and cDNA were pooled and gel purified using the Montage Gel Extraction Kit (Millipore Corporation, Bedford, MA, USA) prior to TRFLP analysis. The purified PCR products were digested with Mung Bean nuclease (New England Biolabs, Frankfurt am Main, Germany) to remove single-stranded DNA and reduce the probability of pseudo-terminal restriction fragments [9]. The restricted DNA was purified using the Millipore Multiscreen 96-well Filtration System (Millipore Corporation, Bedford, MA, USA). *mcrA* PCR products were digested with the restriction enzyme *Hinf*I (New England Biolabs, Frankfurt am Main, Germany). Polyacrylamide gel electrophoresis was performed as described previously [48]. Terminal restriction fragments (TRFs) were assigned to *mcrA* sequences via *in silico* TRF analysis in MEGA 5.0.

3 Results

3.1 Effect of experimental drought on methanogenic potentials in acidic fen soil

In microcosms with soil from 0 to 10 cm depth, methanogenic potentials were similar in control and drought plots before the onset of experimental drought (Figure 1). An initial lag phase before the onset of CH_4 production was observed in all microcosms (Figure 1). Such a lag phase has also been observed in previous studies [65]. The length of the initial lag phase was 5 days with soil from both plot types. After the experimental drought, a higher CH_4 production was observed in control than in drought plot microcosms (Figure 1 A). 4.7 and 2.0 μmol $CH_4 \cdot g_{DW}^{-1}$ were produced in microcosms with control and drought plot soil, respectively. Moreover, the initial lag phase before the onset of CH_4 production was longer in microcosms with soil from drought plots (7 days) than in microcosms with soil from control plots (5 days).

Methanogenic potentials were lower in microcosms with soil from 30 to 40 cm depth than in microcosms with soil from 0 to 10 cm depths. This is in good agreement with previous studies, that detected highest methanogenic potentials in surface layer fen soil [8, 65]. In microcosms with soil from 30 to 40 cm depth, CH_4 production was highly similar in control and drought plot microcosms before the onset of the experimental drought, and 0.1 μmol $CH_4 \cdot g_{DW}^{-1}$ were produced within 21 days of anoxic incubation (Figure 1 1B). After the drought period, CH_4 production in microcosms with soil from drought plots was strongly reduced, amounting to 0.05 μmol $CH_4 \cdot g_{DW}^{-1}$, whereas CH_4 production in microcosms with soil from control plots was higher than before the drought period, amounting to 0.3 μmol $CH_4 \cdot g_{DW}^{-1}$ (Figure 1). The initial lag phase before the onset of CH_4 production was 8 days in microcosms with soil from both plot types before the drought period, and 5 and 14 days in microcosms with soil from control and drought plots, respectively, after the drought period.

3.2 Effect of oxygen on methanogenic potentials of fen soil

CH_4 concentrations increased linearly in anoxic microcosms with 0 to 20 cm fen soil from day 0 to day 15 by approximately 60 μmol $CH_4 \cdot g_{DW}^{-1}$, while no CH_4 production was observed in microcosms with an O_2 headspace concentration of 23% (Figure 2). In microcosms with an O_2 headspace concentration of 0.17%, minor CH_4 production was observed. Approximately 9 μmol $CH_4 \cdot g_{DW}^{-1}$ accumulated within 15 days of incubation

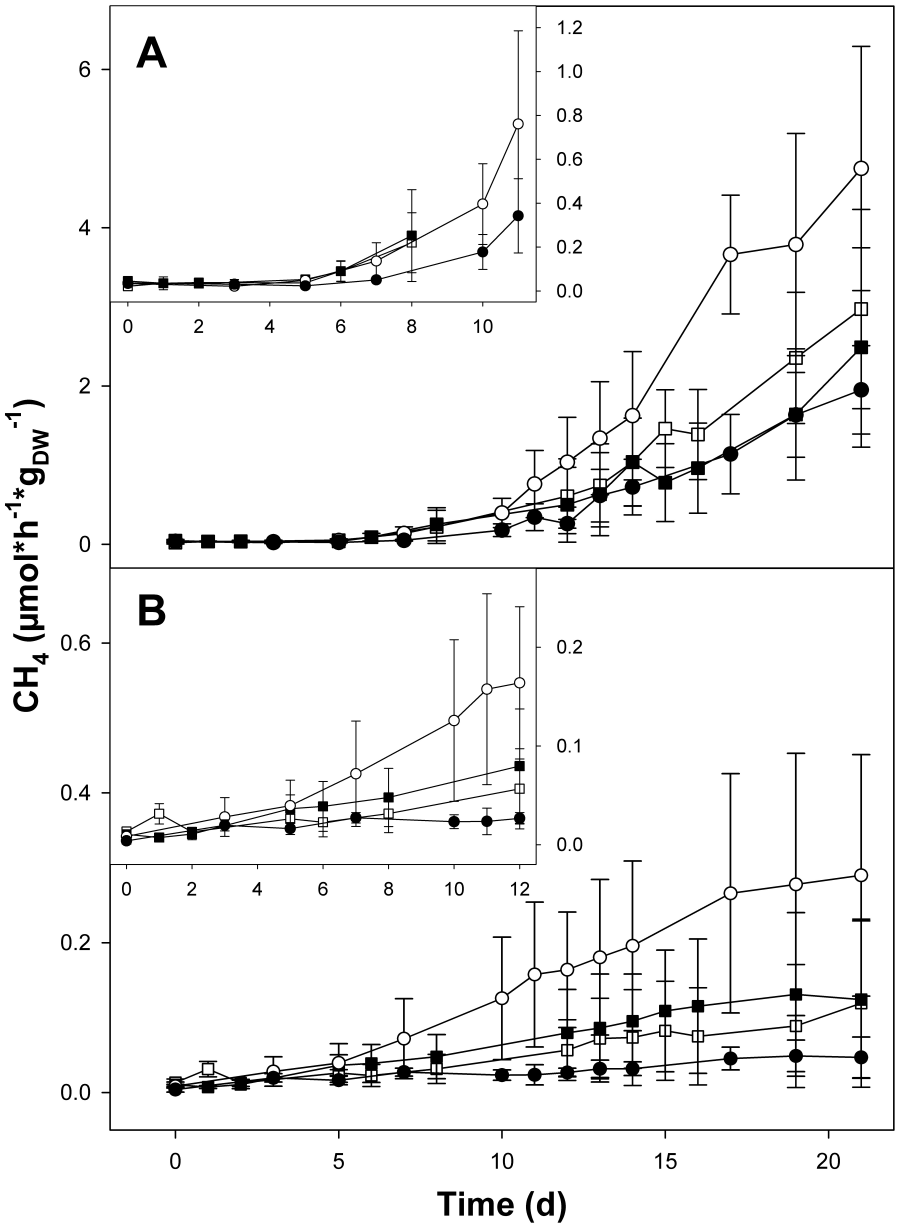

Figure 1: Effect of drying and rewetting on methanogenic potentials in the fen Schlöppnerbrunnen. Mean values and standard errors of three replicate microcosms are shown. Squares and circles represent microcosms with soil sampled before the drying period (August 14th 2006) and after rewetting (September 27th 2006), respectively. Open and closed symbols represent control and drought plots, respectively. A: 0 to 10 cm fen soil, B: 30 to 40 cm fen soil.

(Figure 2). However, the observed CH_4 production might also result from short anoxic periods, as it proved difficult to keep up constant O2 headspace concentrations due to O_2 consumption. 0.6% of O_2 in the headspace sufficed to completely inhibit CH_4 production in fen soil microcosms (Figure 2), indicating that even very small O_2 concentration sufficiently inhibited methanogenesis in fen soil.

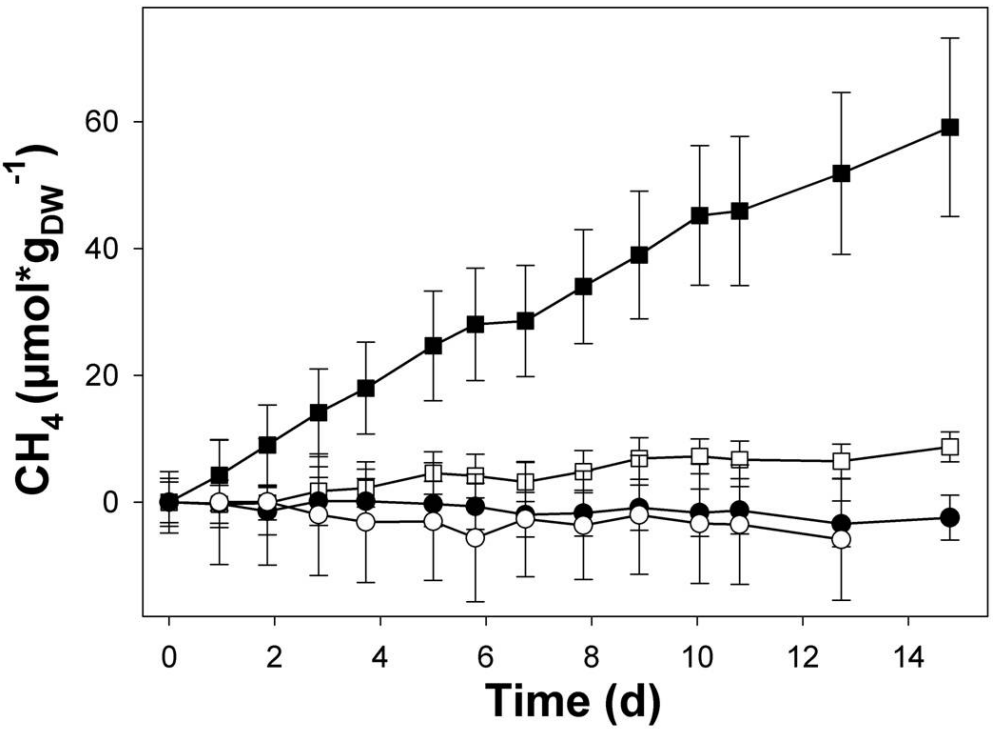

Figure 2: Effect of O_2 on methanogenic potentials in fen soil microcosms. Mean values and standard errors of three replicate microcosms are shown. Closed squares represent anoxic microcosms, open squares represent microcosms with 0.17% headspace concentration of O_2, closed circles represent microcosms with 0.6% headspace concentration of O_2, open circles represent microcosms with 23% headspace concentration of O_2. All microcosms contained difluoromethane (1.5% [vol/vol]) to inhibit methanotrophy. 100 μM formate were added at day 0 to microcosms that were preincubated for 7 days under anoxic conditions.

3.3 Phylogeny of detected fen methanogens

306 quality-checked *mcrA* sequences were obtained from six clone libraries yielding 8 species-level OTUs (Table 1). Library coverages ranged between 97 and 100% (Table 1), indicating that the number of sampled clones was sufficient. The highest number of OTUs was detected in soil from control plots before the drought, while the lowest number of OTUs was detected in soil from drought plots after drought (Table 1). Most OTUs were only distantly related to *mcrA* from cultured organisms, indicating species-level novelty of fen methanogens (Figure 3). OTUs 1, 2, 6, and 7 (i.e., 64% of all sequences) affiliated with sequences of the *Methanocellaceae*, and OTU 7 was closely related to the *mcrA* of *Methanocella paludicola*. OTUs 3 and 8 (i.e., 17% of all sequences) affiliated with *mcrA* of the *Methanosarcinaceae*, OTU 4 (11% of all sequences) and OTU 5 (8% of all sequences) affiliated with *mcrA* of *Methanomicrobiales* in general and the family of *Methanobacteriaceae*, respectively (Figure 3, Figure 4).

Table 1: Analyses of *mcrA* derived from fen soil.

Treatment	Timepoint	No. of sequences	Library coverage (%) [1]	No. of OTUs observed	No. of OTUs estimated (Chao1)	H [2]	E [3]
Control	t_0	81	98.8	8	8	1.71	0.82
	t_1	35	97.1	7	8	1.64	0.84
	t_2	49	100	7	7	1.77	0.91
Drought	t_0	50	98.0	6	6	1.45	0.81
	t_1	53	98.1	4	4	1.11	0.80
	t_2	38	97.4	7	7	1.84	0.94

[1] Percent library coverage. $C = (1 - \frac{n_s}{n_t}) \cdot 100$ (n_s=sequences that occur only once, n_t=total number of sequences)

[2] Shannon-Weaver diversity index.

[3] Species Evenness.

The Shannon Weaver diversity index was significantly lower in the drought plot at the end of the drought period (Table 1), while it did not change significantly in the control plot. After rewetting, *mcrA* diversity in the drought plot was in the same magnitude as before the drought period, and was similar to the *mcrA* diversity in the control plot.

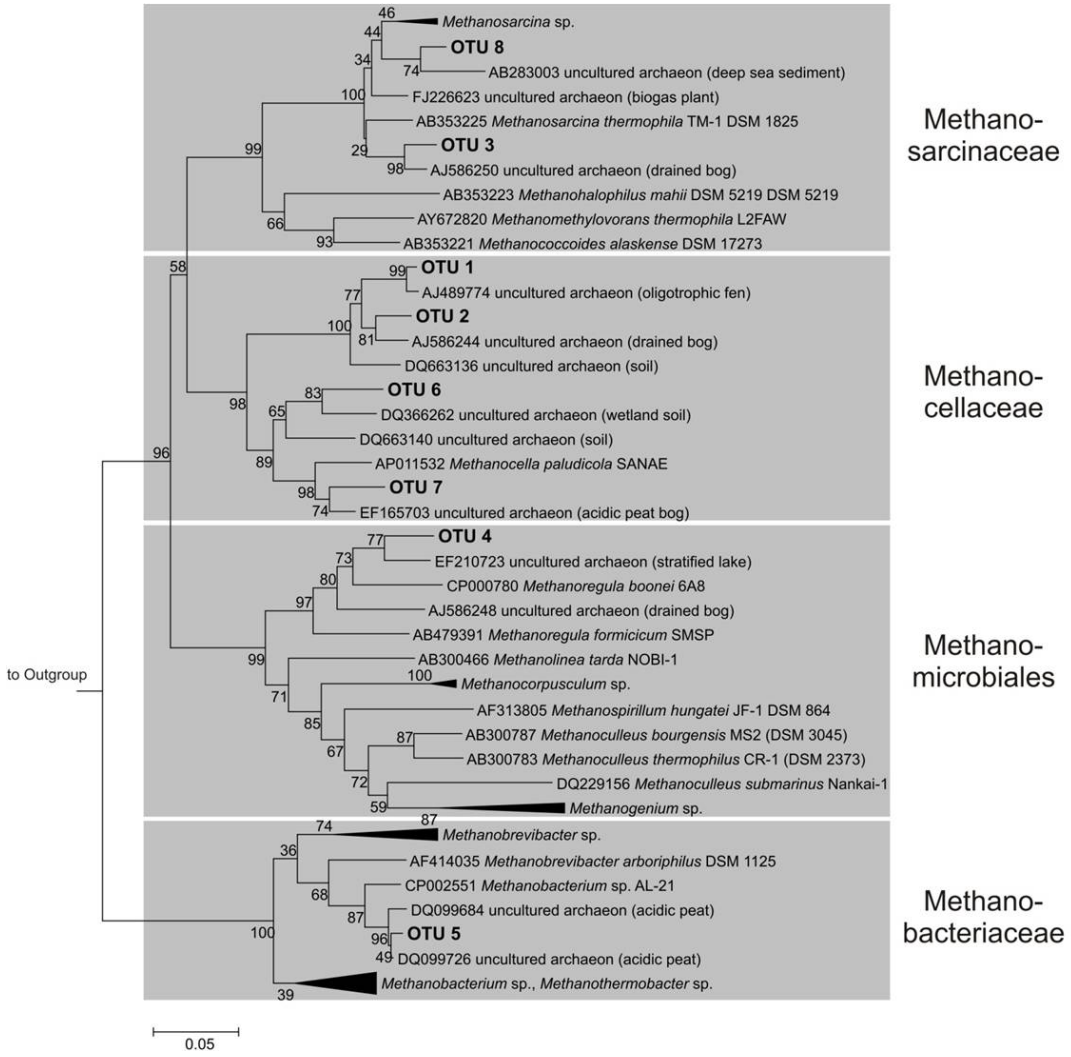

Figure 3: Phylogenetic tree of *mcrA* retrieved from the Schlöppnerbrunnen fen. The tree is based on translated amino acid sequences. One representative sequence per OTU is shown. Codes preceeding sequences names represent sequence accession numbers in public databases. Grey boxes indicate branches where the majority of sequences are derived from reference strains of a certain phylogenetic group. 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 *mcrA* of *Methanococcus maripaludi*s S2 (NC 005791).

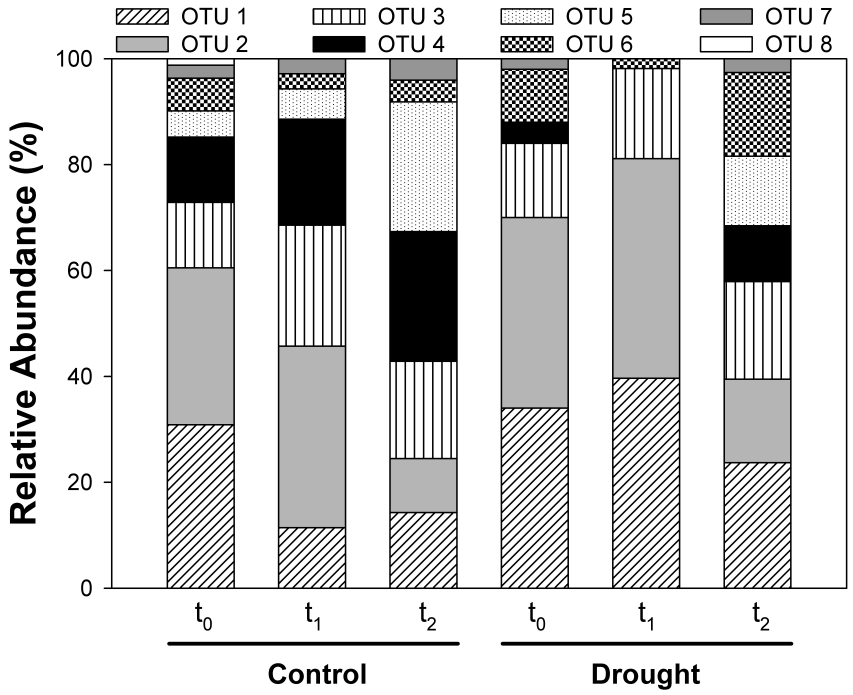

Figure 4: Relative abundance of *mcrA* derived OTUs retrieved from one control and one drought plot at three timepoints of the experimental drought in 2008. Sequences were assigned to OTUs using a sequence similarity threshold of 84%. t_0=before drought (June 09th 2008), t_1=after drought (July 27th 2008), t_2=after rewetting (August 11th 2008).

3.4 Effect of experimental drought on abundance of mcrA genes and transcripts in the acidic fen

Before the experimental drought, detected mcrA gene copy numbers were about 1.7% and 4.1% of detected archaeal 16S rRNA gene copy numbers in control and treatment plots, respectively (Figure 5), indicating a higher relative abundance of methanogens in drought plots before the onset of experimental drought. At the end of the drought period, the relative abundance of *mcrA* genes was significantly lower in drought plots than before the onset of drought (1.5%), while it had remained rather similar in control plots (1.2%), suggesting that the relative abundance of methanogens was lowered by the experimental drought. After rewetting, relative abundances of *mcrA* remained in the same range in the control plots (1.4%), while the relative abundance of *mcrA* genes in the drought plots increased to approximately 3.1%, indicating that rewetting led to increased relative abundances of methanogens in fen soil.

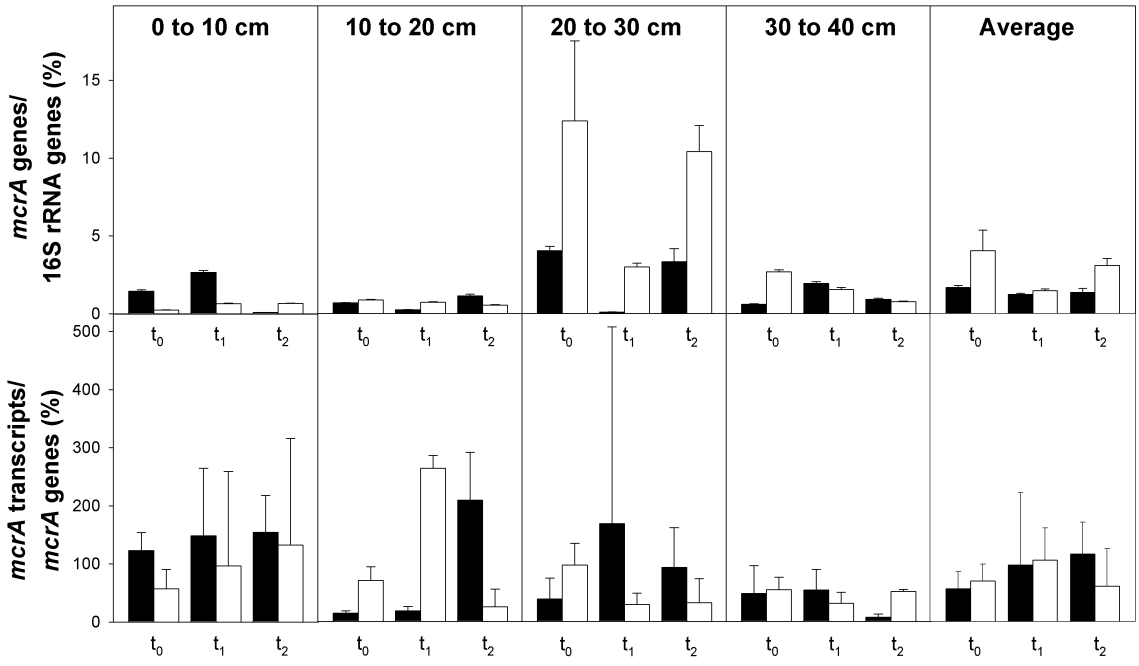

Figure 5: Effect of experimental drought on the abundance of mcrA genes and transcripts. Black bars represent control plots, white bars represent drought plots. Mean values of three plots and four soil layers per plot type and standard errors are shown. t_0=before drought (June 09th 2008), t_1=after drought (July 27th 2008), t_2=after rewetting (August 11th 2008).

20 to 30 cm soil from both plot types yielded the highest relative abundances of *mcrA* (Figure 5). Experimental drought decreased the relative abundance of *mcrA* in 20 to 30 cm depth-soil. After rewetting, relative abundance of *mcrA* amounted to 10.4% in the drought plots, in the control plots they were about 3.3%. In the other soil layers relative *mcrA* gene copy numbers were below 5%, and changes in the relative abundance of *mcrA* were less pronounced than in the soil layer from 20 to 30 cm, indicating that this soil layer was more sensitive to experimental drought than the other soil layers.

Transcript to gene ratios of *mcrA* (i.e., ratios of *mcrA* copies obtained from cDNA to *mcrA* copies obtained from DNA) were highly variable between plots and timepoints, ranging from 0.5% to 500%. The average transcript to gene ratio of *mcrA* (i.e., the average of all 3 plots and all 4 soil depths) was about 50% in both control and drought plots before the onset of drying (Figure 5). At the end of the drought period, the transcript to gene ratio of *mcrA* was about 100% in both plot types, indicating increased activity of fen methanogens in both plot types. In the control plots, the transcript to gene ratio of *mcrA* stayed at about 100% at the time after rewetting, while in the drought plots the expression dropped again to 50%, indicating a reduced activity of methanogens in drought plots. Transcript to gene ratios of *mcrA* were highest in the soil layers from 0 to 20 cm in both plot types (14 to 260%), and lowest in 30 to 40 cm soil (3 to 7%) (Figure 5), indicating a higher acitivity of methanogens in the upper soil layers.

3.5 Effect of experimental drought on the community composition of methanogens

The effect of the experimental drought on the community composition of fen methanogens was assessed by *mcrA* TRFLP analysis from all plots on gene and transcript level. On gene level, 8 to 10 TRFs were found in each sample (Figure 6). The same TRFs were found on transcript level with the exception of the 230 bp TRF, which was absent in the transcript level TRFLP profiles.

The comparison of the average gene-level TRFLP profiles (i.e., the average of all sampled soil depths from all three plots of a plot type) in control and drought plots revealed slight differences in the community composition of fen methanogens at the different timepoints and between the two plot types (Figure 6 A). Differences in *mcrA* community composition before the onset of experimental drought and after the drought period were observed in the drought plots, while *mcrA* community composition at those two timepoints was more similar in the control plots. This indicated a slight effect of

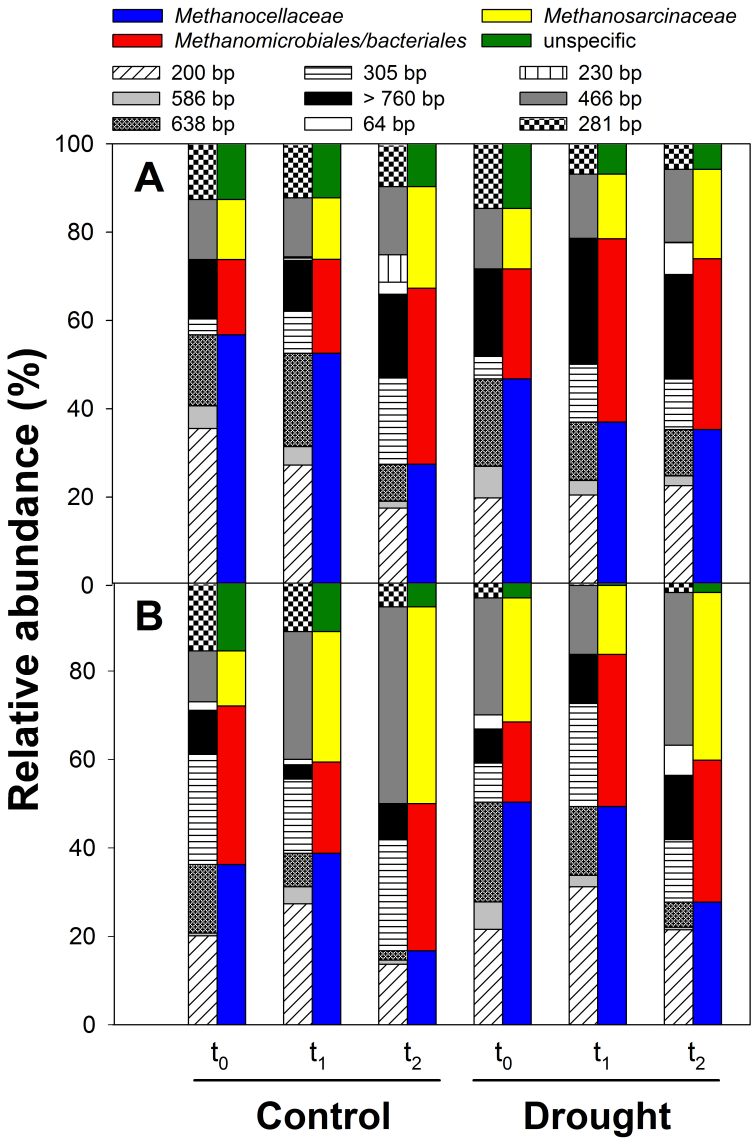

Figure 6: Effect of experimental drought on the community composition of *mcrA* genes (A) and transcripts (B). Comparative TRFLP analysis of *mcrA* amplified from DNA and cDNA of control and drought plots. PCR products were digested with *Hinf*I. Combined relative abundances of the major phylogenetic groups are shown in color (right bars), relative abundances of individual TRFs are in black/white (left bars). Mean values of three plots and four soil layers per plot type are shown. t_0=before drought (June 09th 2008), t_1=after drought (July 27th 2008), t_2=after rewetting (August 11th 2008).

experimental drought on the community structure of fen methanogens. Seven major TRFs (i.e, TRFs with a relative abundance >1%) were observed in both plot types before the experimental drought. The community was dominated by TRFs indicative of

Methanocellaceae, i.e., TRFs of 200 bp length (indicative of OTU 1 and 6), 586 bp length (indicative of OTU 2), and 638 bp length (indicative of OTU 2 and 7), with a combined relative abundance of 57% and 47% in control and treatment plots, respectively. After the drought period, the combined relative abundance of those TRFs was 53% and 37% in control and treatment plots, respectively, indicating a drought induced shift in community composition. Moreover, the relative abundance of TRFs indicative for *Methanomicrobiales/Methanobacteriales*, i.e., the 305 bp and >760 bp TRFs (indicative of OTUs 4 and 5) increased in the treatment plots, and 2 additional TRFs were detected in the control plots (64bp and 230 bp length, indicative of OTU 3 and 4 and of OTU 8, respectively) and one in the drought plots (64bp length). After rewetting, TRFLP profiles of control and treatment plots were again similar to each other, with combined relative abundances of TRFs indicative of *Methanocellaceae* (i.e., TRFs of 200, 586 and 638 bp length) of 27% and 35% in control and treatment plots, respectively, and increased abundances of TRFs indicative for *Methanomicrobiales/Methanobacteriales* (i.e., TRFs of 305 and >760 bp length) of 40% and 39%, respectively. TRFs indicative of Methanosarcinaceae (i.e., TRFs of 230 and 466 bp lengths) had similar relative abundances in control and drought plots at all timepoints, equaling 14-15% before and after the drought period and 20-23% after rewetting. This indicates that (i) a shift in community composition occured in both control and drought plots throughout the experimental period, (ii) drought reduced the relative abundance of *Methanocellaceae*, (iii) rewetting might equalize differences in community composition that were observed after the drought period, and (iv) *Methanosarcinaceae* are least affected by differences between the two plot types.

At the transcript level, 8 major TRFs (i.e, TRFs with a relative abundance >1%) were found in the TRFLP profiles of control and treatment plots (Figure 6 B). Community composition of *mcrA* transcripts differed between control and treatment plots before the onset of drought. The combined relative abundances of TRFs indicative of *Methanocellaceae* (i.e, the 200 bp, 586 bp, and 638 bp length TRFs) amounted to 36% and 50% in control and drought plots, respectively, and the relative abundance of TRFs indicative of *Methanomicrobiales/Methanobacteriales* (i.e., TRFs of 305 and >760 bp length) was twice as high in control as in drought plots (36% and 18%, respectively). After the drought period, combined relative abundances of TRFs indicative of *Methanocellaceae* were 39% and 49% in control and drought plots, respectively. The relative abundance of TRFs indicative of *Methanomicrobiales/Methanobacteriales* was 21% and 34% in control and drought plots, respectively, after the experimental drought. The relative

abundance of TRFs indicative of *Methanosarcinaceae* increased in the control plots from 12% to 30%, while it decreased in the drought plots from 28% to 16%. This indicates that the expression of *mcrA* (i) of *Methanocellaceae* is rather stable under experimental drought conditions, (ii) of *Methanomicrobiales/Methanobacteriales* is upregulated as a result of drought conditions, and (iii) of *Methanosarcinaceae* is downregulated as a result of drought conditions. After rewetting, *Methanosarcina*-related TRFs had the highest relative abundance in both plot types (45% and 38% in control and drought plots, respectively). The relative abundance of *Methanocellaceae*-related TRFs was low in both plot types after rewetting (17% and 28% in control and drought plots, respectively), while the relative abundance of *Methanomicrobiales/Methanobacteriales*-related TRFs was around 30% in both plot types. The data indicates (i) that rewetting restored similar expression patterns in both plot types and (ii) a shift in the active methanogenic community from *Methanocellaceae* in early summer to *Methanosarcinaceae* in late summer.

TRFLPs profiles on transcript level differed from TRFLP profiles on gene level (Figure 6). *Methanosarcina*-related TRFs generally had a higher relative abundance on transcript than on gene level, while *Methanomicrobiales/Methanobacteriales*-related TRFs were generally more abundant on gene than on transcript level. The ratios of relative abundances of TRFs indicative for certain phylogenetic groups on gene and transcript level indicated that *Methanosarcinaceae* were inhibited by experimental drought, while *Methanomicrobiales/Methanobacteriales* were stimulated.

4 Discussion

4.1 Effect of drying and rewetting on the methanogenic community in the acidic fen

In the course of climate change, a higher frequency of more extreme weather events like prolonged drought periods or heavy precipitation events are anticipated [61]. Prolonged drought reduces methanogenic activity in the fen Schlöppnerbrunnen [33]. This is attributed to regeneration of electron acceptors due to higher soil aeration [33]. Thus, when conditions become anoxic methanogenesis competes with other anaerobic redox processes for electrons [8]. Indeed, after experimental drought methanogenic potentials of 0 to 10 and 30 to 40 cm soil were lower and the initial lag phases were longer in drought than in control plots (Figure 1). In an acidic fen soil in Finland, reduced CH_4 emissions are observed in drained sites as compared to the wetter sites along a drainage gradient near a groundwater extraction plant [67]. Reduced CH_4 emissions from drained wetlands are also caused by methanotrophy occuring when O_2 penetrates into deeper soil layers [67]. In mesocosm studies with Schlöppnerbrunnen fen soil, CH_4 emissions decrease due to methanotrophy in more aerated parts above the water table when the water table is experimentally lowered, while the methanogenic pathways are mainly unaffected [32]. Methanotrophic activity has also been detected in microcosm studies with soil from Schlöppnerbrunnen fen, and methanotrophic taxa are detected in the fen soil [64]. After rewetting of peatland soil, recovery of methanogenesis is ofter delayed as a result of increased availability of alternative electron acceptors [7, 32, 33].

Methanogenesis is controlled by *in situ* parameteres such as temperature and the level of the ground water table [5, 31, 32, 57]. A water table drawdown like the one achived during the experimental drought results in higher soil aeration and a rise concomitant rise in the redox potential [33]. Methyl-coenzyme M reductase is inactivated during periods of raised O_2 concentrations [28]. Decreased abundances of methanogens were observed in drought plots after the drought period (Figure 5), however, the composition of the methanogenic community was not significantly affected. Detected *mcrA* were mainly affiliated with *mcrA* of *Methanocella paludicola* (*Methanocellaceae*), representative of 'Rice Cluster I' [55], moreover *mcrA* affiliated with *mcrA* of the *Methanomicrobiales*, *Methanobacteriaceae* and *Methanosarcinaceae* were detected (Figure 3, Figure 4). Even though small changes in community composition were observed (Figure 4, Figure 6), the relative abundances of the detected groups were not significantly affected by experimental drought. This is in good agreement with studies from rice field soils, where

drainage and O_2 exposure reduce the abundance of methanogens while the community composition of methanogens is not affected [38, 39, 68]. Thus, the observed changes in the methanogenic potential of fen soil after drying and rewetting might be caused by reduced abundances of methanogens.

Transcript to gene ratios of *mcrA* ranged from 50 to 110% in fen soil (Figure 5) and were thus of a similar magnitude as ratios of *mcrA* in British peatlands [13]. However, this ratio might be underestimated due to dead or inactive methanogens [13]. *mcrA* expression was similar in drought and control plots after the drought period (Figure 5). Peat methanogens maintain high abundances of *mcrA* transcripts, even when their activity is low, and similar gene and transcript abundances have been detected in peat soil with methanogenic potential reduced due to long-term soil storage in the lab [13]. Thus, lower methanogenic potentials after prolonged experimental drought are likely caused by post-transcriptional effects on *mcrA*, and might also be affected by other parameters than O_2. Moreover, methanogens as well as copies of *mcrA* were also found in the upper soil layers in the acidic fen Schlöppnerbrunnen, even though more oxic conditions are found there troughout the year [23]. Methanogens are also detected in other rather oxic systems such as desert soils [50], and minor CH_4 production and emission is known from oxic upland soils [27, 66]. Reduced viability of methanogens is attributed to the effect of desiccation rather than to the effect of O_2 itself [10]. The mechanism of the survival or even growth under oxic conditions remains unresolved, since there is no evidence for the existence of resting stages in methanogens [10]. However, enzymatic protection against oxidative stress by the enzymes superoxide dismutase and katalase has been reported in methanogenic species like *Methanobacterium bryantii* and *Methanobacterium thermoautotrophicum* [29, 60]. Some *mcrA* sequences obtained from the acidic fen were related to those species, and it seems thus likely that they also harbor the capacities to produce protective enzymes. Additionally, wet and anoxic microenviroments in drought plots might protect methanogens from O_2 during drought periods [32]. However, O_2 inhibited methanogenesis in fen soil microcosms even at very low O_2 concentrations (Figure 2), thus it is likely that fen soil methanogens are inactive during periods of drought stress.

The composition of *mcrA* transcripts was more variable than that of *mcrA* genes (Figure 6). However, differences were more pronounced between the different sampling timepoints than between the two plot types, even though transcript to gene ratios of TRFs suggest inhibitory and stimulatory effects of experimental drought on *Methanosarcinaceae* and *Methanomicrobiales/Methanobacteriales*, respectively. O_2 exposure alters the composition of *mcrA* transcripts in rice field soil [69]. In August, a

greater abundance of *Methanosarcina*-related *mcrA* transcripts than in June or July was detected, indicating higher activity of *Methanosarcina*-related methanogens in late summer. Methanogens of the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanocellales* use hydrogenotrophic methanogenesis for energy conservation [55, 63], while *Methanosarcinales* conserve energy via acetoclastic and hydrogenotrophic methanogenesis [43]. It is thus feasible that the contribution of acetoclastic methanogenesis is variable troughout the year and is of greater influence in the late summer.

4.2 Conclusions and limitations

Future climate change will likely increase the frequency of drought events in peatlands [61]. Experimental drought in the acidic fen Schlöppnerbrunnen decreases methane emissions [33], and reduced methanogenic potentials were observed in microcosm studies with fen soil. The abundance of methanogens was reduced in the drought plots while the composition of the methanogenic community was rather stable throughout the drought experiment based on analyses of *mcrA* gene and transcript abundances and TRFLP profiles. Unfortunately, owing to the experimental design, the effect of experimental drought on methanogenic potentials in microcosm incubations and on the abundance and community structure of fen methanogens were studied in two different years. It is therefore not possible to directly link the observed effects of experimental drought on process level to the observed changes in the methanogenic community.

Within these limitations the data collected in this study nonetheless indicate that experimental drought (i) impacts on methanogenic potentials in the acidic fen, and (ii) moreover influences the abundance but not the community composition of fen methanogens. Further investigation would be needed to establish the link between observed effects on methanogenic potential and observed effects on community composition of fen methanogens.

5 Acknowledgments

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6 Supplemental Figures

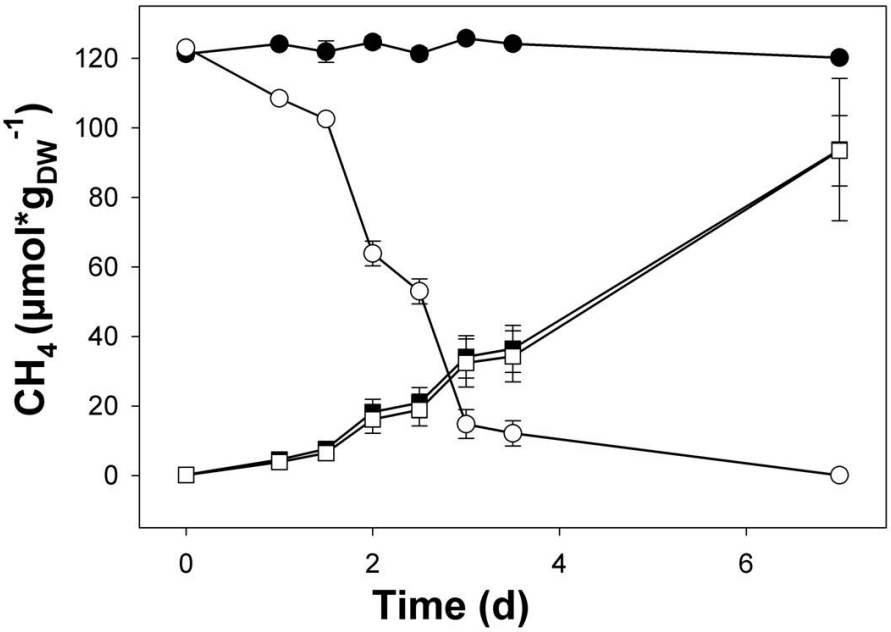

Figure S1: Effect of difluoromethane on methanogenesis and methanotrophy in fen soil microcosms. Mean values and standard errors of three replicate microcosms are shown. Squares represent anoxic microcosms, circles represent oxic microcosms. Open symbols represent microcosms without difluoromethane, closed symbols represent microcosms with difluoromethane.

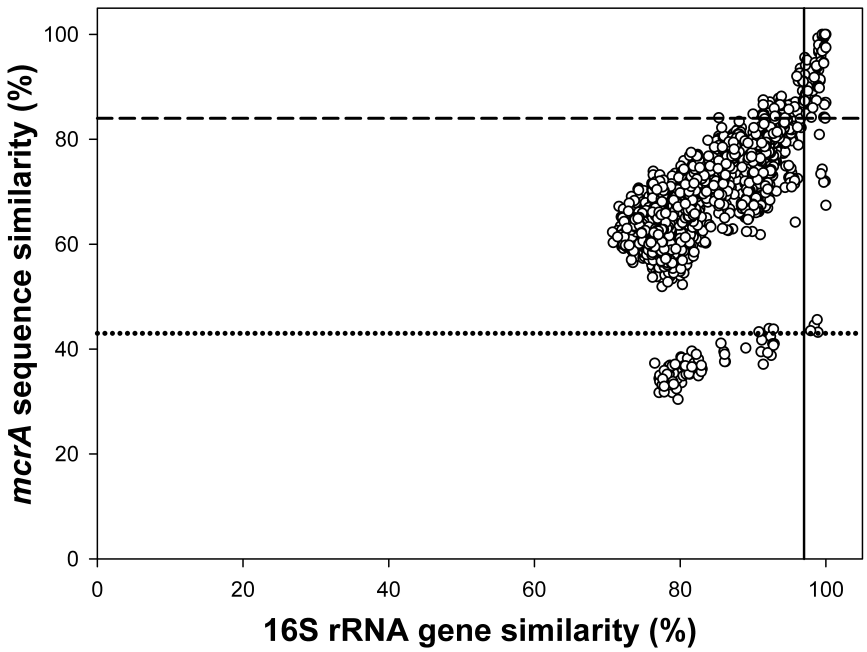

Figure S2: Correlation of DNA sequence similarities of mcrA versus 16S rRNA gene similarity. Dotted lines represent the similarity values, below which two sequences always had less than 97% 16S rRNA gene sequence similarity. The dashed lines represent the 90% quantile of pairwise sequence comparisons with a 16S rRNA gene sequence similarity of 97% (i.e., threshold similarity). The solid lines mark the 97% 16S rRNA gene similarities.

Denitrifier communities in an acidic fen are stable during experimental drought

Manuscript in preparation

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Wetlands can act as sources or sinks of the greenhouse gas nitrous oxide (N_2O). The acidic fen Schlöppnerbrunnen emits denitrification derived N_2O and is also capable of N_2O consumption. Global warming is predicted to cause more extreme weather events in future years, including prolonged drought periods, which will influence the denitrifier community in the acidic fen. Thus, the abundance and community composition of denitrifiers and their reaction to enhanced drought were investigated. Pyrosequencing of the structural gene markers *narG*, *nirK*, *nirS*, and *nosZ* revealed a high denitrifier diversity and 6, 13, 18 and 6 species-level operational taxonomic units (OTUs). Detected species-level OTUs were mainly related to *Actinobacteria*, *Alpha-*, *Beta-*, and *Gammaproteobacteria*. An experiment to simulate prolonged drought was conducted in summer 2008 by experimentally lowering the water table in 3 drought plots for 8 weeks followed by subsequent rewetting of the drought plots. 3 undrained plots served as controls. Samples were taken before and after drought and after rewetting. *narG* and *nosZ* were used to see assess changes in the nitrate reducer and denitrifier community to experimental drought. Higher copy numbers of *narG* and *nosZ* (2x and 1.5x, respectively) were detected after the experimental drought in drought but not in control plots by quantitative PCR (qPCR), indicating that nitrate reducers and denitrifiers are positively influenced by the drought treatment. Rewetting restored the copy numbers of *narG* and *nosZ* to the original level. Terminal restriction fragment length polymorphism (TRFLP) patterns of *narG* and *nosZ* were similar before and after the drought period, indicating a high stability of nitrate reducers and denitrifiers in the fen. The collective data indicate that (i) a high denitrifier diversity exists in the acidic fen Schlöppnerbrunnen, (ii) experimental drought increases denitrifier abundances, and (iii) the community composition is unaffected by experimental drought.

1 Introduction

Extreme weather events such as prolonged drought periods or heavy rainfalls are predicted to increase in frequency as well as in intensity in future years due to global warming [38]. This will affect the water table in wetland soils and thus many biological processes in those soils, including processes involved in the turnover of nitrous oxide

(N_2O) [12, 33, 39]. N_2O is a major greenhouse gas with a global warming potential that is 300 times higher than that of CO_2 and the major ozone-depleting substance in the atmosphere [9, 32]. The atmospheric concentration of N_2O increased from 270 ppb to 319 ppb in the years 1750 to 2005 [9]. Soils are major sources of N_2O, which can be nitrification- or denitrification derived [6, 7, 30]. Moreover, such wetland soils can be sources or sinks (transient or permanent) of atmospheric N_2O [11, 10, 27, 36]. In water-logged wetland soils including fens denitrification is the main source of N_2O [6, 27, 30]. Denitrification is the sequential reduction of nitrate or nitrite to N_2 via nitric oxide (NO) and N_2O [41]. The denitrification processes is catalyzed by the enzymes nitrate reductase Nar or Nap (encoded by *narGHJI* or *napEDABC*, respectively), copper- or cytochrome-dependent nitrite reductases NirK or NirS (encoded by *nirK* or *nirS*, respectively), NO recutase Nor (encoded by *norBC*), and N_2O reductase Nos (encoded by *nosZ*) [41]. Nitrate reductases are moreover found in many non-denitrifying nitrate-reducing microorganisms [41].

narG, *nirK*, *nirS*, and *nosZ* are structural gene markers commonly used in the analysis of nitrate reducer and denitrifier community composition [4, 15, 24, 29]. *narG* and *nosZ* from the acidic fen Schlöppnerbrunnen were studied earlier by conventional cloning based sequencing, revealing novel nitrate reducers and denitrifiers in the acidic fen [27]. However, new sequencing methods including amplicon pyrosequencing allow more in-depth analysis of nitrate reducers and denitrifier communities. Thus, one aim of the present study was to reassess the diversity of *narG* and *nosZ* from Schlöppnerbrunnen fen soil along with the diversity of the nitrite reductase encoding *nirK*/*nirS* using amplicon pyrosequencing.

In the context of global warming, more extreme drought periods are expected to occur, which will effect water tables in fen soils [38]. However, it is so far unresolved how fen nitrate reducer and denitrifier communities react to changing water tables. Thus, a further aim of the study was to assess the effect of simulated drought conditions on nitrate reducer and denitrifier abundance and diversity in acidic Schlöppnerbrunnen fen soil to gain a better understanding of possible feedback effects of global warming.

2 Material and Methods

2.1 Study site and experimental setup

The minerotrophic fen Schlöppnerbrunnen is located in the Lehstenbach catchment in the Fichtelgebirge, Bavaria, Germany (50° 07' 53" N, 11° 52' 51" E), at approximately 700 m above sea level. The sampling site is described in more detail in [14, 25, 27]. In 2008, the water table was artificially lowered in 3 treatment (i.e., drought) plots (size 7.2 m x 5 m). Roofs were erected to shield the plots from rain water, and additional drainage was accomplished by ditches. The roofs had large open side walls to minimize temperature and wind speed effects. Additionally, 3 untreated plots served as control sites. Roofs and drainage on the treatment plots were installed on June 10th 2008 and kept in place until August 7th. On average, experimental drought lowered the water table to 0.60 m below the surface. After the experimental drought period, roofs and drainage were removed to allow rewetting of the drought plots. For more detailed description of the experimental setup refer to [25]. Soil was sampled for molecular analyses before the start of the drought period (June 09th), at the end of the experimental drought period (July 27th), and 4 days after rewetting (August 11th). Soil samples were taken from each plot from soil depths 0 to 40 cm at 10-cm intervals with a soil corer. Soil samples were quick-frozen in liquid nitrogen and stored at -80°C until further processing.

2.2 Extraction of nucleic acids

Nucleic acids from all sampled timepoints, plots, and soil layers were extracted using a bead-beating protocol [13] followed by separation of DNA and RNA using the Qiagen RNA/DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) to the manufacturer's instructions.

2.3 Barcoded amplicon pyrosequencing of narG, nirK, nirS, and nosZ

narG, *nirK*, *nirS*, and *nosZ* were amplified from pooled DNA extracts (i.e., from all timepoints, plots, and soil layers) using published primers tagged with barcodes, purified and pyrosequenced as previously described [26]. Quality filtering of sequences included denoising (i.e., removal of pyrosequencing and PCR-amplification errors by PyroNoise and SeqNoise algorithms [31]) as described [28]. Denoised sequence data sets

were grouped into species-level operational taxonomic untis (OTUs) at threshold similarities of 67%, 83%, 82%, and 80% for *narG*, *nirK*, *nirS*, and *nosZ*, respectively. For *narG* and *nosZ* only sequences obtained from forward reads were used, as mean sequence lenghts of approximately 400 bp yielded to little overlap of forward and reverse reads, while forward and reverse reads of *nirK* and *nirS* were combined [26]. OTU representatives were aligned with sequences of cultured and uncultured references (determined by BLAST analysis [1]) using ClustalW and phylogenetic trees were constructed using the neighbor joining algorithm [34] based on pairwise sequence distances in MEGA 5.0 [22]. The stability of tree topologies was tested using the bootstrap method with 10,000 replications.

2.4 Quantitative PCR

Quantitative PCR (qPCR) of *narG* and *nosZ* was modified from [26]. Reactions were set up in triplicate reactions per DNA extract. The forward primers were fluoresecently labeled to allow the use of the products from qPCR in downstream TRFLP analyses. This approach allows direct coupling of quantification and analysis of community composition of a specific gene marker. Inhibition of qPCR assays by contaminating humic substances was conducted as described in [26].

2.5 Terminal restriction fragment length polymorphism analysis (TRFLP)

Triplicate qPCR reactions of *narG* and *nosZ* were pooled and gel purified using the Montage Gel Extraction Kit (Millipore Corporation, Bedford, MA, USA) prior to TRFLP analysis. The purified PCR products were digested with Mung Bean Nuclease (New England Biolabs, Frankfurt am Main, Germany) to remove single stranded DNA and reduce the probability of pseudo-terminal restriction fragments [8]. The restricted DNA was purified using the Millipore Multiscreen 96-well Filtration System (Millipore Corporation, Bedford, MA, USA). PCR products of *narG* were digested with the restriction enzyme *Cfo*I, *nosZ* PCR products were digested with *Fnu4*HI. Polyacrylamide gel electrophoresis was performed as described previously [27]. Terminal restriction fragments (TRFs) were assigned to *narG* and *nosZ* sequences via *in silico* TRF analysis with sequences obtained from the fen Schlöppnerbrunnen [27].

3 Results

3.1 Diversity of denitrification-associated genes in Schlöppnerbrunnen fen soil

Diversity of Schlöppnerbrunnen fen denitrifiers was assessed by barcoded amplicon pyrosequencing of denitrification-associated genes. Amplicon pyrosequencing of *narG* forward reads yielded 1,325 quality filtered (i.e., denoised) sequences. Sequences were grouped into 6 species-level OTUs, 3 OTUs had relative abundances greater than 1%. Of those 3 OTUs, OTU 1 had a relative abundance of 71% and thus dominated detected *narG* in Schlöppnerbrunnen fen soil (Figure 1). Representative sequences of OTU 1 affiliated with the *Actinobacteria* and were distantly related to *narG* of *Actinosynnema mirum* and *Streptomyces coelicolor* (Figure 1). OTU 2 accounted for 22% of *narG*, affiliated with the *Gammaproteobacteria*, and was related to *narG* of *Pseudomonas* spp. (Figure 1). OTU 3 accounted for 6% of *narG* and was distantly related to the *Deinococcal* genera *Thermus*, *Oceanithermus*, and *Marinithermus* (Figure 1).

2,401 quality filtered sequences were obtained for *nirK*. Sequences grouped into 13 species-level OTUs, and 7 OTUs had a relative sequence abundance greater than 1% (Figure 2). Those OTUs occured with relative abundances of 55%, 21%, 11%, 7%, 2%, 2%, and 1% (OTUs 1, 2, 3, 4, 5, 6, and 7, respectively; Figure 2). Sequences affiliated with *Proteobacterial nirK* (Figure 2). OTU representatives of OTUs 1 and 6 were distantly related to *nirK* of *Enterococcus* sp., OTUs 3 and 5 were related to *nirK* of *Bradyrhizobium* sp., OTU 2 was related to *Rhizobial nirK*, while OTUs 4 and 7 did were not clearly related to published *nirK* (Figure 2).

334 quality filtered sequences were obtained for *nirS* which grouped into 16 species-level OTUs. 6 OTUs had relative sequence abundances greater than 1%. OTUs 1 and 2 dominated *nirS* in Schlöppnerbrunnen fen soil with relative abundances of 52% and 33%, respectively, while OTUs 3, 4, 5, and 6 were detected with relative abundances of 4%, 3%, 2%, and 2%, respectively (Figure 3). OTU representatives affiliated with *Alpha-*, *Beta-*, and *Gammaproteobacteria*. Representative sequences of OTUs 1 and 2 were related to *nirK* of *Bradyrhizobium* sp., while representative sequences of OTUs 3, 4, 5, and 6 were related to *Pseudomonas* spp., *Ralstonia* sp., *Thiobacillus denitrificans*, and *T. denitrificans*, respectively (Figure 3).

384 quality filtered forward read sequences were obtained for *nosZ* which grouped into 7 species-level OTUs, of which 3 had relative sequence abundances greater than 1%. OTU 1 dominated *nosZ* in Schlöppnerbrunnen fen soil with relative abundances

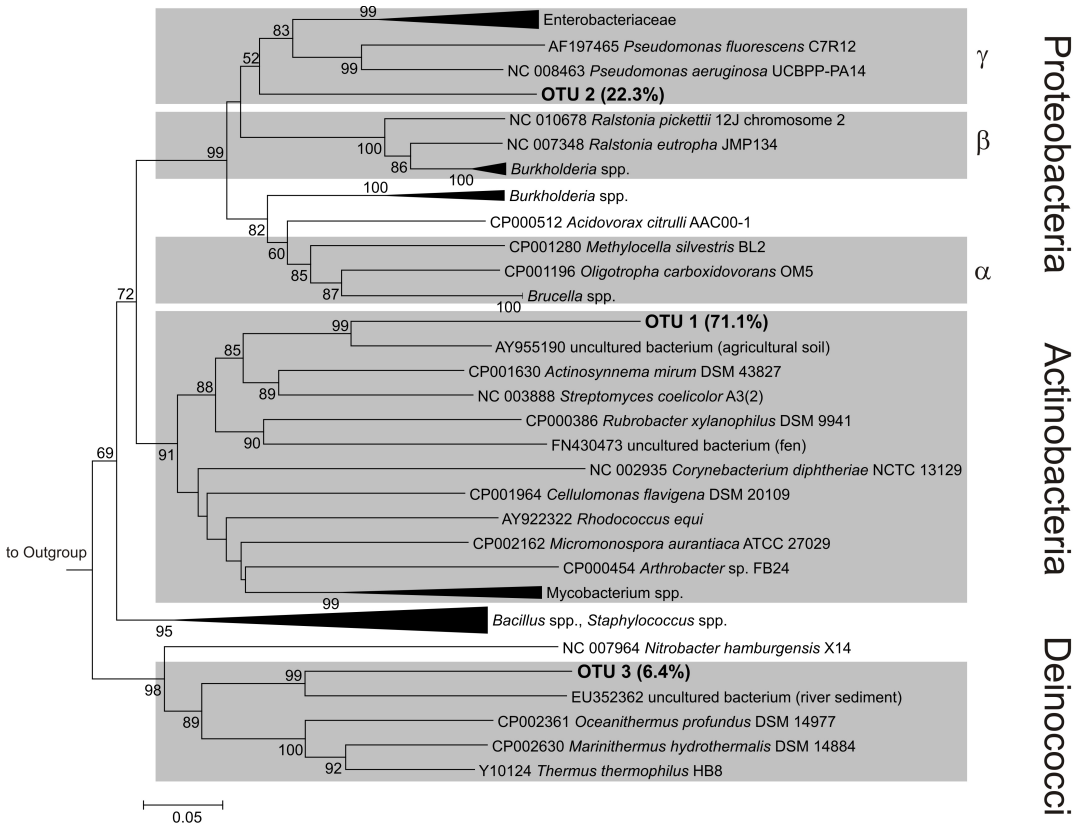

Figure 1: Phylogenetic tree of *narG* OTU representatives. Sequences were retrieved from pooled DNA extracts. The tree is based on *in silico* tranlated amino acid sequences. OTUs were calculated at a species-level threshold similarity of 67% from a total of 1,325 forward read sequences. Only OTUs that contain $\geq$ 1% of sequences are displayed, relative abundances of OTUs are given in parentheses. Codes preceeding sequence names represent sequence accession numbers of reference sequences in public databases. Grey boxes indicate branches were the majority of the sequences belong to a given phylogenetic group (indicated on the right). The percentage of replicate trees that produced the same clustering in the bootstrap analysis (10,000 replications) are shown next to the branches, bootstrap support values below 50% are omitted. The tree was rooted using *narG* of *Haloarcula marismortui* ATCC 43049 as the outgroup (NC 006396).

of 92%, while OTUs 2, and 3 were detected with relative abundances of 6%, and 1%, respectively (Figure 4). OTU representatives affiliated with *Alphaproteobacterial nosZ*. Representative sequences of OTUs 1, 2, and 3 were related to *nosZ* of *Achromobacter* sp., *Mesorhizobium* sp., and *Azospirillum* sp., respectively (Figure 4).

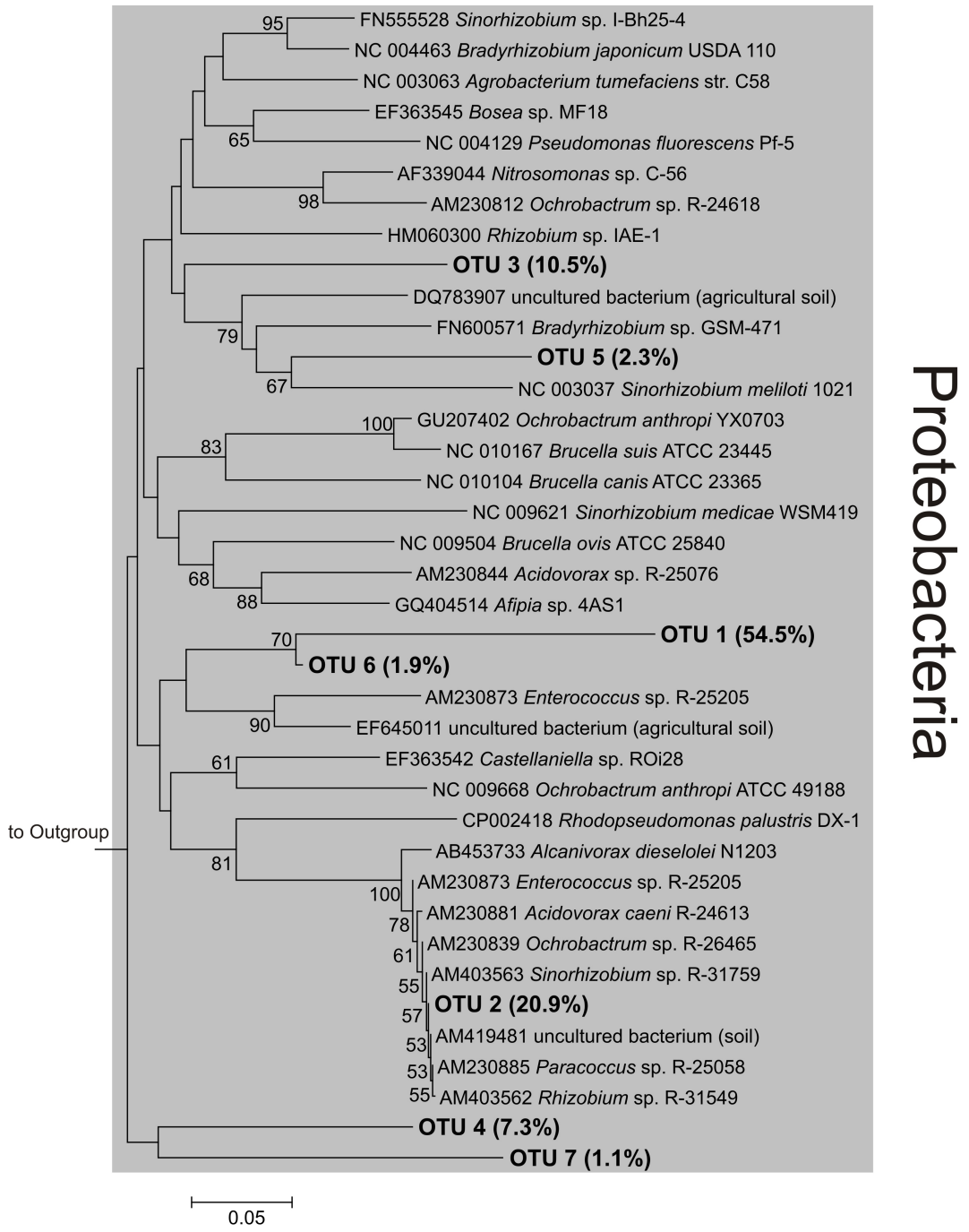

Figure 2: Phylogenetic tree of *nirK* OTU representatives. Sequences were retrieved from pooled DNA extracts. The tree is based on *in silico* tranlated amino acid sequences. OTUs were calculated at a species-level threshold similarity of 83% from a total of 2,401 sequences. Only OTUs that contain $\geq$ 1% of sequences are displayed, relative abundances of OTUs are given in parentheses. Codes preceeding sequence names represent sequence accession numbers of reference sequences in public databases. Grey boxes indicate branches were the majority of the sequences belong to a given phylogenetic group (indicated on the right). The percentage of replicate trees that produced the same clustering in the bootstrap analysis (10,000 replications) are shown next to the branches, bootstrap support values below 50% are omitted. The tree was rooted using *nirK* of *Alcaligenes* sp.CJANPY1 as the outgroup (EF202175).

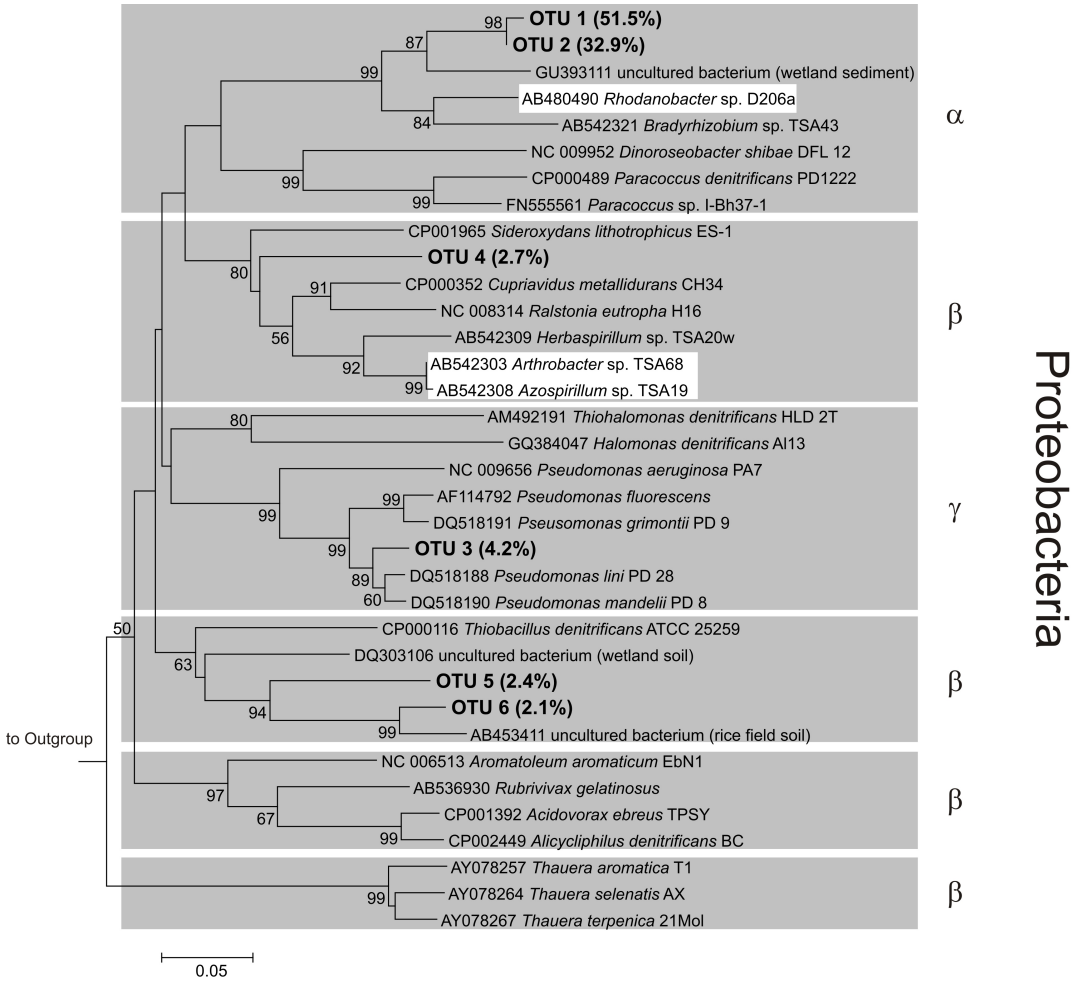

Figure 3: Phylogenetic tree of *nirS* OTU representatives. Sequences were retrieved from pooled DNA extracts. The tree is based on *in silico* tranlated amino acid sequences. OTUs were calculated at a species-level threshold similarity of 82% from a total of 334 sequences. Only OTUs that contain $\geq$ 1% of sequences are displayed, relative abundances of OTUs are given in parentheses. Codes preceeding sequence names represent sequence accession numbers of reference sequences in public databases. Grey boxes indicate branches were the majority of the sequences belong to a given phylogenetic group (indicated on the right), white boxes indicate minority sequences that do not belong to the indicated group. The percentage of replicate trees that produced the same clustering in the bootstrap analysis (10,000 replications) are shown next to the branches, bootstrap support values below 50% are omitted. The tree was rooted using *nirS* of *Rhodothermus marinus* DSM 4252 as the outgroup (CP001807).

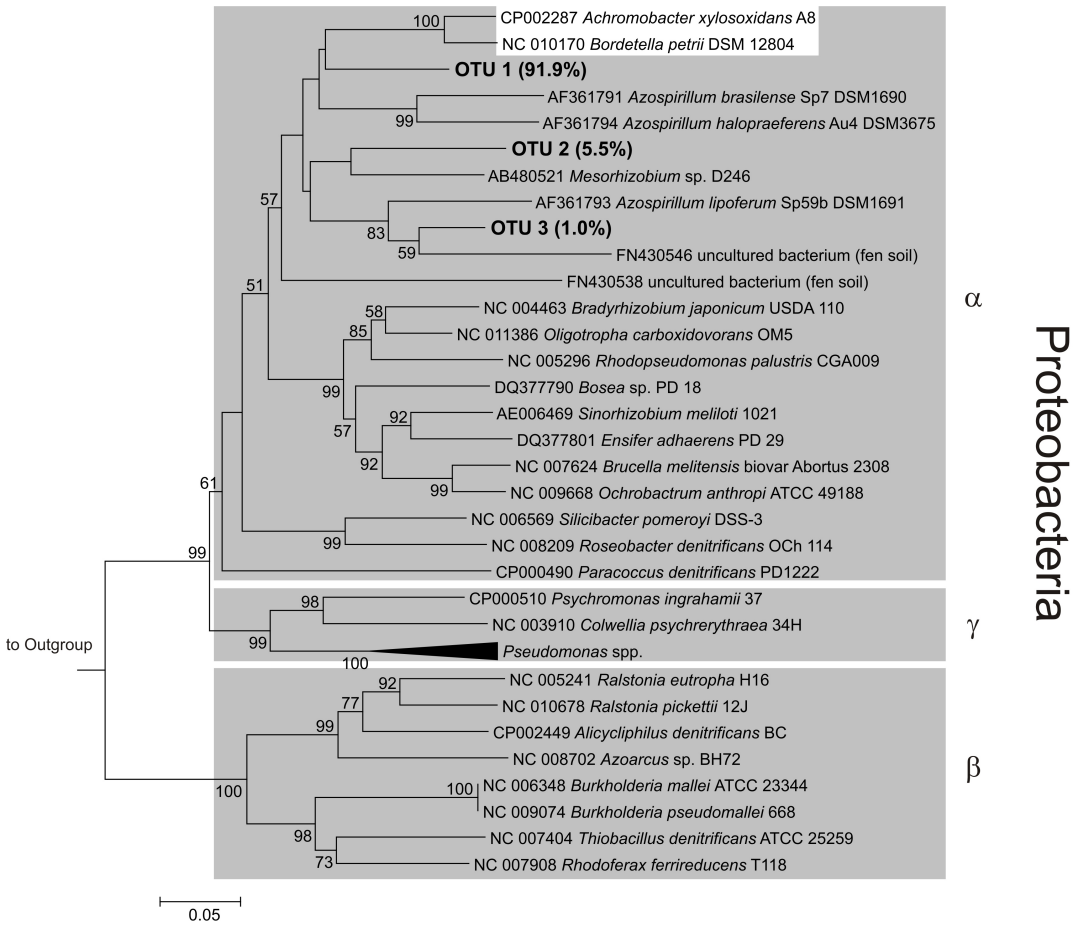

Figure 4: Phylogenetic tree of *nosZ* OTU representatives. Sequences were retrieved from pooled DNA extracts. The tree is based on *in silico* tranlated amino acid sequences. OTUs were calculated at a species-level threshold similarity of 80% from a total of 384 forward read sequences. Only OTUs that contain $\geq$ 1% of sequences are displayed, relative abundances of OTUs are given in parentheses. Codes preceeding sequence names represent sequence accession numbers of reference sequences in public databases. Grey boxes indicate branches were the majority of the sequences belong to a given phylogenetic group (indicated on the right), white boxes indicate minority sequences that do not belong to the indicated group. The percentage of replicate trees that produced the same clustering in the bootstrap analysis (10,000 replications) are shown next to the branches, bootstrap support values below 50% are omitted. The tree was rooted using *nosZ* of *Haloarcula marismortui* ATCC 43049 as the outgroup (NC 006396).

3.2 Effect of experimental drought on abundance of narG and nosZ in the acidic fen

The effect of experimental drought on the relative abundance of nitrate reducers and denitrifiers was assessed by qPCR of *narG* and *nosZ* at different timepoints of the drought experiment (i.e., before and after the drought period and after rewetting). Detected *narG* copy numbers varied between 6 and 14% of bacterial 16S rRNA gene copies (Figure 5). Before the experimental drought, detected *narG* copy numbers were 11.7% and 6.6% of detected bacterial 16S rRNA gene copy numbers in control and treatment plots, respectively (Figure 5). At the end of the drought period, the relative abundance of *narG* in treatment plots was twice as high as before the drought (Figure 5), while the relative abundance of *narG* in control plots was slightly lower than before the drought period. After rewetting, relative *narG* copy numbers were in the same magnitude as before and after the drought period in treatment and control plots, respectively (Figure 5).

Detected *nosZ* copy numbers varied between 0.05 and 0.2% of 16S rRNA gene copy numbers (Figure 5). *nosZ* copy numbers were similar in control and treatment plots before the start of the drought period (0.09 and 0.13% of bacterial 16S rRNA genes, respectively). At the end of the drought period, *nosZ* copy numbers were higher and lower than before the drought period in treatment and control plots, respectively (0.18% and 0.05% of bacterial 16S rRNA genes, respectively) (Figure 5). After rewetting, relative *nosZ* copy numbers in treatment plots had decreased by more than 50% as compared to the end of the drought period, while relative *nosZ* copy numbers in control plots were in the same range as before the drought period (Figure 5).

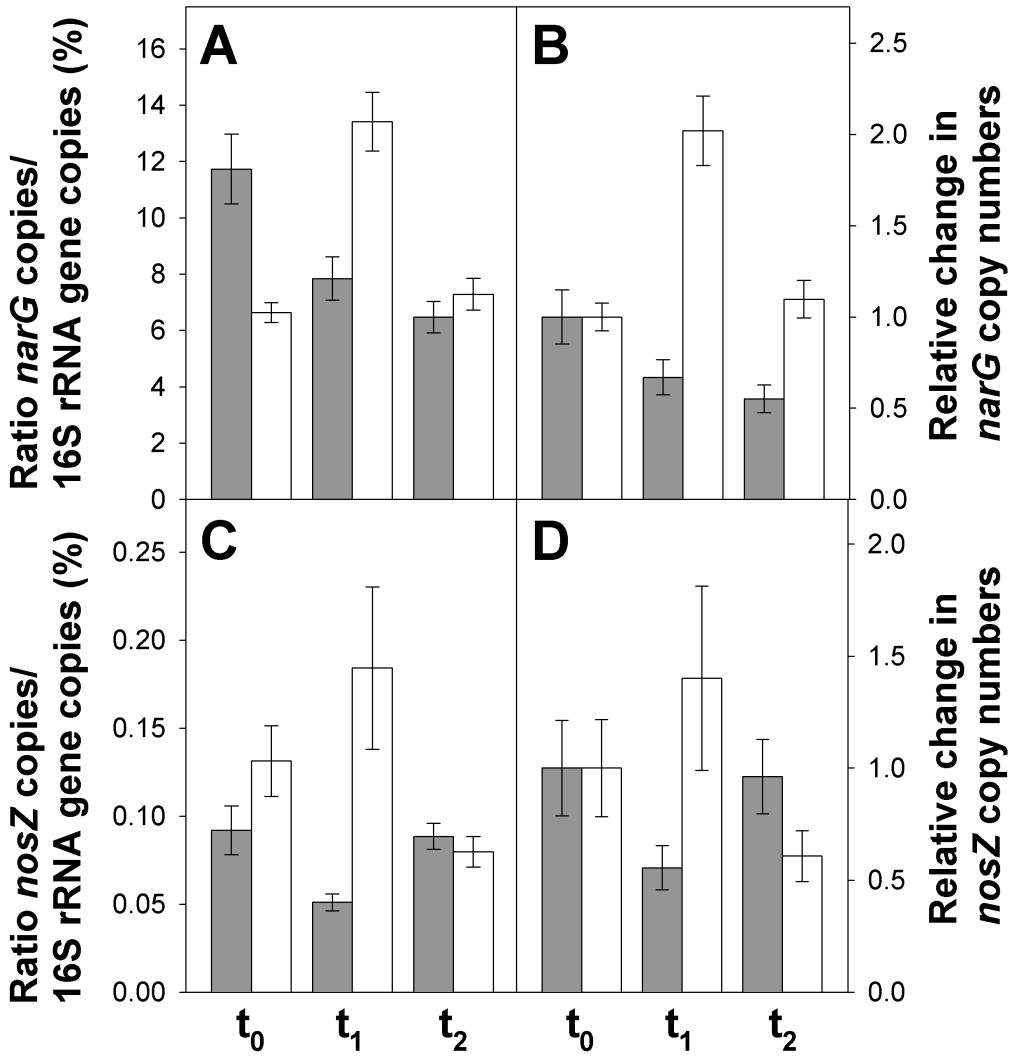

Figure 5: Effect of experimental drought on the abundance of *narG* and *nosZ* genes. Grey bars represent control plots, white bars represent drought plots. Mean values of three plots and four soil layers per plot type and standard errors are shown. t_0=before drought (June 09th 2008), t_1=after drought (July 27th 2008), t_2=after rewetting (August 11th 2008).

3.3 Effect of experimental drought on community composition of narG and nosZ

The effect of the experimental drought on the community composition of fen denitrifiers was assessed by TRFLP analysis of *narG* and *nosZ* at different stages of the experimental drought. 6 and 12 TRFs with relative abundances >1% were detected for *narG* and *nosZ*, respectively (Figure 6). The comparison of the average *narG* TRFLP profiles (i.e., the average of all sampled soil layers from all three plots of a plot type) in control and drought plots revealed only minor differences in the community composition of fen nitrate reducers at the different time points and between the two plot types (Figure 6 A). The 23 bp, 57 bp and 128 bp TRFs were most abundant(Figure 6 A). The average TRFLP profiles of *nosZ* likewise showed only minor differences in community composition of fen nitrous oxide reducers (Figure 6 B). The 165 bp, 215 bp and 296 bp TRFs were most abundant. During the course of the experiment, the relative abundance of the 296 bp TRF decreased from 38% to 21% and from 48% to 25% in control and drought plots, respectively, while the relative abundance of the 165 bp and 215 bp TRFs showed a slight increase in both plot types. This indicates a slight seasonal shift in the community composition of nitrous oxide reducing denitrifiers from the beginning of June to mid August.

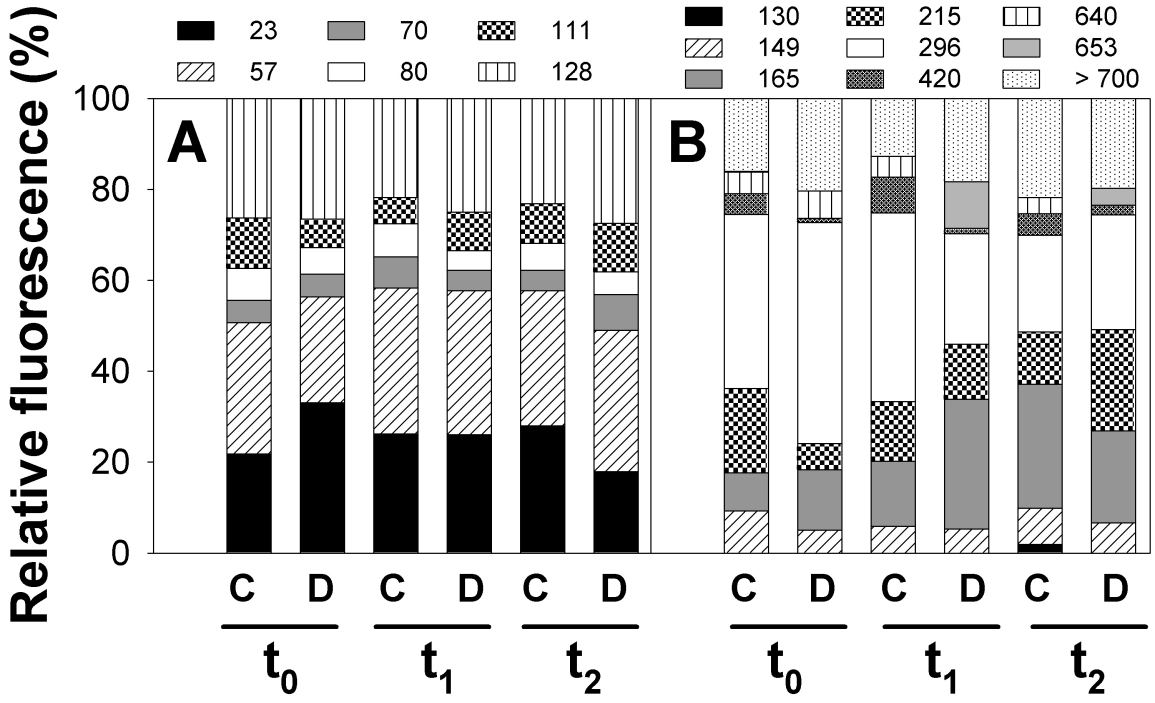

Figure 6: Effect of experimental drought on the community composition of nitrate reducers and denitrifiers. Comparative TRFLP analysis of *narG* (A) and *nosZ* (B) from DNA of control (C) and drought (D) plots. PCR products were digested with *Cfo*I (*narG*) and *Fnu4*HI (*nosZ*). Mean values of three plots and four soil layers per plot type are shown. t_0=before drought (June 09th 2008), t_1=after drought (July 27th 2008), t_2=after rewetting (August 11th 2008). Length of detected TRFs (in basepairs) are indicated above the graph.

4 Discussion

4.1 Detected diversity of nitrate reducers and denitrifiers in acidic Schlöppnerbrunnen fen soil

Up to 16 species-level OTUs of denitrification-associated genes were detected in the acidic fen Schlöppnerbrunnen (Figure 1, Figure 2, Figure 3, Figure 4), indicating a high species-level diversity of fen denitrifiers. OTUs of detected *narG* affiliated with *Actinobacteria*, *Gammaproteobacteria*, and *Deinococci* (Figure 1). Earlier cloning-based studies of *narG* from Schlöppnerbrunnen fen soil also indicate the presence of the mentioned groups, however also *Alpha-* and *Betaproteobacterial narG* are detected by cloning-based approaches, indicating that the detected diversity varies between the 2 approaches, likely because of the shorter fragment length obtained from amplicon pyrosequencing as well as by variability in DNA extraction. The noise-removal algorithms employed in the present study remove errors caused by pyrosequencing (PyroNoise) as well as by PCR-amplification (SeqNoise) [31]. As PCR-amplification errors affect conventional sequencing and pyrosequencing in a simialr magnitude, it is moreover feasible that earlier cloning based sequencing efforts overestimate the genetic diversity of *narG* because of those PCR-amplification errors. Sequences related to *Actinobacterial narG* dominated amplicon libraries (Figure 1). *Actinobacterial* predominance is observed in many other wetland soils including peatland soils [26, 28, 29].

Sequences of *nirK* and *nirS* affiliated with sequences of *Alpha-*, *Beta-* and *Gammaproteobacterial* nitrite reductases (Figure 2,Figure 3). *nirK* was dominated by sequences distantly related to *Enterococcus* sp., indicating that denitrifiers harboring new NirK-type nitrite reductases occur in Schlöppnerbrunnen fen soil. *nirS* was dominated by sequences related to *Bradyrhizobium* sp., indicating the presence of this common soil bacterium in Schlöppnerbrunnen fen soil. Detected *nosZ* affiliated with *Alphaproteobacterial nosZ*, confirming the results of earlier cloning based sequencing efforts [27].

4.2 Relative abundance of denitrifiers in the acidic fen

The relative *narG* gene copy number ranged between 6 and 13% (Figure 5 A). The detected *narG* copy numbers are in the same range as *narG* copy numbers in permafrost affected peat peat soil in Russia, agricultural soils of the Burgundy region in France, freshwater habitats such as rivers and floodplains as well as glacier forelands [2, 3, 19, 26] In some agricultural soils lower *narG* copy numbers of about 1% or even

lower are detected [3, 18]. This indicates a high abundance of nitrate reducers in the fen Schlöppnerbrunnen. Indeed, nitrate-dependent MPNs show similar numbers of nitrate reducers in the acidic fen [27]. On the other hand, relative *nosZ* copy numbers ranged from 0.05% to 3% (Figure 5 C), indicating that about 1% to 10% of nitrate reducers in Schlöppnerbrunnen fen soil possess the genetical potential for complete denitrification. Indeed, culture-dependent studies also reveal a much higher proportion of nitrate reducers than of denitrifiers in soils [5], and many culture-independent studies also show that nitrate reducers are about 100-fold than denitrifiers [2, 26]. Denitrifier numbers from MPN counts from the acidic fen approximated 0.02% of the total cell count [27]. The detectability of denitrifiers by quantitative PCR is therefore slightly higher than the detectability by MPN counts. In glacier foreland soils relative *nosZ* gene copy numbers ranged from about 0.02% to about 1% [19], the denitrifier abundance in the acidic fen is therefore in the same range as in other soils or even higher.

4.3 Effect of drying and rewetting on denitrifier communities in the acidic fen

Climate change models predict a higher frequency of extreme weather events, including prolonged periods of drought [38]. Experimental drought increases fluxes of N_2O from the fen, especially when a significant drawdown of the water table is obtained [10]. Subsequent wetting of the soil increases N_2O emission from previously drained fen soil [10]. The increase in N_2O production after rewetting may be attributed to regeneration of electron acceptors such as nitrate due to higher soil aeration during the preceeding drought [21]. N_2O production is most strongly affected in the top soil of the fen [10]. Long-term water table drawdown does not lead to a strong increase in the yearly N_2O emissions in nutrient-poor peatlands, while it increases N_2O emissions from nutrient-rich peatlands [23], but short-term drainage of peatland mesocosms leads to increased N_2O emission from the soil [33]. Short-term drainage mostly effects upper soil layers of fen soil and leads to a more similar microbial community composition in different soil layers [16]. Water table drawdown in wetlands does not affect the community composition of denitrifiers, even though pronounced impacts on the denitrification process are observed [20, 35]. Actinobacterial community composition in a mesotrophic pine fen is relatively drought-resistant, while drought increases the overall biomass and C-turnover [17]. Indeed, the community composition of *narG* did not change during the drought treatment (Figure 6 A), as the *narG* community in the fen Schlöppnerbrunnen is dominated by

Actinobacterial narG [27].

The response of denitrifier abundance to drought differs between different wetland types, ranging from a decrase in abundance in bogs and fens to no changes in riparian wetlands and marshes [20, 35]. Indeed, the observede increase in *nosZ* abundance was smaller than the increase in *narG* abundance, suggesting that nitrate reducers are favoured more strongly by drought conditions than denitrifiers. Denitrifiers are facultative aerobes that generally prefer oxygen as a terminal electron acceptor [41]. Thus, the growth of denitrifiers is likely favoured when lowering of the water table allows the diffusion of oxygen into the soil. However, denitrification can also occur in the presence of oxygen, and some organisms simultaneously use nitrate or oxygen as terminal electron acceptor [37].

Warming experiments in arctic soil show that denitrifier community composition is more strongly influenced by site characteristics than by warming, even though warming did not lead to significant changes in soil water content [40]. Moreover, denitrifier community composition in salt marshes and riparian wetlands is not significantly affected by water table alterations [20, 35]. Long-term water table drawdown in boreal peatlands does not significantly affect *Actinobacterial* community composition [17]. Indeed, most detected *narG* from Schlöppnerbrunnen fen soil affiliated with *Actinobacterial narG* (Figure 1), indicating that *Actinobacteria* in Schlöppnerbrunnen fen might be one major component affecting the observed stability of the nitrate reducer community composition in Schlöppnerbrunnen fen soil.

4.4 Conclusions and limitations

Prolonged periods of drought are anticipated in future years due to climate warming [38]. Experimental drought and subsequent increase *in situ* emissions of N_2O from the acidic fen Schlöppnerbrunnen [10]. The abundance of nitrate reducers and denitrifiers was increased after the drought period and decreased after the rewetting, indicating that the more oxic conditions favour growth of denitrifiers. The composition of the denitrifier community remained largely unaltered. However, the direct link between *in situ* emissions and the molecular data obtained in this study is not possible, as N_2O emissions of the precise stop and timepoint are unresolved, and laboratory incubations of sampled soil are likewise missing. Within these limitations it is still possible to conclude that the community composition of nitrate reducers and denitifiers in the acidic fen Schlöppnerbrunnen is not severely affected by experimental drought.

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Association of Novel and Highly Diverse Acid-Tolerant Denitrifiers with N_2O Fluxes of an Acidic Fen▽†

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Wetlands are sources of denitrification-derived nitrous oxide (N_2O). Thus, the denitrifier community of an N_2O-emitting fen (pH 4.7 to 5.2) was investigated. N_2O was produced and consumed to subatmospheric concentrations in unsupplemented anoxic soil microcosms. Total cell counts and most probable numbers of denitrifiers approximated 10^{11} cells · g_{DW}^{-1} (where DW is dry weight) and 10^8 cells · g_{DW}^{-1}, respectively, in both 0- to 10-cm and 30- to 40-cm depths. Despite this uniformity, depth-related maximum reaction rate (v_{max}) values for denitrification in anoxic microcosms ranged from 1 to 24 and −19 to −105 nmol N_2O h^{-1} · g_{DW}^{-1}, with maximal values occurring in the upper soil layers. Denitrification was enhanced by substrates that might be formed via fermentation in anoxic microzones of soil. N_2O approximated 40% of total nitrogenous gases produced at *in situ* pH, which was likewise the optimal pH for denitrification. Gene libraries of *narG* and *nosZ* (encoding nitrate reductase and nitrous oxide reductase, respectively) from fen soil DNA yielded 15 and 18 species-level operational taxonomic units, respectively, many of which displayed phylogenetic novelty and were not closely related to cultured organisms. Although statistical analyses of *narG* and *nosZ* sequences indicated that the upper 20 cm of soil contained the highest denitrifier diversity and species richness, terminal restriction fragment length polymorphism analyses of *narG* and *nosZ* revealed only minor differences in denitrifier community composition from a soil depth of 0 to 40 cm. The collective data indicate that the regional fen harbors novel, highly diverse, acid-tolerant denitrifier communities capable of complete denitrification and consumption of atmospheric N_2O at *in situ* pH.

Nitrous oxide (N_2O) is a potent greenhouse gas with a global warming potential that is 300-fold higher than that of CO_2, and its concentration increased from 270 ppb in 1750 to 319 ppb in 2005 (17). N_2O can be produced in soils during denitrification, nitrification, the dissimilatory reduction of nitrate to nitrite and/or ammonium (hereafter referred to as dissimilatory nitrate reduction), or the chemical transformation of nitrite or hydroxylamine (5, 7, 49). The percentage of N_2O produced in any of these processes is variable, depending mainly on the redox potential, pH, and C/N ratio (49). In anoxic ecosystems such as waterlogged soils, most of the N_2O is considered to be denitrification derived (7, 9). Complete denitrification is the sequential reduction of nitrate to dinitrogen (N_2) via nitrite, nitric oxide (NO), and N_2O (75). The main product of denitrification varies with the organism and *in situ* conditions and is usually either N_2O or N_2 (68). N_2O can occur as a by-product during dissimilatory nitrate reduction when accumulated nitrite interacts with nitrate reductase to form N_2O (59). The production of N_2O by dissimilatory nitrate reducers is favored in environments with large amounts of readily available organic carbon (65). Thus, their contribution to nitrate-dependent production of N_2O in soils is likely insignificant compared to that of denitrifiers.

The oxidoreductases involved in denitrification are termed dissimilatory nitrate reductase (Nar, encoded by *narGHJI*, or Nap, encoded by *napEDABC*), nitrite reductase (Nir, encoded by *nirK* and *nirS*), NO reductase (cNor and qNor, encoded by *norBC* and *norB*, respectively), and N_2O reductase (Nos, encoded by *nosZ*) (75). Nitrate reductase is also found in dissimilatory nitrate reducers (60). *narG* can therefore be used as a molecular marker to assess both denitrifiers and dissimilatory nitrate reducers, whereas *nosZ* is specific for the assessment of denitrifiers (25, 43, 48).

Denitrification in soils is regulated by temperature, pH, substrate (i.e., carbon) availability, and water content (10, 24, 66). Although denitrification increases with increasing temperature, it can still occur at temperatures below 0°C (10, 24). Low temperatures appear to limit the activity of N_2O reductase more severely than other enzymes involved in denitrification and thus yield higher relative amounts of denitrification-derived N_2O (24). Although denitrification activity usually decreases under acidic conditions, the relative percentage of N_2O to total denitrification-derived nitrogenous gases increases with increasing acidity, a result attributed to the sensitivity of N_2O reductase to low pH (27, 70). However, denitrifier communities can be adapted to the *in situ* pH of the system (40, 58, 73).

Wetlands are ecosystems in which denitrification is likely a dominant source of emitted N_2O (7, 44, 45). The identification and analysis of main drivers for N_2O production (i.e., the microbiota catalyzing N_2O production and consumption) is thus of major concern in such environments. Fens are specialized wetlands characterized by soil acidity (67). However, information on acid-tolerant denitrifier communities of such wetlands is scarce. It is hypothesized that fens harbor a diverse, hitherto unknown, denitrifier community that is adapted to *in situ* conditions and associated with N_2O fluxes (i.e., fen deni-

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† Supplemental material for this article may be found at http://aem.asm.org/.

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trifiers are acid tolerant and have a high affinity for nitrate and N_2O). Thus, the main objectives of the present study were to evaluate the capacities of denitrifier communities of an N_2O-emitting fen (20) to produce or consume N_2O and to determine if a novel and diverse denitrifier community was associated with these capacities.

MATERIALS AND METHODS

Sampling site. The minerotrophic fen Schlöppnerbrunnen is located in the Lehstenbach catchment in the Fichtelgebirge, Bavaria, Germany (50°07′53″N, 11°52′51″E), at approximately 700 m above sea level. The fen has a mean annual air temperature of 5.3°C and a mean annual precipitation of 1,160 mm (16). The fen soil is a fibric histosol on granite bedrock and was described previously (42, 72). Vegetation consists mainly of *Molinia caerulea*, *Eriophorum vaginatum*, *Carex canescens*, and *Juncus effusus* (42). Total carbon is 140 to 410 mg of C g_{DW}^{-1} of soil (where DW is dry weight), and total nitrogen is 4 to 24 mg of N g_{DW}^{-1} (42, 72). Two independent soil samples per sampling were taken from soil layers at 0 to 40 cm at 10-cm intervals with a soil corer. Soil was transported on ice in air-tight plastic bags and stored at 2°C for no longer than 4 days before processing. Soil for DNA extraction was stored immediately at −80°C.

Soil parameters. Nitrate and nitrite concentrations as well as soil pH were determined in 2 M KCl extracts (3 g of soil in 7 ml KCl; extraction for 16 h at 2°C). Nitrate was measured by flow injection analysis (FIA-LAB; MLE, Dresden, Germany); nitrite was determined colorimetrically (23). pH was determined with a pH electrode (InLab 422; Mettler Toledo GmbH, Gießen, Germany) as a water-soil solution (1:4 dilution of fen soil with double-distilled water). Moisture content was determined by weighing the soil before and after drying at 40°C for 1 week.

Assessment of denitrification in fen soil microcosms. Approximately 16 g of homogenized soil from four depths and two soil cores was diluted with 3 volumes of sterile water and placed into 125-ml infusion flasks that were then sealed with gas-tight rubber stoppers. The gas phase was sterile argon. Experiments were done in triplicate. Preliminary tests with unsupplemented microcosms (i.e., fen soil slurries) assessed denitrification potentials from *in situ* nitrate and nitrite. Microcosms were preincubated for 14 to 18 h at 15°C before being supplemented with nitrate (provided as $NaNO_3$) or N_2O; this preincubation was designed to consume traces of nitrate and nitrite by denitrification prior to the addition of nitrate or N_2O.

Nitrate concentrations of 0 to 100 μM were used for apparent Michaelis-Menten kinetics, as up to 130 μM *in situ* nitrate has been observed (42, 55). N_2O ranged from 0 to 56 μM (based on the volume of the aqueous phase), covering atmospheric N_2O concentrations as well as concentrations where N_2O consumption rates were maximal. The incubation time was 2 to 14 h, depending on the rate of N_2O production or consumption. Acetylene blocks the reduction of N_2O at the level of N_2O reductase (74), and parallel nitrate-supplemented microcosms with and without acetylene (15% [vol/vol] in the headspace) were used to differentiate total denitrification and N_2O production, respectively. Microcosms were incubated at 15°C at *in situ* pH in the dark unless otherwise indicated. Kinetic parameters (K_m and the maximum reaction rate [v_{max}]) for nitrate-dependent denitrification were based on the production of N_2O in microcosms supplemented with both nitrate and acetylene. Headspace concentrations of N_2O were determined at three time points via gas chromatography for determining rates of N_2O production or consumption. N_2O was quantified with a Hewlett-Packard 5980 series II gas chromatograph equipped with an electron capture detector, 3396 series II integrator, and a Porapak Q-80/100 (Supelco, Bellefonte, Pa.) column (length, 4 m; inner diameter, 3.2 mm) with Ar-CH_4 (95:5) as the carrier gas (40 or 20 ml per min); the injector temperature was 150°C, the column temperature was 60°C, and the detector temperature was 300°C (modified from reference 26).

Apparent Michaelis-Menten kinetics were fitted to the data points using the program SigmaPlot 2000 (SPSS Science Software GmbH, Erkrath, Germany) for calculation of K_m and v_{max} according to the following equation (56): $v = (v_{max} \cdot [S])/(K_m + [S])$.

The influence of temperature, pH, and electron donors was tested at initial nitrate or N_2O concentrations of 100 μM or 0.1 μM, respectively. Sodium salts of acetate, formate, succinate, or butyrate were supplied at 300 μM each, whereas ethanol was supplied at 500 μM. Consumption of electron donors was assessed by high-performance liquid chromatography (8, 31, 34).

Temperature and pH optima were calculated with SigmaPlot, version 10.0 (Systat Software Inc., San Jose, CA), based on the observed denitrification rates at different incubation temperatures (i.e., at 0.5, 5, 15, 27, 38, 48, 59, and 69°C) or incubation pH values. Temperature optima were approximated by the maximum of the following Ratkowsky equation (46): $\sqrt{r} = a(T - T_{min})[1 - e^{b(T-T_{max})}]$, where r is the rate of N_2O production or consumption at a given temperature T (in K); T_{min} and T_{max} are the minimal and maximal temperatures (in K), respectively, at which N_2O production or consumption approximates zero; and a and b are fit parameters. pH optima were approximated with a Gaussian model: $y = ae^{-0.5(x-x_0)^2}$, where y is the observed N_2O production rate at a given pH x, x_0 is the optimum pH, and a and b are fit parameters.

Total cell counts and enumeration of denitrifiers. One milliliter of homogenized samples of 10^{-3} or 10^{-4} soil dilutions was incubated with 0.5 ml of phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer [pH 7.4] containing 130 mM NaCl) containing 1 μg of 4′,6-diamidino-2-phenylindoldihydrochloride (DAPI) for 20 min on ice. Cells were fixed on polycarbonate filters (pore size, 0.2 μm; GTTP 4700; Millipore, Eschborn, Germany) via vacuum filtration and washed with PBS and then with 96% ethanol to reduce background fluorescence. Cells were quantified using a fluorescence microscope (Zeiss Axioscope 2; Carl Zeiss AG, Oberkochen, Germany) equipped with mercury vapor lamp HBO100. Cells were counted in 11 randomly chosen squares (square size was 15.25 mm^2).

Most probable numbers (MPN) of denitrifiers were determined in triplicate (11). The mineral salts medium contained the following (in mg/liter) (modified from references 1 and 28): $(NH_4)_2SO_4$, 12.6; Na_2SO_4, 13.5; $CaCl_2 \cdot 2\,H_2O$, 10.0; $MgCl_2 \cdot 2\,H_2O$, 10.0; KH_2PO_4, 0.4; $FeCl_2 \cdot 4\,H_2O$, 10; $MnSO_4 \cdot 1\,H_2O$, 5; $FeSO_4 \cdot 7\,H_2O$, 1; $CoCl_2 \cdot 6\,H_2O$, 1; $CaCl_2 \cdot 2\,H_2O$, 1; $ZnSO_4 \cdot 7\,H_2O$, 1; $CuSO_4 \cdot 5\,H_2O$, 0.1; $AlK(SO_4)_2 \cdot 12\,H_2O$, 0.2; H_3BO_3, 0.1; $Na_2MoO_4 \cdot 2\,H_2O$, 0.1; and nitrilotriacetic acid, 15; the medium also contained vitamins ([μg/liter] biotin, 2; folic acid, 2; pyridoxine-HCl 10; thiamine-HCl, 5; riboflavin, 5; niacin, 5; DL-Ca-pantothenic acid, 5; vitamin B_{12}, 0.1; *p*-aminobenzoic acid, 5; lipoic acid, 5). This medium was supplemented with nutrient broth (0.27 g/liter), $NaNO_3$ (final concentration of 5 mM), and glutamate, succinate, butyrate, and ethanol (final concentration of 1.5 mM each). Anoxic medium were prepared using modified Hungate techniques (72); the pH was 5, and the headspace was sterile argon. Tubes were incubated in the dark at 15°C for 3 months. Denitrifiers were scored positive for growth when the optical density (at 660 nm) of culture tubes was greater than 0.04 and either N_2 (at values 5-fold greater than that in uninoculated control tubes) or N_2O (at values 5-fold greater than that of air) was produced.

Extraction of nucleic acids. Nucleic acids were extracted using a bead-beating protocol (21), followed by separation of DNA and RNA using a Qiagen RNA/DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Amplification of *narG* and *nosZ*. *narG* and *nosZ* were amplified using the primer pair narG1960f (TAY GTS GGS CAR GAR AA) and narG2650r (TTY TCR TAC CAB GTB GC) (43) and the pair nosZF (CGC TGT TCI TCG ACA GYC AG) and nosZR (ATG TGC AKI GCR TGG CAG AA), respectively (48). Each PCR was preceded by an initial denaturation (95°C for 5 min). Denaturation and elongation were at 95°C and 72°C, respectively. Seven precycles with annealing at 55°C were followed by 26 cycles with annealing at 51°C for *narG*; denaturation, annealing, and elongation were for 1, 1, and 2 min, respectively. Annealing was lowered stepwise from 58°C to 52°C in 10 precycles for *nosZ*, followed by 30 cycles with annealing at 52°C; denaturation, annealing, and elongation were for 0.5, 1, and 1 min, respectively. Final elongation was for 10 min at 72°C.

Cloning, screening, and sequencing. PCR products of *narG* and *nosZ* were purified using a MinElute gel extraction kit (Qiagen GmbH, Hilden, Germany). Purified PCR products were ligated into vector pGEM-T (Promega, Mannheim, Germany) and transformed into competent *Escherichia coli* JM109 cells (Promega) according to the manufacturer's protocol. Clones were screened for insert-positive vectors via amplification with primers $M13_{uni}$/$M13_{rev}$ (35). Gene libraries were screened by restriction fragment length polymorphism (RFLP) analysis. Products from PCRs using the M13 primers (M13-PCR) were digested with HaeIII and CfoI (2 U at 4 h at 37°C). Restriction fragments were separated on a 3% agarose gel (1 h at 100 V). One to two representative clones of each RFLP pattern were sequenced. M13-PCR products were purified prior to sequencing with a Millipore Multiscreen 96-well filtration system (Millipore Corp., Bedford, MA). Sequencing was done commercially by Macrogen (Seoul, South Korea).

Sequence analyses. Sequence analyses were done with MEGA, version 4.0 (http://www.megasoftware.net/) (29). Sequences were edited, translated *in silico*, and aligned with reference sequences using the ClustalW algorithm implemented in MEGA, version 4.0. Sequences that had the same RFLP patterns with sequenced gene fragments were represented by the sequenced fragments. The abundance of each sequence in the alignment was adjusted according to the

number of observations of the corresponding RFLP pattern in the gene library. The alignments were refined manually. Phylogenetic trees were constructed from *in silico* translated sequences. Each neighbor-joining (52) tree was constructed with 1,000 bootstrap replicates. Uncorrected distance matrices with sequences from different soil layers were created from the translated amino acid alignment and used for diversity analyses in DOTUR (54). Grouping of sequences into operational taxonomic units (OTUs), as well as estimations of species richness and species diversity (expressed by the Shannon diversity index), was conducted at sequence differences of 0 and 41% for *narG* and 0 and 14% for *nosZ* to assess maximal diversity as well as the species-level diversity of denitrifiers. The thresholds for estimating species-level diversity were obtained from comparisons of 16S rRNA similarities and structural gene similarities of cultured denitrifiers (39). Coverage (C) is the number of the detected genotypes relative to their expected total number in a gene library, and was calculated as follows: $C = (1 - n \times N^{-1}) \times 100$, where n is the number of genotypes that occurred only once, and N is the number of clones screened (54).

TRFLP analyses. *narG* and *nosZ* were amplified with fluorescently labeled primers (narG1960f-DY681/narG2650r-DY781 and nosZF-DY681/nosZR-DY781) for terminal RFLP (TRFLP) analysis. Fluorescently labeled DNA was digested with mung bean nuclease (New England Biolabs, Frankfurt am Main, Germany) to remove single-stranded DNA and reduce the probability of pseudo-terminal restriction fragments (14). Digested DNA was purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany). PCR products of *narG* were digested with the restriction enzymes CfoI, HaeIII, or XhoI (New England Biolabs, Frankfurt am Main, Germany), and PCR products of *nosZ* were digested with BtgI, NlaIV, or PvuI plus SacI (New England Biolabs, Frankfurt am Main, Germany) (double digest). Gel electrophoresis was performed with an NEN model 4300 DNA analyzer (Licor, Lincoln, NE). The polyacrylamide gel consisted of 15 g of urea, 3.75 ml of 40% acrylamide-bisacrylamide solution (37.5:1; Bio-Rad, Hercules, CA), 6 ml of 5× Tris-borate-EDTA buffer (AppliChem GmbH, Darmstadt, Germany), and 9.25 ml of double-distilled H_2O. A bind-silane solution (1:1 bind-silane [PlusOne; GE Healthcare, Piscataway, NJ] and 10% acetic acid) was applied to the glass plates for stabilizing the comb region of the gel. The gel was poured according to the manufacturer's protocol (Licor, Lincoln, NE). Electrophoresis was performed for 3 h at 1,500 V and 45°C. Gels were analyzed with GelQuest (Sequentix, Klein Raden, Germany). Terminal restriction fragments (TRFs) were assigned to *narG* or *nosZ* sequences via *in silico* TRF analysis in MEGA, version 4.0. Principal component analyses of the combined TRFLP profiles were conducted using RapidMiner (http://rapid-i.com/) for *narG* and *nosZ*.

Nucleotide sequence accession numbers. Sequences are deposited in EMBL under accession numbers FN430426 to FN430490 (*narG*) and FN430491 to 430566 (*nosZ*).

RESULTS

Soil parameters. Soil moisture content ranged from 37 to 90%, was highest in the 0- to 10-cm-depth soil, and decreased with increasing soil depth. Soil pH varied between 3.4 and 3.7 in KCl extracts and between 4.7 and 5.2 in water extracts (sampling dates, 2 July 2007 and 28 February 2008). Nitrate and nitrite concentrations were below the detection limit of 0.4 μM and 5 μM, respectively (sampling date, 2 July 2007).

Depth-related denitrification in acidic fen soil. Unsupplemented anoxic fen soil produced only minor amounts of N_2O (up to 40 nmol · g_{DW}^{-1} after 3 days), which was in agreement with the low nitrate and nitrite concentrations in the soil. The N_2O that was produced initially was completely consumed after 3 days in unsupplemented microcosms, demonstrating the potential of the acidic fen soil to consume N_2O. Supplemental nitrate stimulated the production of N_2O in all soil layers without apparent delay (Fig. 1A). The amount of N_2O that accumulated in nitrate-supplemented (20 μM nitrate or less) microcosms leveled off or started to decrease after 2 h of incubation in microcosms without acetylene (data not shown), suggesting that N_2O was subject to consumption under these conditions. The production of N_2O was linear at all supplemental nitrate concentrations in microcosms with acetylene. The ratios of N_2O to total N gases (i.e., N_2 plus N_2O) of 0- to 20-cm-depth soils were lower than those of 20- to 40-cm-depth soils; increased nitrate concentrations yielded increasingly higher ratios with 0- to 20-cm-depth soils (see Fig. S1 in the supplemental material).

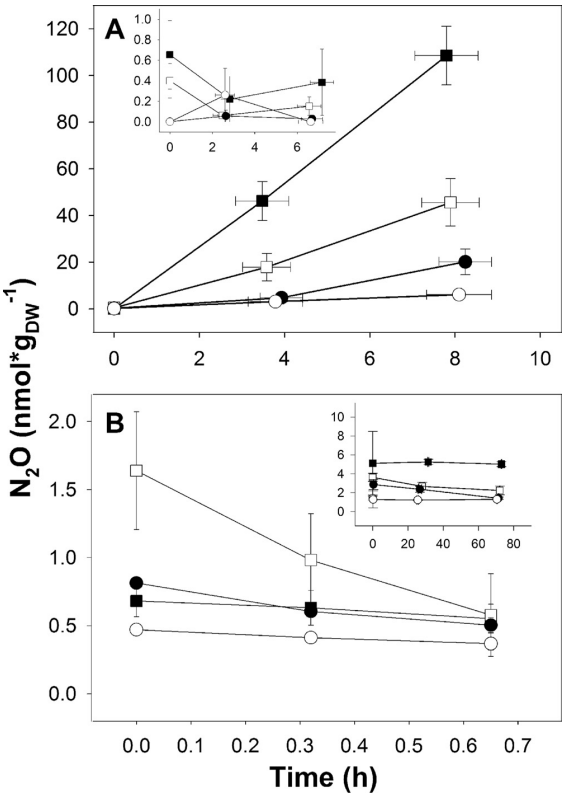

FIG. 1. Production (A) or consumption (B) of N_2O by fen soil after the addition of 40 μM nitrate (A) or 0.02 μM N_2O (B). Microcosms used for the experiments shown in panel A were supplemented with acetylene. Mean values and standard errors of three replicates are shown (sampling dates were 10 July 2007 for panel A and 17 July 2007 for panel B). The inset in panel A shows the control without supplemental nitrate; the inset in panel B shows the control treated with acetylene. Closed squares represent the soil layer at 0 to 10 cm, open squares represent the soil layer at 10 to 20 cm, closed circles represent the soil layer at 20 to 30 cm, and open circles represent the soil layer at 30 to 40 cm.

The consumption of supplemental N_2O by fen soil was linear and without apparent delay and was blocked by acetylene (Fig. 1B). N_2O was consumed to less than 100 ppb, a concentration less than that of atmospheric N_2O, which approximates 319 ppb (18), indicating that acid-tolerant fen denitrifiers in all fen soil layers were capable of consuming atmospheric N_2O. Initial nitrate-dependent N_2O production rates and N_2O-dependent N_2O consumption rates followed apparent Michaelis-Menten kinetics (Table 1; see also Fig. S2 in the supplemental material). v_{max} values were highest in upper soil layers and decreased with soil depth, indicating that denitrification potentials in upper soil layers were greater than those of lower soil layers.

TABLE 1. Kinetics, temperature optima, and pH optima of denitrification by denitrifiers in fen soil microcosms

Depth of soil layer (cm)	*In situ* pH	Value in nitrate-amended soil				Value in N_2O-amended soil		
		v_{max} (nmol · h^{-1} g_{DW}^{-1})	K_m (μM)	Optimum temp (°C)	Optimum pH	v_{max} (nmol · h^{-1} g_{DW}^{-1})	K_m (μM)	Optimum temp (°C)
0–10	5.2	24 ± 2	19 ± 3	34	5.5	83 ± 13	47 ± 13	20
10–20	4.8	9 ± 1	15 ± 4	34	4.5	105 ± 5	10 ± 2	20
20–30	4.7	3 ± 1	9 ± 6	46	4.2	40 ± 4	15 ± 4	30
30–40	4.8	1 ± 0	6 ± 0	38	4.3	19 ± 4	24 ± 9	6

Effects of temperature, pH, and electron donors on fen denitrifiers. Denitrification, including the capacity to consume N_2O, occurred at temperatures ranging from 0.5°C to 70°C. The optimal temperatures for nitrate-dependent N_2O production and the consumption of N_2O ranged from 34°C to 46°C and 6°C to 30°C, respectively (Table 1). Denitrification rates at temperatures above 60°C were minimal, a trend similar to that observed with other soils (33).

Denitrification occurred at pH 2 to 6.6 in all soil layers. Denitrification was observed at pH 7.5 only with 0- to 10-cm-depth soil. Highest denitrification rates were observed at *in situ* pH (i.e., 4.7 to 5.2). The ratio of N_2O to total N gases tended to decrease with increasing pH (Fig. 2). At *in situ* pH, N_2O approximated 40% of total N gasses in 0- to 30-cm-depth soils and around 80% in 30- to 40-cm-depth soils. N_2O constituted nearly 100% of total N gases produced at pHs 3.1 and 2.2.

Denitrification rates of formate-supplemented microcosms were up to 2.5-fold higher than those of unsupplemented microcosms (see Fig. S5 in the supplemental material). Denitrification rates of acetate- and ethanol-supplemented microcosms were up to 1.5 times higher than those of unsupplemented microcosms. These enhancements of denitrification were concomitant to the net consumption of up to 150 μM formate, 50 μM acetate, and 600 μM ethanol. Succinate and butyrate did not significantly augment denitrification (Fig. S5).

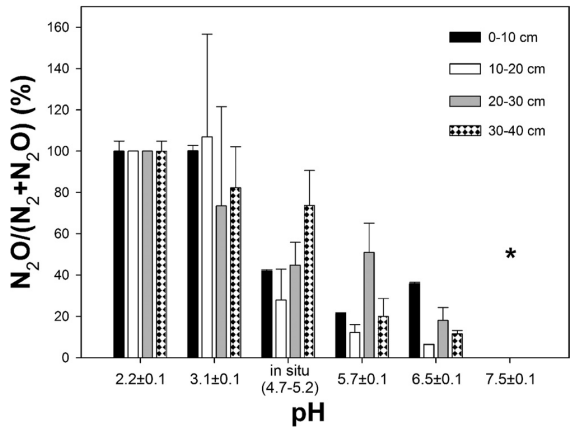

FIG. 2. Effect of pH on the relative amount of N_2O produced by fen soil. The asterisk indicates that N_2O was detected in the presence but not in the absence of acetylene in 0- to 10-cm-depth soil and was not detected in 10- to 40-cm-depth soil microcosms. Mean values and standard errors of three replicates are shown (sampling date, 28 February 2008).

Enumeration of fen microbes and denitrifiers. Total cell numbers (DAPI counts) approximated $3.2 \times 10^{11} \pm 0.5 \times 10^{11}$ cells · g_{DW}^{-1} and $1.0 \times 10^{11} \pm 0.5 \times 10^{11}$ cells · g_{DW}^{-1} in 0- to 10-cm and 30- to 40-cm-depth soils, respectively. Denitrifier counts were similar at different soil depths, approximating 7.5×10^7 (3.2×10^7 to 1.7×10^8) cells · g_{DW}^{-1} and 5.9×10^7 (2.5×10^7 to 1.4×10^8) cells · g_{DW}^{-1} in 0- to 10-cm and 30- to 40-cm-depth soils, respectively.

Phylogenetic analysis of fen denitrifiers. A total of 191 *narG* and 196 *nosZ* clones were analyzed by RFLP, and sequences were obtained from 64 and 75 clones, respectively. Translated amino acid sequences of representative *narG* and *nosZ* sequences were assigned to 15 and 18 OTUs, respectively (Table 2). The library coverages were 87 to 99% for *narG* at a sequence dissimilarity of 41% and 87 to 97% for *nosZ* at a sequence dissimilarity of 14% (39) (Table 2), indicating that the numbers of clones sampled were sufficient. *narG* and *nosZ* sequences from the acidic fen formed seven and five distinct clusters in the phylogenetic trees, respectively (Fig. 3 and 4). Most *narG* sequences were related to uncultured soil bacteria. Cultured relatives of fen sequences included *Comamonas nitrativorans* and species of *Brucella* and *Hydrogenophaga* (Fig. 3). *nosZ* sequences of clusters 1, 3, and 4 were closely related to *Bradyrhizobium japonicum*, *Azospirillum lipoferum*, and *Azospirillum irakense*, respectively, while *nosZ* sequences of cluster 2 were not closely related to cultured denitrifiers (Fig. 4).

The Shannon-Weaver diversity index for translated *narG* and *nosZ* as well as their estimated species richness values was highest in the upper soil layers and tended to decrease with increasing soil depth (Table 2).

Depth-resolved TRFLP fingerprints of fen denitrifiers. Different soil layers yielded dissimilar relative *narG* TRF intensities (Fig. 5). *narG* OTUs 1, 3, and 4 were consistently identified in the TRFs generated with the three restriction digests (see Table S1 in the supplemental material). A 57-bp TRF (TRF 57 bp) and TRF 128 bp (digestion with CfoI) were dominant in all soil layers. The relative abundance of TRF 57 bp increased from 40% in 0- to 10-cm-depth soil to 55% in 30- to 40-cm-depth soil, while the relative abundance of TRF 128 bp decreased from 40% in 0- to 10-cm-depth soil to 25% in 30- to 40-cm-depth soil, indicating a depth-related decrease of a portion of sequences belonging to *narG* OTUs 3 and 4 and a depth-related increase of another portion of sequences belonging to *narG* OTUs 3 and 4. TRFs 80 bp and 169 bp were not detected in 30- to 40-cm-depth soil, indicating the absence of a portion of sequences belonging to OTU 3 at this soil depth. The number of TRFs detected with HaeIII increased from two in 0- to 10-cm-depth soil to four in 30- to 40-cm-depth soil. The relative fluorescence of TRF 56 bp decreased significantly

from 65% with 0- to 10-cm-depth soil to 15 to 30% with lower soil layers, indicating a decrease of a portion of sequences belonging to OTUs 1 and 3. TRF 159 bp was detected only in 20- to 30-cm-depth soil, while TRF 292 bp was detected only in 30- to 40-cm-depth soil, where it accounted for 30% of the relative fluorescence, indicating that a significant portion of sequences belonging to OTU 4 occurred only in those soil layers. Digestion with XhoI yielded only two TRFs (i.e., TRF 268 bp and TRF 700 bp). TRF 268 bp was detected at upper and middle soil depths and accounted for 35% relative fluorescence with 10- to 20-cm-depth soil, indicating the presence of a portion of sequences belonging to OTU 3 in this layer. Principal component analysis of the combined *narG* TRFLP profiles indicated that the nitrate reducer communities of 0- to 10-cm-, 20- to 30-cm-, and 30- to 40-cm-depth soils were similar and might differ from the community of 10- to 20-cm-depth soil (Fig. 5A to C; see also Fig. S3 in the supplemental material).

Different soil layers yielded dissimilar relative *nosZ* TRF intensities (Fig. 5). *nosZ* OTUs 1, 3, and 11 were consistently identified in the TRFs generated with the three restriction digests (see Table S1 in the supplemental material). BtgI yielded five TRFs, four of which occurred in all soil layers. TRF 614 bp had the highest relative fluorescence (65%) in 30- to 40-cm-depth soil, indicating a dominance of organisms associated with this TRF (belonging to a portion of sequences from OTU 1) in that soil layer. NlaIV yielded three TRFs, with TRF 700 bp being dominant (80 to 90% relative fluorescence) in 0- to 30-cm-depth soils, indicating that a portion of sequences from OTU 3 occurred more frequently in upper soil layers. TRF 514 bp was dominant (70% relative fluorescence) in 30- to 40-cm-depth soil, indicating that a portion of the OTU 1-affiliated sequences was dominant in deeper soils. Both PvuI and SacI yielded four TRFs. TRF 310 bp could not be assigned to any OTU. TRF 700 bp was dominant in all soil layers (55 to 80% relative fluorescence), indicating that a portion of sequences from OTUs 1 and 3 was dominant. TRF 646 bp decreased with increasing soil depth from approximately 25% to 2% relative fluorescence, indicating that a portion of OTU 11-affiliated sequences was less dominant in deeper soil layers. Principal component analysis of the combined *nosZ* TRFLP profiles indicated that minimal differences in the denitrifier community composition of the sampled soil layers might have occurred, with gradual changes between soil layers (Fig. 5D to F; see also Fig. S4 in the supplemental material).

DISCUSSION

Phylogenetically novel fen denitrifiers. Cultured denitrifier numbers approximated 10^7 cells per gram of dry weight. Similar numbers of cultured fermenters and cultured aerobes occur in the Schlöppnerbrunnen fen (72), whereas the numbers of cultured Fe(III) reducers and cultured methanogens are lower, approximating 10^5 to 10^6 cells per gram of fresh weight and 10^4 to 10^5 cells per gram of dry weight, respectively (47, 72). Thus, denitrifiers appear to be a relatively abundant bacterial group capable of anaerobiosis in Schlöppnerbrunnen fen soil.

Novel *narG* and *nosZ* genotypes indicate that hitherto unknown denitrifiers occur in the Schlöppnerbrunnen fen, and statistical analyses verified a high phylogenetic diversity of the

TABLE 2. Analyses of translated amino acid sequences of *narG* and *nosZ* derived from fen soil

Depth of soil layer (cm)	*narG*									*nosZ*								
	No. of clones[a]	% Dissimilarity[b]	% Library coverage	No. of OTUs observed	No. of OTUs estimated by:[c]				H'[d]	No. of clones[a]	% Dissimilarity[b]	% Library coverage	No. of OTUs observed	No. of OTUs estimated by:[c]				H'
					ACE	Bootstrap	Chao1	Jacknife						ACE	Bootstrap	Chao1	Jacknife	
0–10	51	0	53	36	68	46	64	63	3.5	61	0	77	26	45	32	22	43	3.0
		41	90	12	18	14	14	17	1.8		14	95	10	13	11	12	13	1.8
10–20	30	0	40	24	60	31	46	44	3.1	52	0	77	22	41	27	35	35	2.8
		41	87	8	14	10	10	12	1.4		14	95	8	13	9	10	11	1.6
20–30	80	0	81	33	56	40	41	48	3.0	47	0	60	27	82	34	61	62	3.0
		41	99	8	9	9	8	9	1.6		14	87	12	21	14	20	19	2.0
30–40	22	0	59	14	30	18	21	23	2.5	38	0	76	16	31	20	25	25	2.4
		41	95	5	6	5	5	6	1.4		14	97	4	5	4	4	5	0.8
Total	191	0	94	62	66	70	65	73	3.9	196	0	87	51	96	62	84	88	3.3
		41	98	15	19	19	18	20	2.0		14	97	18	25	21	22	24	2.1

[a] Number in the library.
[b] Percent sequence dissimilarity needed for OTU definition.
[c] Number of genotypes estimated using the ACE, bootstrap, Chao1, and Jackknife richness estimators.
[d] Shannon-Weaver diversity index.

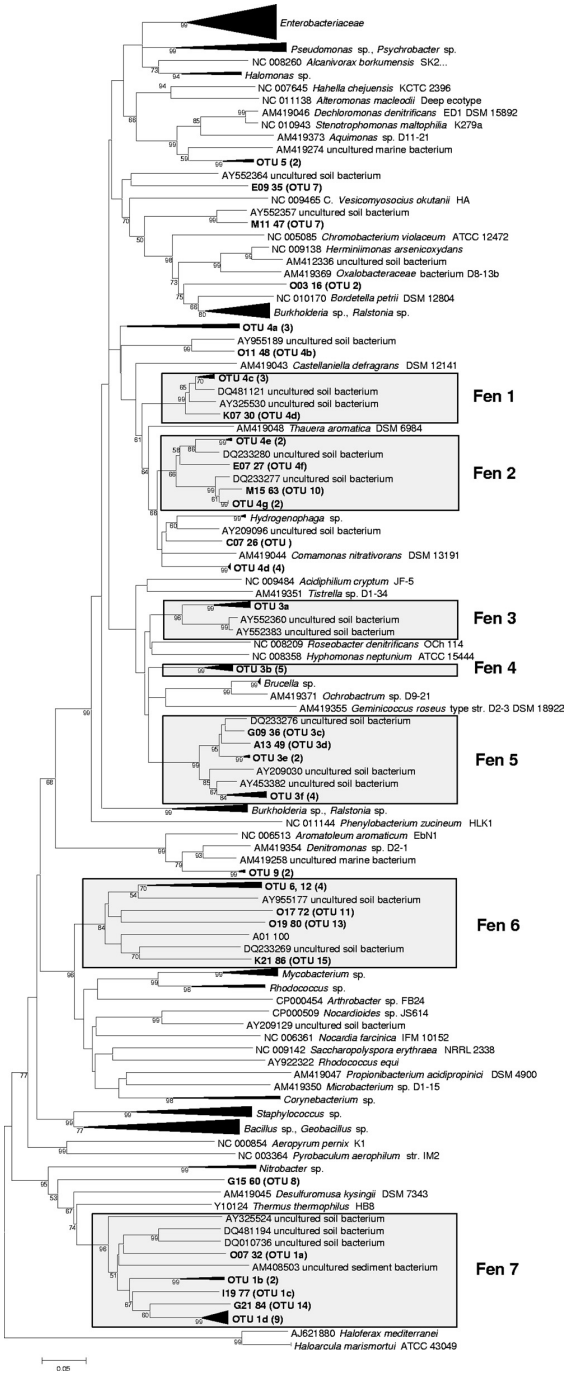

FIG. 3. Phylogenetic tree of *narG* sequences retrieved from the Schlöppnerbrunnen fen. The tree is based on translated amino acid sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

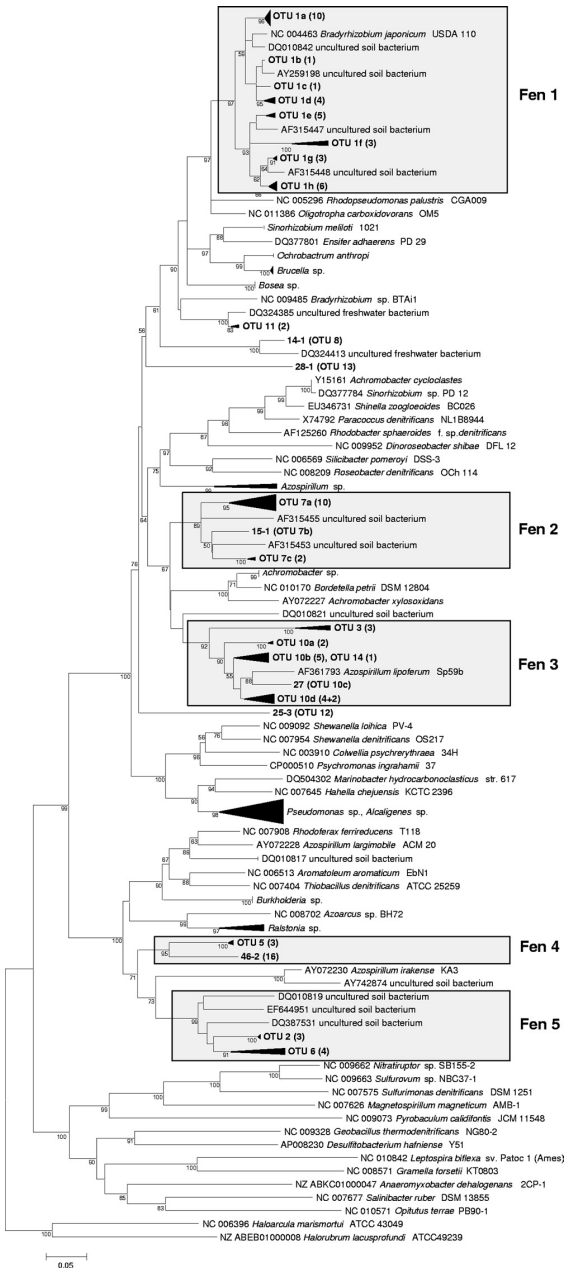

FIG. 4. Phylogenetic tree of *nosZ* sequences retrieved from the Schlöppnerbrunnen fen. The tree is based on translated amino acid sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

denitrifier community. Although novel genotypes were detected, some *nosZ* and *narG* sequences were related to sequences indicative of known soil genera (e.g., *Azospirillum*, *Ralstonia*, and *Bradyrhizobium*) (15, 25, 36, 43), indicating that

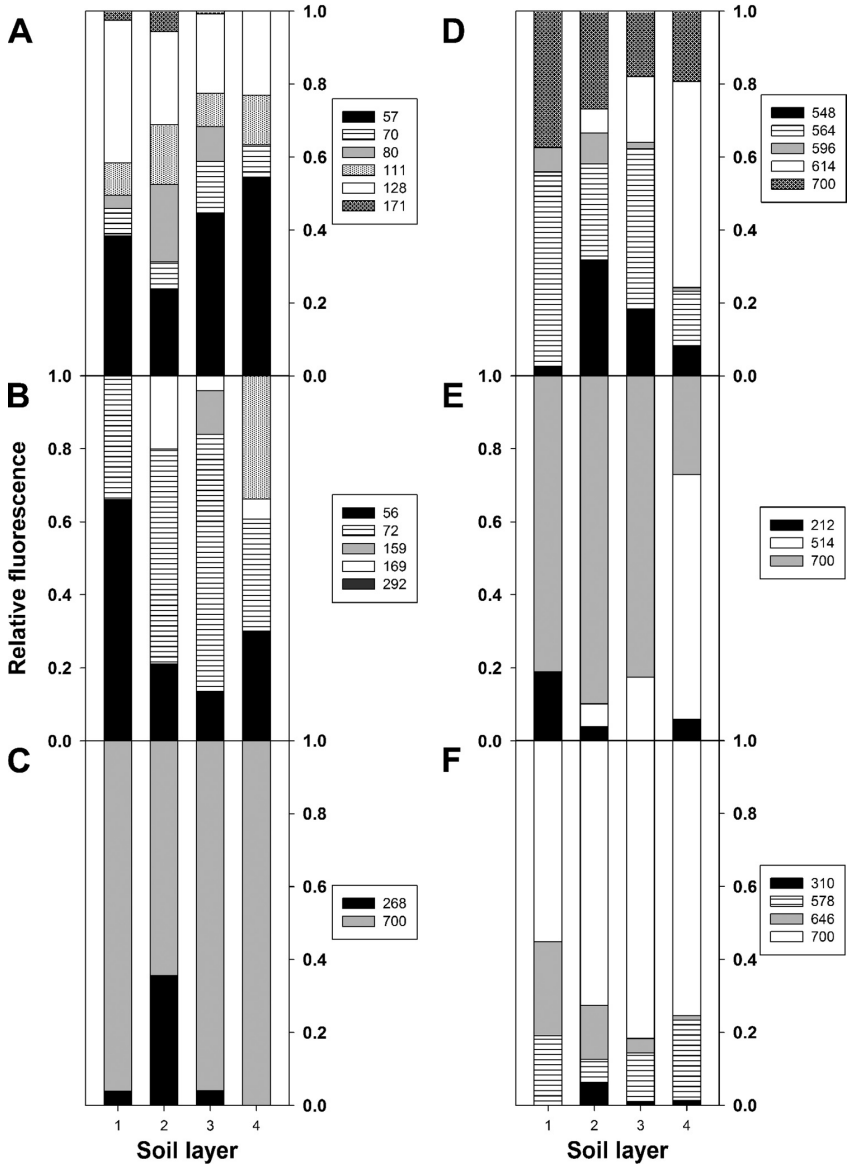

FIG. 5. Comparative TRFLP analyses of *narG* (A to C) and *nosZ* (D to F) amplified from different soil layers of the acidic fen. PCR products were digested with CfoI (A), HaeIII (B), XhoI (C), BtgI (D), NlaIV (E), and PvuI and SacI (F). Mean values of three replicates are shown. Detected TRFs could be assigned to sequences from OTUs 1, 3, and 4 for *narG* and OTUs 1, 3, and 11 for *nosZ*. Soil layers 1, 2, 3, and 4 refer to soil depths of 0 to 10 cm, 10 to 20 cm, 20 to 30 cm, and 30 to 40 cm, respectively. Lengths of detected TRFs (in base pairs) are given in boxes next to the diagrams.

part of the fen denitrifier community is similar to previously resolved genera. Communities of different soil layers were phylogenetically similar, indicating that fen denitrifiers were derived from the same pool of microorganisms. TRFLP analysis of *nosZ* revealed only minor differences between different soil layers. In contrast, *narG*-associated differences were more pronounced, suggesting that the detected dissimilatory nitrate reducers (which have *narG* but lack *nosZ* [60]) in the fen are more dissimilar between soil layers than are detected denitrifiers. The largest difference observed in the *narG* TRFLP profiles was between the profile of 10- to 20-cm-depth soil and profiles of the other soil layers, indicating that 10- to 20-cm-depth soil harbors a nitrate-reducing population that is not identical to the populations of the other soil layers.

***In situ* consequences of denitrifier activity.** Nitrate concentrations in the Schlöppnerbrunnen fen are generally low and

often below the detection limit but can be as high as 0.13 mM in the upper 20 cm of soil (42, 55). Unsupplemented fen soil produced minor amounts of N_2O, reflecting the low *in situ* nitrate concentrations in the fen (30, 42, 55). Supplemental nitrate caused a rapid increase in the production of N_2O without apparent delay, indicating that (i) fen denitrifiers are poised to respond rapidly to nitrate and have a high potential to denitrify, and (ii) *in situ* denitrification is likely limited by nitrate availability. Increased concentrations of nitrate caused an increase in the relative proportion of N_2O in total N gases, a phenomenon that has been observed with other soils (3, 18). The fen Schlöppnerbrunnen is a net source of N_2O, and N_2O concentrations of up to 100 ppm occur in the pore water (20).

There is a large difference between nitrate input and detected nitrate concentrations in the fen soil as nearby oxic soils receive the same amount of nitrate input but have nearly 100-fold higher nitrate concentrations (42). This difference is suggestive of a high turnover of nitrate that is due in part to denitrification. The K_m values (<20 μM) (Table 1) for nitrate are well below the maximum nitrate concentrations found *in situ*, indicating that fen denitrifiers have a high affinity for nitrate and can cope with low nitrate concentrations. Higher nitrate concentrations increased the relative amount of N_2O formed by 0- to 20-cm-depth soils (see Fig. S1 in the supplemental material). Nitrate concentrations above 40 μM are rarely encountered *in situ* (30, 42, 55). Thus, the complete reduction of nitrate to N_2 might occur under most *in situ* conditions, and the relative emissions of N_2O versus N_2 might increase when nitrate concentrations are periodically elevated. The rapid increase of N_2O production in response to nitrate and the capacity of fen soil to consume supplemental N_2O without apparent delay (Fig. 1) suggest that denitrifiers are active *in situ*.

High denitrification potentials in upper soil layers (0 to 20 cm) are coincident with higher concentrations of nitrate in those layers (42, 55). Rain events likely contribute to the larger amounts of nitrate in surface soils. The percentage of N_2O in total N gases formed by fen soils increased with increasing soil depth, a trend that might be due to the limitation of readily available organic carbon in deeper layers in the Schlöppnerbrunnen fen (72). Electron donor limitation can enhance the percentage of N_2O in total N gases produced by pure cultures of denitrifiers (53).

Nitrification versus denitrification as possible sources of N_2O. Isotope signatures of the N_2O indicate that denitrification is the main source of the N_2O emitted from the Schlöppnerbrunnen fen (20). Although denitrification tends to be the dominant source of N_2O under water-saturated conditions (44), nitrification likely occurs in the acidic fen when oxic conditions are augmented during dryer periods. Nitrification during dryer and more oxic conditions would theoretically provide additional nitrate for denitrification in anoxic microzones or subsequent to a rain event. In this regard, N_2O emissions from the fen increase after rewetting events following periods of drought (19).

Denitrification as an N_2O sink. Wetlands can consume N_2O (4). The capacity of wetlands to consume N_2O is influenced by environmental factors such as pH and temperature, as well as the composition of the microbial community (6). The capacity of Schlöppnerbrunnen fen soil to consume N_2O to subatmospheric levels under anoxic conditions and the periodic occurrence of N_2O at subatmospheric concentrations in fen pore water (20) are indirect evidence that N_2O consumption occurs *in situ*. Isotope signatures of N_2O from the fen indicate that the upward diffusion of the N_2O produced in lower soil layers is subject to reduction to N_2 in the upper soil layers (20). Indeed, N_2O consumption rates were higher in upper soil layers than in lower soil layers. These collective findings suggest that the Schlöppnerbrunnen fen functions as not only an N_2O source but also an N_2O sink.

Ecophysiology of fen denitrifiers. K_m values for denitrification ranged from 6 to 19 μM nitrate, indicating that fen denitrifiers had a high affinity for nitrate. K_m values were in the same range or lower than those of other soil types (32, 37, 62) and in the range of those of pure cultures that display a high affinity for nitrate (e.g., species of *Alcaligenes*, *Pseudomonas*, and *Flavobacterium*) (2, 41, 64).

Denitrification rates of different fen soil layers were optimal at 34 to 46°C, optimal temperatures that approximate those of many model soil denitrifiers (e.g., *Pseudomonas denitrificans*, which denitrifies optimally at 38°C) (69). In contrast, the highest denitrifier activity and highest numbers of cultured denitrifiers of different soils occur between 25 and 30°C (50, 51), indicating that denitrifiers in the Schlöppnerbrunnen fen have a temperature optimum that is slightly higher than the optima of denitrifiers from other soils. Enhanced denitrification capacities at temperatures that exceed most *in situ* conditions are a common phenomenon, and higher rates of denitrification in soils in summer can be attributed to increased soil temperatures (10, 24, 33).

The capacity of fen soil to consume N_2O under anoxic conditions was blocked by acetylene (Fig. 1B) and is therefore assumed to be due to the reduction of N_2O to N_2 by N_2O reductase (74). The consumption of N_2O by different soil layers was highest between 6 and 30°C, temperatures lower than those for nitrate-dependent denitrification. These contrasting temperature optima suggest that different denitrifier subpopulations in the acidic fen have different temperature and electron acceptor (i.e., nitrate or N_2O) preferences. The terminal reaction of the denitrification pathway appears to be more adapted to *in situ* temperatures than the preceding reactions. The degree to which N_2O reductase of soil denitrifiers is inhibited by lower temperatures varies, with effects ranging from no inhibition to almost complete inhibition (12).

Denitrification rates were highest at *in situ* pH, indicating that Schlöppnerbrunnen fen denitrifiers are well adapted to the moderately acidic fen environment. Denitrification activities of acidic agricultural soils can be highest at *in situ* pH even though denitrification capacities might be higher in more pH-neutral soils (40), suggesting that soils of different pH values harbor distinct denitrifier communities adapted to *in situ* pH. Many pure cultures of denitrifiers (e.g., *Pseudomonas* sp.) have nearly neutral to slightly alkaline pH optima (63). Denitrification also occurred at very low pH by all fen soil layers, whereas only the upper 10-cm soil layer was capable of denitrification under slightly alkaline conditions. Therefore, alkaline conditions appear to be more limiting for fen denitrifiers than acidic conditions, which is consistent with the *in situ* conditions fen denitrifiers are subjected to. Acidic pH increases the percentage of N_2O in total N gases (57, 70). Denitrification by *Para-*

coccus denitrificans yields nitrite and nitrous oxide as transient intermediates at pH 5.5, whereas the amounts of these intermediates are low or not detectable at pH 8.5 (64). Up to 5 μM N_2O occurs in the fen pore water (20), indicating that those intermediates occur *in situ*. The relative percentage of N_2O in total N gases formed by Schlöppnerbrunnen fen soil was highest at pHs of 2 to 3 but was similar at *in situ* pH to values obtained at pH 7, indicating that the N_2O reductases of fen denitrifiers are not inhibited by the moderately acidic *in situ* conditions.

Conclusions. Schlöppnerbrunnen fen soil produces formate, ethanol, and acetate under anoxic conditions via fermentation (22, 72). Such substrates are utilized by pure cultures of denitrifiers such as *Pseudomonas denitrificans*, *Pseudomonas stutzeri*, and *Paracoccus denitrificans* (38, 61); formate and acetate are detectable in the fen pore water (22, 30, 72); and the augmentation of denitrification in fen soil microcosms by these substrates suggests that fen denitrifiers might form trophic links to fen fermenters. Denitrification optima by fen denitrifiers at moderately acidic pH are dissimilar to those of model denitrifiers (such as those listed above). That the temperature optima of fen denitrifiers are above temperatures usually occurring *in situ* indicates that the fen denitrifiers are prone to respond to global warming with increased activity. Thus, the source and sink functions of the fen for N_2O might be enhanced. These physiological findings and the novel phylogeny of denitrifier community members indicate that the fen contains heretofore unknown denitrifiers that are adapted to *in situ* conditions and are integrated in the intermediary ecosystem metabolism (i.e., processes that link input and output) of the fen (13).

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Katharina Palmer

Dipl. Biol.

Ausbildung

2008–2012 **Promotion**, *Lehrstuhl für Ökologische Mikrobiologie (Betreuung: PD Dr. Marcus A. Horn)*,
Universität Bayreuth.
Thema: Greenhouse gas metabolizing prokaryotes in peatlands

2007–2008 **Diplomarbeit**, *Lehrstuhl für Ökologische Mikrobiologie (Betreuung: PD Dr. Marcus A. Horn, Prof. Harold L. Drake*,
Universität Bayreuth.
Thema: Phylogenetische und funktionelle Diversität N_2O-produzierender und -verbrauchender Prokaryoten in einem sauren, N_2O-emittierenden Niedermoor

2003–2008 **Studium der Biologie**,
Universität Bayreuth.

2006–2007 **Auslandssemester (ERASMUS)**,
Oulun yliopisto, Oulu, Finnland.

2002–2003 **Studium der Physik und Geowissenschaften**,
Ruhruniversität Bochum.

Berufserfahrung

Juli-August 2010 **Forschungsaufenthalt im Ausland (Finnland)**, *im Rahmen des EU-Projektes „Lapland Atmosphere-Biosphere Facility“ (LAPBIAT)*, Forschungsstation Kevo (University of Turku), Forschungsstation Oulanka (University of Oulu),
Thema: Effect of high latitude on denitrification-dependent N_2O-fluxes and denitrifier community structure in acidic mires.

2006–2007 **Projektarbeit**, *Department of Biology, Plant physiology, Dr. Anna Maria Mattila*,
Oulun yliopisto, Oulu, Finnland.
Themen:
1. ENDIS: Discovery and development of new antibacterial compounds from endophytes
2. Role of endophytic *Methylobacterium* in plant tissue

2004–2006 **Studentische Hilfskraft**, *Lehrstuhl für Pflanzensystematik, Abteilung Mykologie, Prof. Dr. Gerhard Rambold*,
Universität Bayreuth.

Erhaltene Förderungen

Kurzstipendium für Doktoranden,Deutscher Akademischer Austauschdienst (DAAD), Juli-August 2010:
Unterstützung des Forschungsaufenthaltes in Finnland (Aussetzung der DFG-Stelle)

Stipendium zur Förderung von Kongressreisen, Deutscher Akademischer Austauschdienst (DAAD), April 2011:
Kongress „Ecology of Soil Microorganisms“, Prag

Reisekostenzuschüsse, Frauenförderung aus den Mitteln des Zentralansatzes zur Gleichstellung, Universität Bayreuth:
Tagungsreisen: BAGECO-10 (2009), ISME-13 (2010), NordSIR-Meeting (2010), Gordon Research Conference on Applied and Environmental Microbiology (2011)

Mitbetreute Abschlussarbeiten

Schulz, K. 2009. Einfluss von Austrocknungsereignissen auf die Methanogenenpopulation im sauren Niedermoor. Bachelor-Arbeit, Universität Bayreuth.

Mundinger, A. 2010. Einfluss von Sauerstoff auf die Methanogenese im sauren Niedermoor. Bachelor-Arbeit, Universität Bayreuth.

Perras, S. 2011. Trophische Interaktionen im mikrobiellen Nahrungsnetz eines Methan-emittierenden pH-neutralen Niedermoores. Bachelor-Arbeit, Universität Bayreuth.

Guttmann, T. 2011 (laufend). (Toxische) Effekte von Eisennanopartikeln auf Bodenmikroorganismen. Bachelor-Arbeit, Universität Bayreuth.

Publikationen

Palmer, K., Drake, H.L., Horn, M.A. 2009. Genome-derived criteria for assigning environmental *narG* and *nosZ* sequences to operational taxonomic units of nitrate reducers. Appl. Environ. Microbiol. 75:5170-5174.

Palmer, K., Drake, H.L., Horn, M.A. 2010. Association of novel and highly diverse acid-tolerant denitrifiers with N_2O fluxes of an acidic fen. Appl. Environ. Microbiol. 76:1125-1134.

Palmer, K., Biasi, C., Horn, M.A. 2011. Contrasting denitrifier communities relate to contrasting N_2O emission patterns from acidic peat soils in arctic tundra. ISME Journal 6: 1058-1077.

Publizierte Abstracts auf Tagungen und Workshops

Palmer, K., Horn, M.A. 2012. Unknown denitrifier diversity in a pH neutral fen soil in Finnish Lapland. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract SMV003, S. 210

Palmer, K., Horn, M.A. 2012. Palsa peats represent hitherto underappreciated reservoirs of new denitrifier diversity associated with N_2O fluxes. International Polar Year 2012 Conference. Abstract online.

Palmer, K., Biasi, C., Drake, H.L., Horn, M.A. 2011. Cryoturbation affects denitrifier communities in N_2O-emitting arctic permafrost peat soil. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract EMP104, S. 116.

Palmer, K., Biasi, C., Drake, H.L., Horn, M.A. 2011. Cryoturbation affects denitrifier communities in N_2O-emitting arctic permafrost peat soil. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract EMP104, S. 116.

Palmer, K., Biasi, C., Drake, H.L., Horn, M.A. 2011. Impact of cryoturbation on denitrifier community structure and activity in N_2O-emitting arctic permafrost peat soil. Ecology of Soil Microorganisms. Abstract 99

Palmer, K., Schulz, K., Horn, M.A., Drake, H.L. 2010. Stability of the methanogenic community in an acidic fen to experimental drought. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract ECV02, S. 81.

Palmer, K., Schulz, K., Horn, M.A., Drake, H.L. 2010. Effects of enhanced drought on the diversity of methanogens in an acidic fen. Bayreuth Center of Ecology and Environmental Research (BayCEER) Workshop 2010, Abstract O 1.5.

Palmer, K., Schulz, K., Horn, M.A., Drake, H.L. 2010. Impact of artificial drought on diversity, abundance, and gene expression of methanogens in an acidic fen. 13th International Symposium on Microbial Ecology (ISME-13), Abstract on disk

Palmer, K., Drake, H.L., Horn, M.A. 2009. Activity and diversity of phylogenetically novel acid-tolerant nitrate reducer and denitrifier communities in an N_2O-emitting fen. Annual Meeting Vereinigung für Allgemeine und Angewandte Mikrobiologie, BioSpectrum. Abstract PN22, S. 127.

Palmer, K., Drake, H.L., Horn, M.A. 2009. Activity and diversity of phylogenetically novel acid-tolerant nitrate reducer and denitrifier communities in an N_2O-emitting fen. Bayreuth Center of Ecology and Environmental Research (BayCEER) Workshop 2009, Abstract O 1.7.

Palmer, K., Drake, H.L., Horn, M.A. 2009. Novel and highly diverse acid-tolerant nitrate reducers are associated with N_2O-fluxes of an acidic N_2O-emitting fen. 10th International Symposium on Bacterial Genetics and Ecology (BAGECO-10), Abstract P197, S. 279.

Sonstige Tagungsbeiträge

Palmer, K., Biasi, C., Horn, M.A. 2011. Denitrifier communitiy composition impacts N_2O emission patterns in acidic tundra permafrost soils. Nordic Network for Stable Isotope Research (NordSIR) Meeting

Palmer, K., Drake, H.L., Horn, M.A. 2011. Effect of high latitude on denitrification-dependent N_2O-fluxes and denitrifier community structure in peatlands. Gordon Research Conference on Applied and Environmental Microbiology

Palmer, K., Drake, H.L., Horn, M.A. 2010. Novel and highly diverse acid-tolerant nitrate reducers are associated with N_2O-fluxes of an acidic N_2O-emitting fen. Nordic Network for Stable Isotope Research (NordSIR) Meeting

Schmidt, O., Horn, M.A., Palmer, K., Drake, H.L. 2010. H_2 - A hidden link between fermentation and methanogenesis in the intermediary ecosystem metabolism of a wetland soil. Gordon Research Conference on the Molecular Basis of Microbial One-Carbon Metabolism