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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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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).

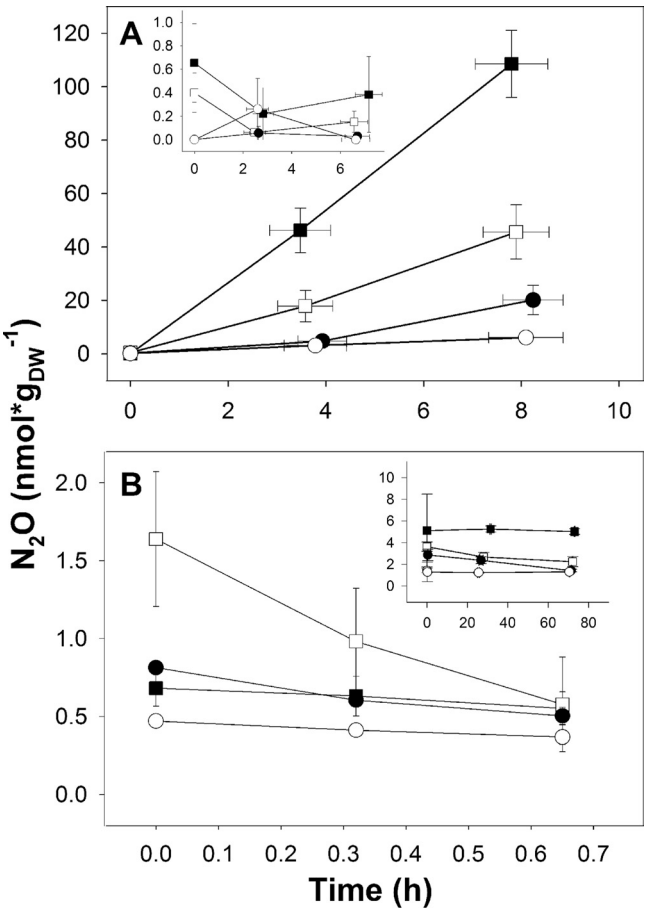

FIG. 1. Production (A) or consumption (B) of N_2O by fen soil after the addition of 40 μM nitrate (A) or 0.02 μM N_2O (B). Microcosms used for the experiments shown in panel A were supplemented with acetylene. Mean values and standard errors of three replicates are shown (sampling dates were 10 July 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).

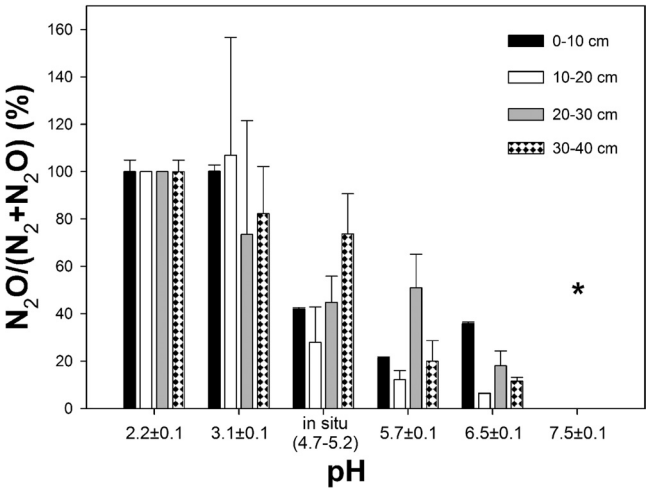

FIG. 2. Effect of pH on the relative amount of N_2O produced by fen soil. The asterisk indicates that N_2O was detected in the presence but not in the absence of acetylene in 0- to 10-cm-depth soil and was not detected in 10- to 40-cm-depth soil microcosms. Mean values and standard errors of three replicates 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'[d]
					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.

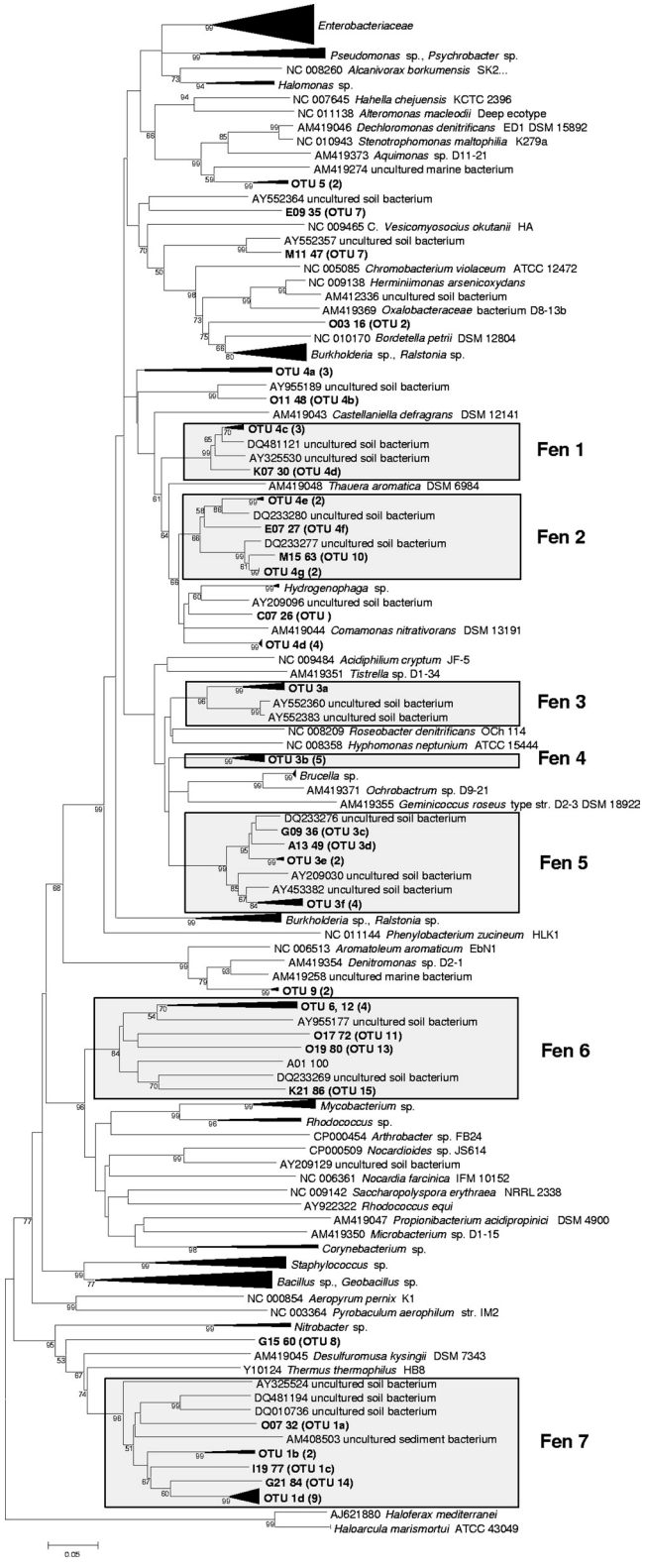

FIG. 3. Phylogenetic tree of *narG* sequences retrieved from the Schlöppnerbrunnen fen. The tree is based on translated amino acid sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

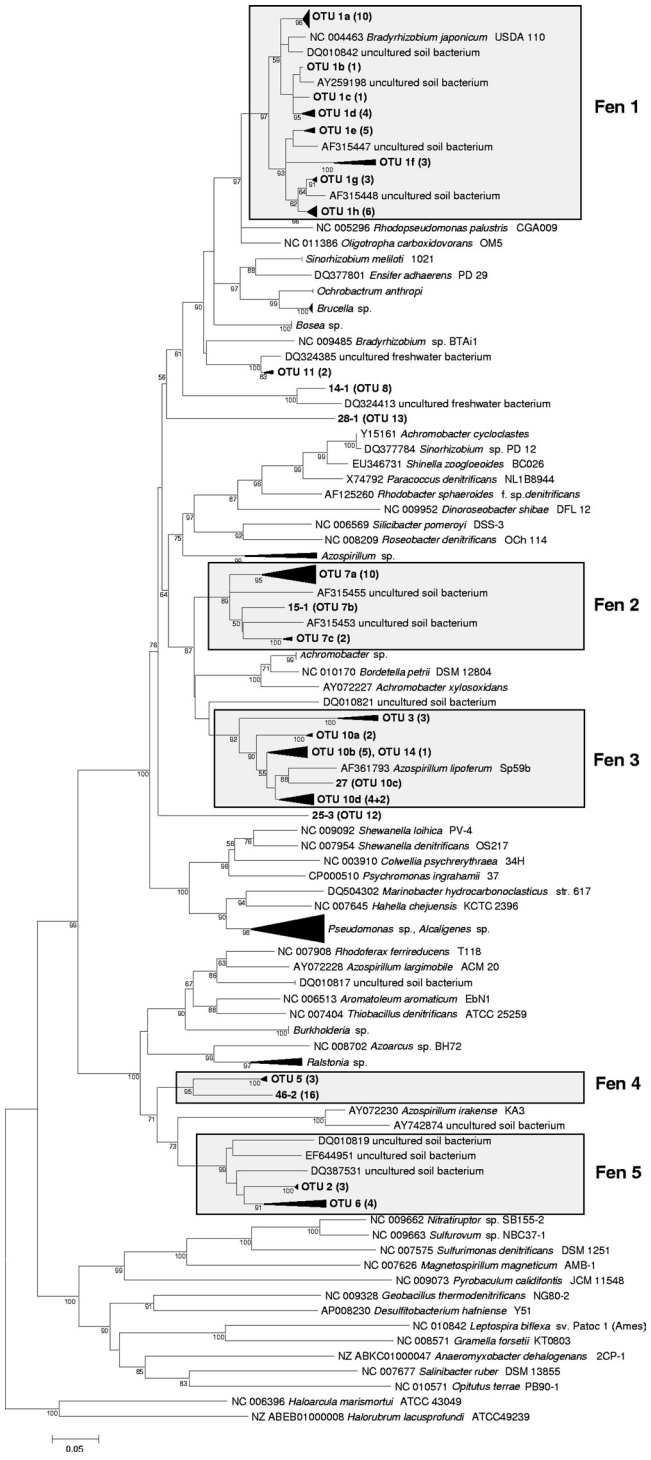

FIG. 4. Phylogenetic tree of *nosZ* sequences retrieved from the Schlöppnerbrunnen fen. The tree is based on translated amino acid sequences. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

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

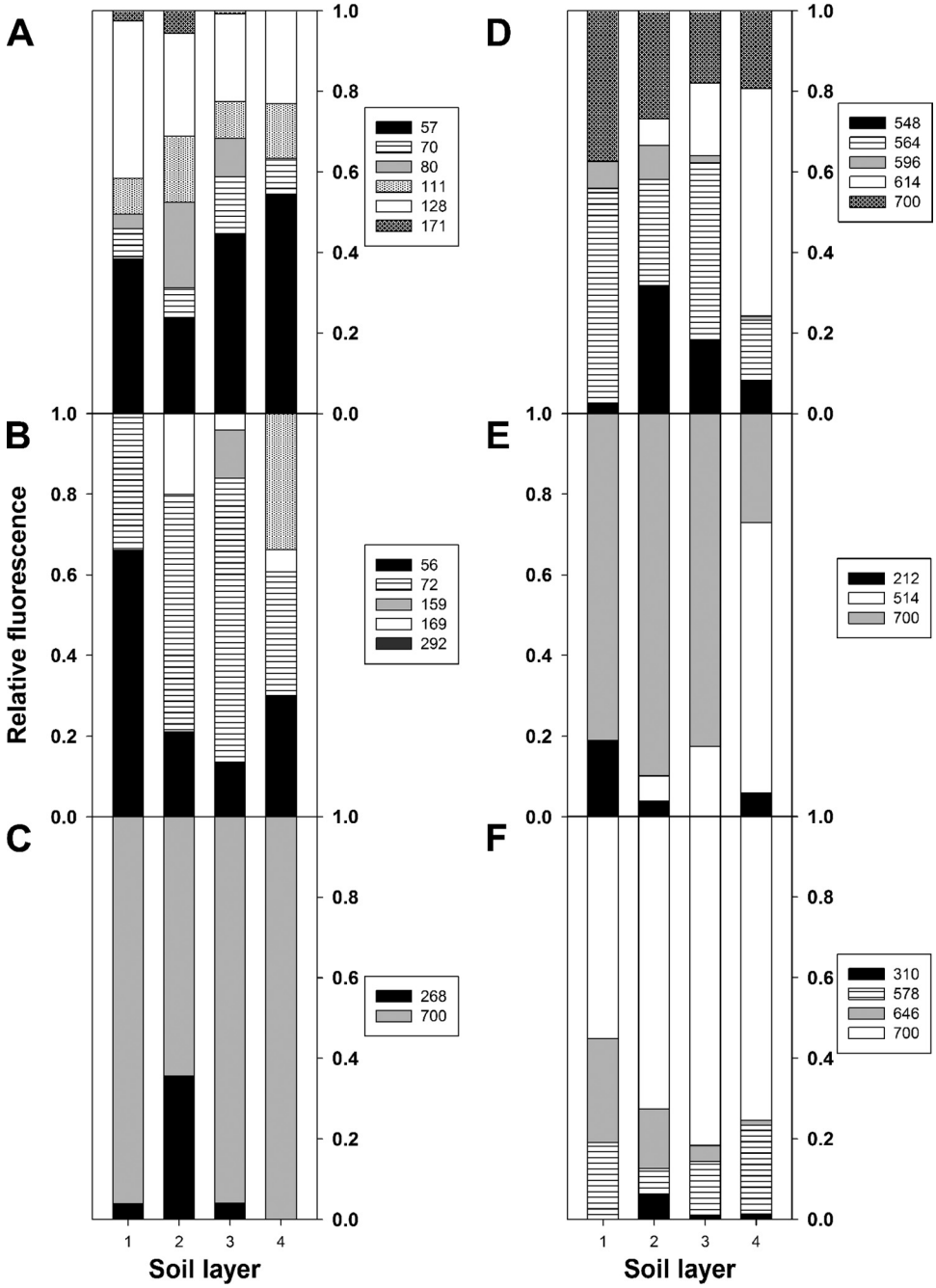

FIG. 5. Comparative TRFLP analyses of *narG* (A to C) and *nosZ* (D to F) amplified from different soil layers of the acidic fen. PCR products were digested with CfoI (A), HaeIII (B), XhoI (C), BtgI (D), NlaIV (E), and PvuI and SacI (F). Mean values of three replicates are shown. Detected TRFs could 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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