RESEARCH ARTICLE

Community-specific pH response of denitrification: experiments with cells extracted from organic soils

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Abstract

Denitrifying prokaryotes are phylogenetically and functionally diverse. Little is known about the relationship between soil denitrifier community composition and functional traits. We extracted bacterial cells from three cultivated peat soils with contrasting native pH by density gradient centrifugation and investigated their kinetics of oxygen depletion and NO_2^-, NO, N_2O and N_2 accumulation during initially hypoxic batch incubations (0.5–1 μM O_2) in minimal medium buffered at either pH 5.4 or 7.1 (2 mM glutamate, 2 mM NO_3^-). The three communities differed strikingly in NO_2^- accumulation and transient N_2O accumulation at the two pH levels, whereas NO peak concentrations (24–53 nM) were similar across all communities and pH treatments. The results confirm that the communities represent different denitrification regulatory phenotypes, as indicated by previous denitrification bioassays with nonbuffered slurries of the same three soils. The composition of the extracted cells resembled that of the parent soils (PCR-TRFLP analyses of 16S rRNA genes, *nirK*, *nirS* and *nosZ*), which were found to differ profoundly in their genetic composition (Braker *et al.*, 2012). Together, this suggests that direct pH response of denitrification depends on denitrifier community composition, with implications for the propensity of soils to emit N_2O to the atmosphere.

Introduction

Denitrification is an important source of N_2O emitted from soils, contributing to global warming (IPCC, 2007) and the destruction of stratospheric ozone (Ravishankara *et al.*, 2009). The rates of denitrification and its N_2O/N_2 product ratio are known to be directly controlled by soil physical and chemical factors (Nommik, 1956; Firestone, 1982). On the other hand, it has been hypothesized that the N_2O/N_2 product ratio of denitrifying communities also depends on the taxonomic composition of denitrifying communities (Schimel & Gulledge, 1998; Wallenstein *et al.*, 2006 and references therein; Philippot *et al.*, 2011), that is, that there exist denitrifying communities with inherently high vs. low N_2O/N_2 product ratios overriding the control of environmental factors.

Denitrification is the stepwise reduction in nitrate via nitrite to the gaseous N species nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2), catalysed by the four enzymes nitrate reductase, nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR) (Zumft, 1997). Denitrifying bacteria react to oxygen depletion by expressing denitrification enzymes, enabling the organisms to sustain respiratory metabolism in the absence of oxygen. Transient accumulation of intermediates (nitrite, NO, N_2O) seems to be inevitable after oxic–anoxic transition (Firestone *et al.*, 1980), but the relative amounts of intermediates accumulated have been found to be highly variable among denitrifying bacteria (Zumft, 1997), most probably due to differences in their regulation of denitrification gene expression. The ability to denitrify is found among a broad variety of microorganisms, including Proteobacteria, Gram-positive bacteria, Archaea and some fungi (Zumft, 1997). Some denitrifiers lack genes coding for one or several of the four reductases, resulting in truncated denitrification pathways. For instance, *Agrobacterium tumefaciens* lacks *nosZ* encoding N_2O reductase and thus produces N_2O as

a final product (Bergaust *et al.*, 2008). Other bacteria exist which can sustain anoxic respiration and growth based on truncated sets of denitrification enzymes (NIR + NOR, NIR + N_2OR or only N_2OR) resulting in various phenotypes either because they lack denitrification genes or because of regulatory deficiencies (Zumft & Kroneck, 2007). Similarly, denitrification enzyme assays have indicated that certain soils harbour distinct denitrifier communities with inherently different propensities for N_2O emissions (Cavigelli & Robertson, 2000; Holtan-Hartwig *et al.*, 2000). Therefore, it appears likely that the property of high vs. low N_2O/N_2 product ratios of microbial communities depends on their species composition, which has been shown to vary greatly among soils (Philippot *et al.*, 2002; Prieme *et al.*, 2002; Rösch *et al.*, 2002; Stres *et al.*, 2004; Cuhel & Simek, 2011; Braker *et al.*, 2012).

A common approach to infer inherent traits of soil denitrifying communities is to assess their kinetics of N-gas production and consumption in anoxic incubations of soils or soil slurries, with or without substrates added. It is then assumed that by controlling for all environmental regulators known to affect denitrifier N_2O production and consumption, the only variable contributing to differences in N_2O/N_2 production would be the intrinsic properties of the denitrifier communities (Holtan-Hartwig *et al.*, 2000; Cavigelli & Robertson, 2001). This somewhat naïve assumption is impossible to prove, however, as long as the soil matrix is present, and easy to refute in the case of Holtan-Hartwig *et al.* (2000) who worked with soils that differed greatly in pH: the German organic soil (GER) had a pH of 5.4, the Finnish organic soil (FIN) had a pH of 6.0, and the Swedish organic soil (SWE) had a pH of 7.1. There is now ample evidence that pH exerts a direct effect on the transient accumulation of N_2O by denitrification in soils (Simek & Cooper, 2002; Liu *et al.*, 2010), and experiments with the model strain *Paracoccus denitrificans* have indicated the mechanisms involved (Bergaust *et al.*, 2010). It appears that pH affects N_2OR post-translatory, resulting in an increasingly dysfunctional enzyme with decreasing pH. This is effectively what Holtan-Hartwig *et al.* (2002) observed; the most acid soil (GER, pH 5.4) accumulated much more N_2O than the soil with intermediate pH (FIN, 6.0), and the lowest amounts were recorded for the neutral soil (SWE, pH 7.1).

We revisited the three soils studied by Holtan-Hartwig *et al.* (2000) to see whether direct pH effects were responsible for the functional contrasts observed previously in soil slurry incubations. A more stringent analysis of the intrinsic functional characteristics of the three denitrifier communities was accomplished by a denitrification bioassay with cells extracted from the three soils by density gradient centrifugation, thus removing any interference by the soil pH (or any other matrix-dependent factor). The experiments were run at two contrasting pH levels, 5.4, which is the native pH of the most acidic soil (GER), and 7.1, which is the native pH of the most alkaline soil (SWE). In addition, we analysed the composition of the extracted communities by PCR-T-RFLP of 16S rRNA genes and *nirK, nirS* and *nosZ* and compared it with that of community DNA extracted from bulk soil to confirm the dissimilarity of extracted communities and to compare them with the community composition of the parent soils studied by cloning-sequencing of PCR products by Braker *et al.* (2012).

Materials and methods

Soils

All three soils are Terric Histosols (FAO) that had contrasting pH presumably because of differences in wetland type and cultivation history. The acidic soil from Germany (pH 5.4) was sampled from an arable field at the 'Donaumoos', a 12 000 ha cultivated fen in southern Germany first drained in 1790–1793. The 200 years of cultivation have resulted in a strongly humidified peat (muck) of 1.2–2.0 m depth with no recognizable remnants of *Phragmites* or *Carex* ssp. The estimated annual N_2O emission at the German site was 14.6 kg N_2O-N ha^{-1} (P. Dörsch, unpublished). The less acidic Finnish soil (pH 6.0) was sampled from an experimental site at the Siikasalmi Research Station in East Finland. The study site is located on an old shore consisting of organic lake sediments drained in 1957 for birch plantation (*Betula pendula*, Roth). Arable cultivation started in 1979. The depth of the organic soil layer is 0.2 m and the estimated annual N_2O emission was 8.4 kg N_2O-N ha^{-1} (Maljanen *et al.*, 2003). The neutral Swedish soil (pH 7.1) was sampled from a farmland near the city of Falköping. This site was reclaimed from a lake bottom around 1920 and used alternately as a ley or for arable cropping. The soil still contains lacustrine limestone, explaining its neutral pH. Estimated annual N_2O emission for the Swedish site was 4.1 kg N_2O-N ha^{-1} (Klemedtsson *et al.*, 2009). The German field had been grown to Triticale and the Finnish and Swedish fields to barley (*Hordeum vulgare* L.) without fertilization during the last 3–4 years prior to sampling. For average climate and water tables see Table 1. Composite soil samples (12 cores, 50 mm diameter) were taken from the plough layer (0–20 cm) in autumn 1998 after harvest from all three sites, sieved (< 6 mm) and stored in polyethylene bags at field moisture and 4 °C. The stored soils were used in a series of experiments (Holtan-Hartwig *et al.*, 2000, 2002; Dörsch & Bakken,

Table 1. Soils and climate

	Position	C (%)	C : N	Bulk density (g cm^{-3})	pH (H_2O)	Annual precipitation (mm)	Average groundwater depth (cm)	Mean temperature (°C)
Finnish	62°31′N, 29°23′E	26	16.7	0.32	6.0	643	106	2.6
German	48°40′N, 11°04′E	35	14.2	0.36	5.4	579	67	7.1
Swedish	58°20′N, 13°30′E	24	9.5	0.27	7.1	645	60	4.9

2004), and the same soil samples were used in 2007 for the pH experiments described here, meaning that the experiments reported here were conducted 9 years after sampling. The prolonged storage may be criticized, but we found in a series of experiments carried out throughout the years (*ibid*) that these organic soils sustained denitrification capacity throughout storage at +4 °C. This was probably due to the ample amount of organic matter present in these soils, sustaining nitrification many years after sampling as judged from steadily increasing nitrate concentrations in the stored soils. One may have expected that prolonged storage at stable temperature and moisture would result in convergent denitrifying communities with respect to taxonomic composition and physiological state. This was not the case, however; functional experiments conducted in Norway showed reproducible kinetic responses, and repeated DNA extractions followed by PCR and T-RFLP analyses based on denitrification genes (one of which as late as in spring 2011) revealed stable *nir* and *nos* community composition (G. Braker, unpublished). We, therefore, believe that the long storage supports our approach to detect soil-specific taxon-function relationships, as it equilibrates for short-term effects of moisture content and nutrient state at the date of sampling.

Cell extraction

A one-step density gradient centrifugation without pH adjustment was used to separate microbial cells from soil particles (Lindahl & Bakken, 1995). Two portions of 50 g fresh weight soil from Finland and Sweden (70 g for German soil because of its lower activity) were dispersed in 500 mL filter-sterilized, precooled (4 °C) double distilled water in sterilized 1000 mL Waring blenders. Subsequently, cells were physically separated from soil particles by blending five times for 1 min at maximum speed (18 000 rpm) with intermittent cooling of the blender (5 min) on crushed ice. After sedimentation of coarse soil particles for 30 min, 4 × 200 mL supernatant was decanted into four sterile 250 mL Nalgene centrifugation tubes. A 40 mL cushion of Nycodenz solution (1.3 g cm^{-3}) was placed underneath the supernatant using a hypodermic needle, and the tubes were centrifuged at 10 000 ***g*** for 1 h (4 °C). The cells floating on top of the Nycodenz cushion were syphoned off with a sterile capillary, and the cell suspensions from four tubes were pooled (*c.* 50 mL) and diluted to 800 mL volume with filter-sterilized double distilled water. This volume was equally distributed into four 250 mL buckets which were centrifuged for 1 h at 10 000 ***g*** (4°C) to remove remnant Nycodenz. The supernatants were discarded, and the pellets were resuspended in a total of 30 mL filter-sterilized water and stirred aerobically for 0.5 h prior to inoculation into a mineral medium.

Evaluation of extraction efficiency and community composition

The efficiency of the cell extraction and its effect on community composition was determined in a separate experiment. Cells from three replicate samples from GER, FIN and SWE soil were extracted as described previously. Total cells in the soil suspensions prior to density gradient centrifugation and in the final suspensions of the extracted cells were counted microscopically after acridine orange staining (Bakken, 1985). Cells for molecular analyses were collected on cellulose acetate filters (0.45 μm pore size) for subsequent DNA extraction, and DNA from these cells as well as from bulk soil DNA was extracted using the Fast DNA® Spin kit for Soil (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. Contaminating humic acids were removed during the extraction procedure by introducing an additional Guanidine-Isothiocyanate purification step (Yeates & Gillings, 1998). After binding onto the matrix, DNA was carefully washed three times with 500 μL each of Guanidine-Isothiocyanate (5.5 M) until returning to its original colour. Subsequently, the DNA was loaded onto the spin filter and eluted from the column in 100 μL DES solution. DNA concentrations of the extracts were determined by spectrophotometry. Ratios of A260/A280 were 1.94, 1.75 and 1.87 for the Finnish, German and Swedish soil, respectively, indicating a high purity of the DNA extracts. There was, however, some contamination with humic acids as indicated by low ratios of A260/A230 that were 0.1, 0.27 and 0.23. The latter may cause inhibition during PCR which we, however, did not observe.

Gene fragments were PCR-amplified from 1 μL of the DNA extracts using either forward or reverse 5′-end 6-carboxyfluorescein-labelled primers (MWG Biotech, Ebersberg, Germany). Primers specific for bacterial 16S rRNA genes (Amann *et al.*, 1995) and for the denitrification genes *nirK*, *nirS* and *nosZ* (Braker *et al.*, 1998; Scala & Kerkhof, 1998) and the respective PCR protocols were used as published previously. Subsequently, amplicons were analysed by T-RFLP analysis according to protocols described previously (Scala & Kerkhof, 2000; Braker *et al.*, 2001; Avrahami *et al.*, 2002). 16S rRNA gene amplicons were cleaved with the restriction endonuclease MspI; the denitrification gene amplicons of *nirK* (515 bp), *nirS* (890 bp) and *nosZ* (1131 bp) were cleaved with HaeIII, MspI and HinPI, respectively. Resulting fragments were separated on a 310 automated sequencer (Applied Biosystems, Darmstadt, Germany). The size of fluorescently labelled terminal restriction fragments was determined by comparison to the internal length standard X-Rhodamine MapMarker® 30–1000 bp (BioVentures, Murfreesboro, TN) using Genescan 3.71 software (Applied Biosystems). As T-RFs can vary slightly in size, T-RFLP patterns were inspected visually and peak size differences of one or two base pairs were confirmed by comparing the respective peaks of all patterns. Peaks with a threshold value of >100 and >50 units above the background fluorescence for 16S rRNA and for denitrification genes, respectively, were normalized by an iterative normalization procedure (Dunbar *et al.*, 2001). The relative abundance of T-RFs was determined as the ratio between the heights of a given peak and the normalized total peak height of each sample. Similarity in T-RFLP profiles for cells extracted from the soils and for communities in the bulk soil was evaluated by principle component analysis using the PCord program version 4.01 (MJM Software, Gleneden Beach, OR) and by calculating the Morisita index for pairwise comparisons. The Morisita index (I_M) was calculated (Eqn. 1), where λ is Simpson's index of dominance (calculated separately for each community), n_i is the number of individuals (abundance) of species i, and N is the total number of individuals sampled. The Morisita index has been applied previously to analyse T-RFLP data based on the assumption that each terminal restriction fragment represents a separate operational taxonomic unit (OTU) and that peak height is a measure of OTU abundance (Dollhopf *et al.*, 2001; Schmitt-Wagner *et al.*, 2003).

$$I_M = \frac{2\sum n_{1i}n_{2i}}{(\lambda_1 + \lambda_2)N_1N_2} \quad (1)$$

Simpson's index of dominance (λ, Eqn. 2) gives the probability that a pair of individuals drawn at random from an infinitely large community belongs to different species; s is the total number of species in the community.

$$\lambda = \frac{\sum_{i-1}^{s}(n_i(n_i - 1)}{N(N-1)} \quad (2)$$

The Morisita index ranges from 0 to 1, with 0 indicating that no species are shared between two communities and 1 indicating complete identity of two communities. Because the index takes species abundance into account, communities that contain the same species but have different species abundance will have an index value of < 1.

Growth conditions and pH adjustment

To experimentally adjust pH, cells extracted from the soils were inoculated into mineral medium that contained (L^{-1}): 200 mg KH_2PO_4, 20 mg $CaCl_2$, 40 mg $MgSO_4$, 3.8 mg Fe-NaEDTA, 0.056 mg LiCl, 0.111 $CuSO_4$, 0.111 mg $ZnSO_4$, 1.222 mg H_3BO_3, 0.111 mg $Al_2(SO_4)_3$, 0.056 mg $SnCl_2$, 0.778 mg $MnCl_2$, 0.111 mg $NiSO_4$, 0.111 mg $Co(NO_3)_2$, 0.111 mg TiO_2, 0.056 mg KI, 0.056 KBr, 0.1 mg $NaMoO_4$. The medium was buffered with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and supplied with KNO_3 and Na-glutamate (2 mM each). Two batches of sterile autoclaved medium were adjusted to pH 5.4 and 7.1, respectively, by adding 1 N NaOH, and distributed as 50 mL into six sterile 120 mL serum flasks (three flasks pH 5.4, three flasks pH 7.1) except for the experiment with SWE where the volume was only 43 mL. Extra flasks were filled for blanks and off-line incubation. The serum flasks were crimp sealed with butyl septa and made near-anoxic by six cycles of evacuation and He-filling using an automated manifold while vigorously stirring the suspensions with magnetic stirrers (Molstad *et al.*, 2007).

Incubation and gas analyses

Each soil denitrifier community was studied by incubation immediately after the extraction of the cells. Incubation bottles (6) prepared with 40–50 mL medium as described previously (depending on the experiment), three blanks, and three calibration standards, were placed on a submersible magnetic stirring board (Variomag HP 15; H+P Labortechnik GmbH, Germany) in a 15 °C water bath, which is an integrated part of the robotized incubation system for the quantification of O_2-, CO_2-NO-, N_2O and N_2-production in denitrifying cultures described by Molstad *et al.* (2007). After temperature equilibration, excess He was released and the bottles were

spiked with 0.5 mL of pure Neon (Ne) used as an inert tracer to calculate dilution from repeated sampling. The O_2 concentrations in the flasks prior to inoculation were around 0.8–1.5 mL L^{-1} in the headspace (equivalent to 0.5–1 μM in the liquid).

Incubation was started by injecting 2 mL of cell suspension, yielding approximate cell numbers of 2×10^9 cells per flask (4×10^7 mL^{-1}). The headspace concentrations of the relevant gases (O_2, N_2, N_2O, NO, CO_2 and Ne) were monitored by repeated sampling (every 3 h) as described by Molstad *et al.* (2007). Briefly, the flasks were placed in a temperature controlled water bath on a submersible magnetic stirring board, and samples were drawn repeatedly by an autosampler (Gilson 222, leBel, France) equipped with a hypodermic needle from the headspace of the bottle via a peristaltic pump (Gilson Minipuls 3) to a Varian CP4900 microGC equipped with two columns (10 m poraPLOT U and 20 m 5 Å Molsieve) with separate injectors and TCD detectors. The outlet from the sampling loop of the GC was coupled to a T-piece with He-flow (15 mL min^{-1}) which carries the gas further to the open inlet of a chemoluminescence NO analyser (Model 200A; Advanced Pollution Instrumentation, San Diego). Each sampling removed app. 3.3% of the headspace, which was replaced by reversing the pump and pumping He into the bottles. The exact dilution was quantified by following the decline of Ne in the bottles over time.

The incubation experiments were terminated after 110–130 h. At this stage, the treatments with the most active denitrification (SWE and FIN with pH 7.1) had depleted the nitrate (*c.* 100% recovery of NO_3-N, no further gas production). We measured pH directly after incubation which showed an average rise of 0.5 pH units for GER and FIN and 1.1 pH units for SWE pH 5.4 treatments, which can be ascribed to the consumption of glutamic acid and protons during denitrification and hence reflects differences in respiration activity between the cultures. Changes in pH were negligible in the pH 7.1 treatments because of more efficient buffering at this pH.

Results

Separation efficiency and community similarity

Microscopic counts of the soil suspensions prior to density gradient centrifugation gave similar cell densities for the three soils (2.1, 1.0 and 2.0×10^{10} cells g^{-1} dry weight in SWE, GER and FIN, respectively) and respectively 14.5%, 12.5% and 15.6% thereof were recovered in the extracted microbial suspensions after density gradient centrifugation. DNA extracted from cells corresponded to 6.6%, 11.3% and 11.7%, respectively, of the amount extracted directly from the soil. Analysis of T-RFLP patterns of amplified *nir*K, *nir*S, *nos*Z and 16S rRNA genes (not shown) revealed the same dominant restriction fragments as DNA from separated cell in bulk soil DNA albeit slightly differing in relative abundance. No *nir*S gene fragments could be amplified from cells extracted from the FIN soil. Principal component analysis showed that T-RFLP profiles of separated cells clustered with those from bulk DNA of a given soil (Fig. 1), suggesting that cell separation did not reduce the taxonomic dissimilarity of the investigated communities. Morisita indices calculated to assess community similarity between bulk soil and separated communities ranged between 0.67 (GER, *nosZ*) and 0.98 (GER, *nir*S) (Fig. 1).

O_2 respiration and CO_2 production

The cell suspensions had initial O_2 concentrations in the liquid ranging from 0.8 to 1.0 μM with the exception of SWE pH 7.1 (0.5 μM O_2) which was He-washed twice because of an initial leak in the flushing system (Table 2). Oxygen was consumed at variable rates, reaching complete O_2 depletion after 90 and 100 h in GER pH 7.1 and pH 5.4, respectively, after 60 h in both pH treatments of SWE and after 80 and 90 h in the FIN pH 7.1 and pH 5.4 treatments, respectively (Supporting information, Fig. S1). Mean initial O_2 respiration in the GER suspension was three times higher at pH 7.1 than at pH 5.4 (Table 2), indicating that respiration was initially inhibited by low pH in GER. Inhibition of initial O_2 respiration by acidity was less pronounced for the SWE and FIN communities (Table 2). O_2 kinetics is given together with O_2 respiration and CO_2 production rates in Fig. S1.

NO_2^- accumulation

Measurements of NO_2^- concentration levels in parallel incubations revealed exponential NO_2^- accumulation in GER at pH 5.4 and 7.1 (Fig. 2). The measurements were performed in extra bottles incubated in a temperature cupboard (two replicates per pH treatment for GER, single bottle values for SWE and three replicates for FIN) and were too infrequent to deduce a clear pH effect on NO_2^- accumulation. The data were, therefore, fitted to an exponential (pH 5.4) or modified Gaussian (pH 7.1) model to obtain similar resolution as for the gas measurements. NO_2^- accumulation in SWE was one to two orders of magnitude lower than in GER (Table 2) and showed some but not complete consumption of accumulated NO_2^- towards the end of the incubation. Like in GER, there was no significant effect of pH on NO_2^- accumulation. The FIN

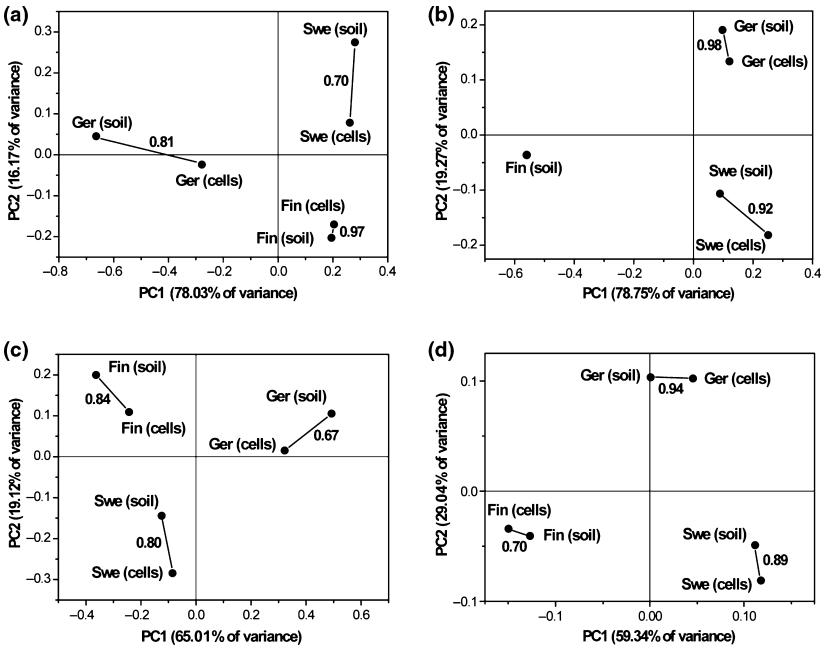

Fig. 1. Principle component analysis of terminal restriction fragment length polymorphism (T-RFLP) fingerprints for gene fragments amplified from soil DNA (soil) and from cells (cells) separated from soil particles by Nycodenz density gradient centrifugation. Soils were from Germany (GER), Finland (FIN) and Sweden (SWE). Numbers given are Morisita similarity indices for pairwise comparison of T-RFLP fingerprints from soil and cells. The four panels show the results for *nirK* (a), *nirS* (b), *nosZ* (c) and 16S rRNA genes (d).

Table 2. Mean values for observed rates of respiration and NO_x accumulation (both rates and peak concentrations) during the incubations. Maximum amounts of intermediate denitrification products (nitrite, NO and N_2O) are reported both as total amounts (nmol per flask) and as concentration in the liquid; (n = 3), SD in parentheses

		Soil					
		GER		SWE		FIN	
Product	Concentrations and accumulation rates	pH 5.4	pH 7.1	pH 5.4	pH 7.1	pH 5.4	pH 7.1
O_2	[O_2] at start (μM)	0.9 (0.02)	0.9 (0.05)	0.8 (0.03)	0.5 (0.04)	1.0 (0.37)	1.0 (0.16)
	Initial O_2 respiration (0–15 h, nmol per flask h^{-1})	5.7 (2.2)	18.7 (5.3)	29.0 (1.3)	37.4 (0.5)	9.4 (1.3)	14.8 (3.7)
	Max O_2 respiration (nmol per flask h^{-1})	48.3 (7.1)	46.8 (10.1)	71.8 (9.5)	40.6 (0.5)	53.1 (5.4)	64.3 (3.7)
NO_2^-	Max NO_2^- (μmol per flask)	16.0	11.7	0.6	0.5	21.5	10.7
	Max [NO_2^-] in liquid (μM)	308	224	13	12	399	198
NO	Initial NO accumulation rate (nmol per flask h^{-1})	1.92 (0.14)	0.06 (0.01)	0.11 (0.02)	0.53 (0.11)	1.83 (0.08)	0.31 (0.14)
	Max NO (nmol per flask)	60 (4.0)	36 (4.1)	32 (1.7)	39 (1.5)	66 (4.5)	30 (2.8)
	Max [NO] in liquid (nM)	49 (3.2)	29 (3.3)	24 (1.3)	29 (1.1)	53 (3.6)	24 (2.3)
N_2O	Initial N_2O accumulation rates (nmol per flask h^{-1})	0.61 (0.12)	0.09 (0.01)	0.27 (0.02)	0.70 (0.11)	1.33 (0.11)	0.52 (0.04)
	Initial N_2O/NO accumulation ratio	0.32	1.69	2.45	1.33	0.73	1.69
	Max N_2O accumulation rate (nmol per flask h^{-1})	29.3 (11.8)	4.3 (1.6)	1.5 (0.4)	9.8 (4.9)	6.1 (0.8)	10.9 (8.7)
	Max N_2O (nmol per flask)	594 (142)	56 (34)	38 (2)	117 (51)	104 (5)	111 (79)
	Max [N_2O] in liquid (μM)	4.4 (1.0)	0.40 (0.25)	0.23 (0.01)	0.86 (0.37)	0.77 (0.04)	0.82 (0.58)
	Max N_2 production rate (nmol per flask h^{-1})	100 (28.9)	2.1 (0.7)	3.7 (0.2)	35.6 (18.5)	7.3 (0.24)	34.2 (23)
N-balance	Final N_2 accumulation (μmol per flask)	3.8 (0.6)	3.2 (0.5)	43.3 (4.5)	41.6 (0.5)	10.3 (1.9)	50.1 (2.7)
	Nitrogenous e-acceptor used (%)	7.7 (1.1)	6.2 (0.9)	94.6 (9.6)	90.4 (1.2)	20.4 (3.8)	98.2 (5.3)
	Electrons produced/electrons consumed	1.0 (0.07)	1.0 (0.13)	0.80 (0.07)	0.80 (0.01)	1.36 (0.08)	1.13 (0.03)

bottles were sampled at a higher temporal resolution and showed a clear pH effect with a more than twice as high transient accumulation of NO_2^- at pH 5.4 as compared to pH 7.1. NO_2^- build-up in GER (pH 5.4 and 7.1) and FIN (pH 5.4) was substantial, accounting at its maximum for 32–43% of the initially supplied NO_3^-.

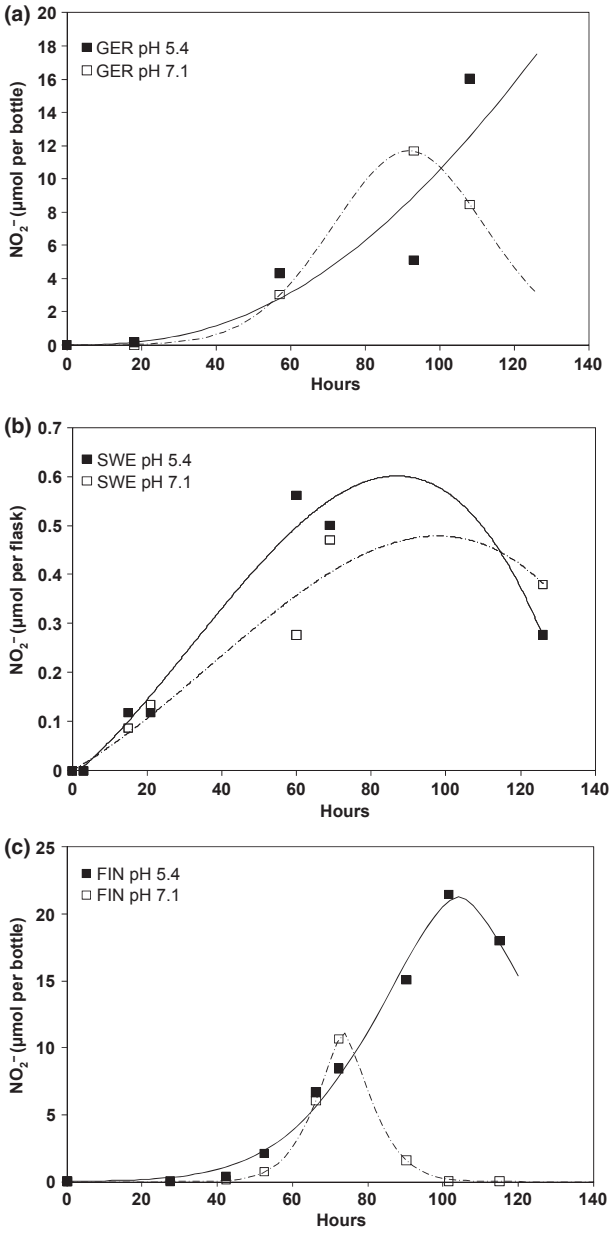

Fig. 2. NO_2^- accumulation in incubations of cell suspensions from GER (a), SWE (b) and FIN (c) at pH 5.4 and 7.1. The data points were fitted to a modified Gaussian model to calculate electron flow. NO_2^- concentrations for GER are based on average values of duplicate flasks for pH 5.4 and 7.1, respectably. For SWE, single flask values at pH 5.4 and 7.1 are shown, whereas average values of three flasks are shown for FIN at each pH.

Kinetics of N-gas accumulation

Total N conversion to NO, N_2O and N_2 and the relative effect of pH thereon differed substantially between the communities (Fig. 3).

Initial accumulation of NO and N_2O

Detectable accumulation of NO and N_2O occurred at an early stage of the incubation for all communities (see inserted panels, Fig. 3). The NO and N_2O accumulation in GER started at time *c.* 0, and the rate of accumulation was clearly higher at pH 5.4 than at pH 7.1 (Table 2). In FIN, NO and N_2O accumulation started after 5–10 h, and the rates were higher for pH 5.4 than for pH 7.1. In SWE, NO and N_2O accumulation started after 10–15 h, but in contrast to GER and FIN, the rates were lower for pH 5.4 than pH 7.1.

NO concentrations

NO fluctuated variably throughout the incubations. In GER, NO was 40–60 nmol per flask throughout most of the incubation at pH 5.4, whereas at pH 7.1, such high levels were only reached after the onset of significant N_2-production. In SWE, NO reached similar maximum levels (30–40 nmol per flask) for both pH levels, coinciding with the onset of significant N_2 production. In FIN, NO concentrations were most variable, at a somewhat higher concentration for pH 5.4 than for pH 7.1 (40–60 nmol per flask for pH 5.4 and 20–30 nmol per flask for pH 7.1).

N_2O concentrations

N_2O concentrations reached distinct maxima (Table 2) that preceded or coincided with the onset of rapid N_2 production. In GER, the N_2O maximum was 500–750 nmol per flask at pH 5.4 vs. 30–80 nmol per flask at pH 7.1. In FIN, the N_2O curves showed variable patterns throughout, but two peaks can be recognized, one preceding the onset of N_2 production and a second during N_2 accumulation (similar concentrations of up to 100 nmol per flask for both pH levels except for the high values of up to 200 nmol for a single flask at pH 7.1). In SWE, N_2O peaked early after the onset of denitrification at 40 nmol per flask (pH 5.4), whereas higher N_2O concentrations were reached at pH 7.1 (80–180 nmol per flask).

N_2 production

N_2 accumulation showed profound contrasts between the communities and in particular in response to pH. N_2 accumulation in GER was extremely slow compared to the two other soils (notice the scale of the right axis of Fig. 3); it started earlier at pH 7.1 than at 5.4, but the cumulated N_2 production reached similar values for the two pH treatments. In contrast, N_2 accumulation in FIN and SWE was initiated approximately at the same time

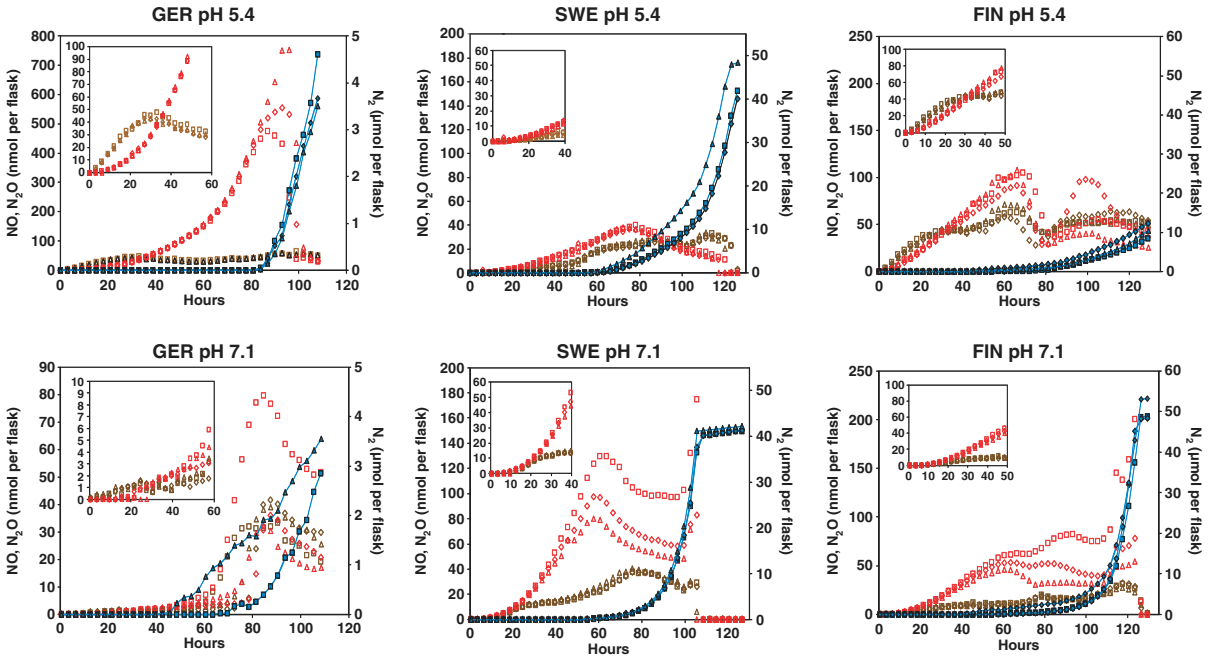

Fig. 3. Accumulation of NO, N_2O and N_2 in cell suspensions from GER, SWE and FIN at pH 5.4 (top panel) and 7.1 (bottom panel). Symbols in brown: NO; in red: N_2O; in blue with line: N_2. For GER pH 7.1, only two of the three N_2 accumulation curves are shown, because one of the bottles was leaky. The N_2 values are corrected for sampling losses, whereas the NO and N_2O concentrations are the measured values. The equilibrium concentration of NO in the liquid is 0.8 nM at 1 nmol NO per flask. Note: scale on left *y*-axis differs by a factor of 10 for GER pH 5.4 and 7.1.

for the two pH levels, but reached higher rates at pH 7.1 than at pH 5.4. For all treatments with high N_2 accumulation rates, N_2 levels reached stable plateaus close to the total amounts of NO_3^--N initially present in the flasks (86 and 100 μmol NO_3^- for SWE and FIN, respectively, equivalent to 43 and 51 μmol N_2 per flask; the difference is because of different medium volumes per flask, see Materials and methods).

In summary, the N-gas kinetics showed a number of apparent contrasts between the communities both regarding the absolute rates of denitrification and the transient accumulation of intermediates (NO and N_2O) and not the least in their response to pH. Table 2 lists some of the variables extracted from the Figs S1, 2 and 3. The comparison of the communities' intrinsic properties based on kinetics of transient NO_2^-, NO, N_2O and N_2 accumulation is complicated by the fact that the communities depleted the traces of oxygen initially present at different pace. In an attempt to normalize the results as to their relative rates of N_2O and N_2 accumulation during denitrification, we plotted the $N_2O/(N_2 + N_2O)$ ratios against total denitrification (sum of all gaseous denitrification products) (Fig. 4). To normalize for denitrification progress, the ratios for the first 8 μmol of denitrified N are shown within which all communities reached N_2O net-uptake (i.e. a nominal $N_2O/(N_2 + N_2O)$ ratio = 0). All communities started with a $N_2O/(N_2 + N_2O)$ ratio = 1 (because N_2O is detectable before N_2 accumulation) and showed a decline in the N_2O/N_2 product ratio with progressing denitrification, reflecting a gradual increase in the relative rate of N_2O reduction. In GER, the N_2O/N_2 product ratio declined at an early stage of denitrification at pH 7.1, while at pH 5.4, this decline did not occur before one μmol of N was denitrified. In FIN, a similar effect of pH was observed, albeit to a smaller extent. In SWE, in contrast, the product ratio started to decline at an earlier stage at pH 5.4 as compared with pH 7.1.

Electron balance

To evaluate whether the differences in denitrifier performance between the communities (in terms of total N conversion, see Table 2) were related to glutamate fermentation, we calculated the cumulative electron balances between CO_2 accumulation (not shown) and O_2 plus NO_x reduction. To estimate the total amount of glutamate mineralized by respiration, we used the final amounts of CO_2 per flask at the end of the incubations, taking the final pH into account to estimate partitioning of the accumulated CO_2 between headspace and liquid. This value was then used to calculate the total amounts of electrons donated per flask (2.2 mole NADH per mole CO_2 produced for glutamate degradation through the TCA-cycle) and compared this number with the cumulated electron

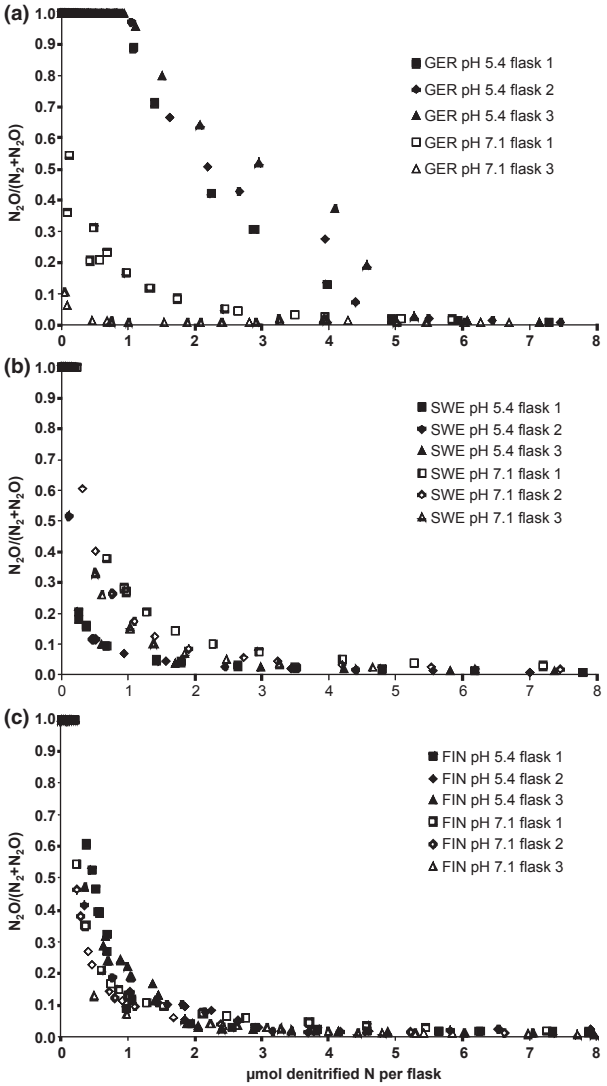

Fig. 4. $N_2O/(N_2 + N_2O)$ ratios as a function of total denitrification and pH in extracted communities from GER (a), SWE (b) and FIN (c). The ratios are calculated from the amount of N_2O and N_2 accumulated in the flasks through the first phase of denitrification (8 μmol N per flask corresponding to *c.* 8% of the total amount of NO_3-N present initially).

transport to the measured electron acceptors O_2 and NO_2^-, NO, N_2O and N_2 (Table 2). The ratio between the estimated amounts of electrons donated and the electron accepted was very close to 1 (± 0.2) for all flasks, indicating that electron flow was mainly driven by denitrification although some accumulation of fermentation products of glutamate fermentation cannot be excluded.

Discussion

The experiments were conducted with extracted denitrifier communities as opposed to soil slurries used previously to characterize functional traits in denitrifier communities (Holtan-Hartwig *et al.*, 2000, 2002; Dörsch & Bakken, 2004). Although the analysis of T-RFLP fingerprints showed that the composition of the extracted communities were different from that of the soils they were extracted from, the contrasts between the three extracted communities were clearly congruent with the contrasts between the respective soil communities (Fig. 1). This is also reflected in the calculated pairwise similarities (Morisita index), which were 70–98% for the contrasts between soils and their extracted communities, but much lower for the contrasts between the different soils and different extracted communities (Fig. 1). Our tentative conclusion is, therefore, that the soils harboured denitrification communities with very different composition, and these contrasts were well represented by the extracted communities. By using extracted cells in a buffered minimum medium, we were able to eliminate any direct effect of soil pH (or any other effects of the soil matrix) and thus to explore the intrinsic properties of the three denitrifier communities, independent of the native pH of the soils. By running the experiments at two pH levels (5.4 and 7.1), we additionally determined the direct effect of pH on the denitrification characteristics of the three distinct communities. The results suggested that the communities responded differently to pH in many respects: (1) in GER, the initial oxygen consumption at pH 7.1 was approximately three times higher than at pH 5.4; the equivalent numbers for FIN were *c.* 1.6 and for SWE *c.* 1.3 (Fig. S1, Table 2), (2) the transient NO_2^- accumulation was high at both pH levels in GER, high only at pH 5.4 in FIN and low at both pH levels in SWE, (3) the transient N_2O accumulation decreased with pH in GER and FIN, but increased in SWE (Figs 3 and 4, Table 2), and (4) the initial ratio between N_2O and NO accumulation (Table 2) increased with pH in GER and FIN, but was reduced in SWE. Together, these results confirmed that pH had some direct effect on oxic respiration, but that the communities retained characteristic patterns of anoxic respiration irrespective of pH. Hence, we refute the hypothesis that previously reported differences in community-specific denitrification patterns were a mere function of soil pH. If this were true, shifting the acid-adapted GER community to neutral pH should have resulted in higher denitrification rates and tighter stoichiometry of denitrification products, whereas shifting the neutral-adapted SWE community to acid conditions would have resulted in a more dysfunctional denitrification as observed in soil slurries of GER. The observed kinetic responses to adjusted pH clearly showed that this was not the case. The GER community pertained many of its dysfunctional traits (low denitrification rates, instant accumulation of NO and N_2O, late induction of

N_2OR) when shifted to neutral (Fig. 3). In contrast, no such pH effect on denitrification rate or product stoichiometry was found for the neutral-adapted SWE community. The SWE community was remarkable in several respects. Shifting pH down to 5.4 did hardly slow down denitrification (Fig. 3), and the accumulation of denitrification intermediates appeared to be even lower at acid pH than under neutral conditions. Together, these data suggest a peculiar functional robustness of the community from the neutral soil (SWE, native pH 7.1) and a consistent dysfunctionality of the community from the most acidic soil (GER, native pH 5.4). The FIN community in turn, which had a native pH of 6.0, was somewhat intermediate between the two others in most respects. In summary, our results lend strong evidence to the notion that direct pH response depends on the communities' regulatory phenotype, which in turn may be a function of how the native pH has affected the community composition (Enwall *et al.*, 2005; Hallin *et al.*, 2009; Philippot *et al.*, 2009). The community-dependent pH response may also explain contradictory findings between pH effects on denitrification stoichiometry and N_2O emission along natural pH gradients (Dannenmann *et al.*, 2008; Philippot *et al.*, 2009; Weslien *et al.*, 2009) and in short-term field manipulations (Baggs *et al.*, 2010; Cuhel *et al.*, 2010).

Soil pH appears to have a pervasive effect on the microbial taxonomic composition in soils. Although soil microbes may be exposed to spatial and temporal pH variations (Zarate-Valdez *et al.*, 2006; Blossfeld *et al.*, 2011), bulk soil pH exerts a strong selection pressure on soil microbes (Alexander, 1980; Rousk *et al.*, 2010). In general, acidic soils support lower microbial diversity than neutral or mildly alkaline soils (Fierer & Jackson, 2006; Wakelin *et al.*, 2008). Microbial communities adapted to acidity, on the other hand, seem to perform reasonably well in broad-scale functions such as growth (Bååth, 1996) or denitrification (Parkin *et al.*, 1985; Simek *et al.*, 2002) at any given pH. In contrast to that, low pH soils are recurrently reported to have lower relative N_2OR activities, resulting in higher N_2O/N_2 product ratios and thus higher potential N_2O emission both in soils (Simek & Cooper, 2002; Cuhel *et al.*, 2010; Liu *et al.*, 2010) and in bacterial culture experiments (Thomsen *et al.*, 1994; Baumann *et al.*, 1997; Saleh-Lakha *et al.*, 2009; Bergaust *et al.*, 2010). Irrespective of the inhibitory mechanism, malfunctioning of an enzyme involved in energy conservation (such as N_2OR) should result in a gradual loss of the gene coding for this enzyme, which could be one reason for the reported instability/lower abundance of *nosZ* genes relative to other denitrification genes in soils (Jones *et al.*, 2009; Philippot *et al.*, 2009). The three soils investigated here represented strong contrasts in origin (acid fen for GER vs. calcareous lake bottom for SWE) and drainage history (220 years in GER vs. 80 years in SWE), suggesting that *nosZ* communities of these two soils should differ in abundance, richness and taxonomic composition. Surprisingly, the similarity of *nosZ* communities between the three soils was greater than the similarity for other functional denitrification genes (Braker *et al.*, this issue), and there was no significant difference in relative *nosZ* abundance between the soils. Our experiments showed that a significant number of organisms able to express *nosZ* genotypes exist in the degraded, acid German soil because there was a rapid uptake of N_2O along with N_2 production after 90 h of incubation. It is unclear, however, whether these genotypes were dominant from the start of the assay, because denitrification kinetics of all cultures indicated exponential growth towards the end of the incubation which may have changed community composition. It is interesting to note that measurable N_2OR in GER appeared earlier at its native pH (5.4) than at neutral pH (7.1), illustrating once again that the acid and degraded GER soil harboured highly adapted denitrifier populations.

Together with the detailed genetic study of Braker *et al.* (2012), our experiments demonstrate that soils harbour structurally dissimilar denitrifier communities that show distinct denitrification regulatory phenotypes with respect to total denitrification and relative product accumulation. Previous experiments with soil slurries of the three soils indicated that the three soils harboured denitrifying communities with profoundly different characteristics with respect to their ability to express N_2OR (Holtan-Hartwig *et al.*, 2000, 2002; Dörsch & Bakken, 2004). This could be due to a direct effect of the soil pH, however. In the current experiment, we avoided any effect of the soils' pH by studying extracted cells. The results largely confirmed our previous evaluation of the three communities and identified a new contrast between them regarding their ability to tackle different pH levels.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effect of pH on O_2 respiration and dissolved O_2 concentrations (left panel) and CO_2 production rates (calculated from pH corrected total inorganic carbon, right panel) in incubations of cell suspensions extracted from GER, SWE and FIN.

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