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THE FUNCTIONAL SIGNIFICANCE OF DENITRIFIER COMMUNITY COMPOSITION IN A TERRESTRIAL ECOSYSTEM

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Abstract. We tested the hypothesis that soil microbial diversity affects ecosystem function by evaluating the effect of denitrifier community composition on nitrous oxide (N_2O) production. Denitrification is a major source of atmospheric N_2O, an important greenhouse gas and a natural catalyst of stratospheric ozone decay. The major environmental controls on denitrification rate and the mole ratio of N_2O produced during denitrification have been incorporated into mechanistic models, but these models are, in general, poor predictors of in situ N_2O flux rates. We sampled two geomorphically similar soils from fields in southwest Michigan that differed in plant community composition and disturbance regime: a conventionally tilled agricultural field and a never-tilled successional field. We tested whether denitrifier community composition influences denitrification rate and the relative rate of N_2O production [$\triangle N_2O/\triangle(N_2O + N_2)$], or rN_2O, using a soil enzyme assay designed to evaluate the effect of oxygen concentration and pH on the activity of denitrification enzymes responsible for the production and consumption of N_2O. By controlling, or providing in nonlimiting amounts, all known environmental regulators of denitrifier N_2O production and consumption, we created conditions in which the only variable contributing to differences in denitrification rate and rN_2O in the two soils was denitrifier community composition. We found that both denitrification rate and rN_2O differed for the two soils under controlled incubation conditions. Oxygen inhibited the activity of enzymes involved in N_2O production (nitrate reductase, Nar; nitrite reductase, Nir; and nitric oxide reductase, Nor) to a greater extent in the denitrifying community from the agricultural field than in the community from the successional field. The Nar, Nir, and Nor enzymes of the denitrifying community from the successional field, on the other hand, were more sensitive to pH than were those in the denitrifying community from the agricultural field. Moreover, the denitrifying community in the soil from the successional field had relatively more active nitrous oxide reductase (Nos) enzymes, which reduce N_2O to N_2, than the denitrifying community in the agricultural field. Also, the shape of the rN_2O curve with increasing oxygen was different for each denitrifying community. Each of these differences suggests that the denitrifying communities in these two soils are different and that they do not respond to environmental regulators in the same manner. We thus conclude that native microbial community composition regulates an important ecosystem function in these soils.

Key words: biodiversity; community composition; denitrification enzyme activity; denitrifier community composition; ecosystem function; Michigan; microbial diversity; nitrate; nitrous oxide; oxygen; pH.

INTRODUCTION

The significance of biodiversity to ecosystem function has emerged as a major ecological issue in recent years, particularly among ecologists working with macroorganisms (e.g., Mooney et al. 1995, Grime 1997, Hooper and Vitousek 1997, Huston 1997, Chapin et al. 1998). At question is the degree to which species diversity or individual species affect overall ecosystem function and the delivery of ecosystem services. This question is equally relevant for microbial taxa (e.g., Schimel 1995, Conrad 1996), especially in light of the tremendous apparent diversity among soil microorganisms. For example, recent estimates of microbial species richness, based on novel molecular techniques, suggest that there can be as many as $4–10 \times 10^3$ different bacterial taxa in a single soil sample (Torsvik et al. 1990, Klug and Tiedje 1994).

Within broad taxonomic bounds, microbial diversity clearly matters a great deal: only certain taxa appear to carry out particular biogeochemical processes. It is unknown, however, whether diversity within the broad functional groups that perform these processes (groups such as nitrifiers, denitrifiers, and methanotrophs) is important. While symbiotic microorganisms can have high host specificity (e.g., Alexander 1985, Allen et al. 1995), diversity within most microbial functional groups is often assumed to be functionally redundant (e.g., Meyer 1993, Beare et al. 1995, Heal et al. 1996). In fact, in both schematic and mechanistic models of

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nutrient cycling, functional groups are depicted and treated as black boxes that transform inputs to outputs at rates determined solely on a specific set of rate-limiting environmental factors.

Enzymological studies of isolated microorganisms, on the other hand, often reveal great variability in enzyme immunochemical cross-reactivity, efficiency, and regulation within functional groups (e.g., Korner 1993, Robertson et al. 1995, Conrad 1996, Ka et al. 1997, Cavigelli 1998). Such results suggest that the regulation of ecosystem processes that are mediated by microbes may in fact be affected by, and reflect, the community composition of functional groups.

Denitrifiers provide an excellent microbial model for studying questions related to the functional significance of biotic diversity: they are among the most diverse groups of bacteria in terrestrial ecosystems and, because of their importance to nitrogen (N) cycling, among the best studied (Tiedje 1988, Zumft 1992). Denitrification, which is carried out solely by denitrifying bacteria, is the conversion of nitrate (NO_3^-) and nitrite (NO_2^-) to the nitrogen gases nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2). Thus, denitrification can have a direct impact on soil nitrogen availability and, ultimately, on net primary production in many terrestrial and coastal margin ecosystems. Denitrification is also a major source of atmospheric N_2O (Firestone and Davidson 1989), an important greenhouse gas and a natural catalyst of stratospheric ozone decay (Rasmussen and Khalil 1986, Cicerone 1987). The atmospheric concentration of N_2O is increasing annually at ~0.8 $m^3/10^9$ m^3 (Houghton et al. 1996) and the global N_2O budget is far from balanced (Davidson 1991, Robertson 1993). Denitrification's role in this increase is unclear.

Denitrifiers are facultative anaerobes, capable of shunting electrons from electron transport phosphorylation (ETP) to nitrogen oxides when oxygen becomes limiting (Tiedje 1988). The four enzymes that link ETP to nitrogen oxide reduction (nitrate reductase, Nar; nitrite reductase, Nir; nitric oxide reductase, Nor; and nitrous oxide reductase, Nos) are usually induced sequentially under anaerobic conditions (Tiedje 1994):

$$NO_3^- \xrightarrow[\text{Nar}]{} NO_2^- \xrightarrow[\text{Nir}]{} NO \xrightarrow[\text{Nor}]{} N_2O \xrightarrow[\text{Nos}]{} N_2.$$

Since N_2O is produced by Nor and consumed by Nos, N_2O accumulates during denitrification under two sets of conditions: (1) after Nor but before Nos is induced (Firestone and Tiedje 1979, Dendooven and Anderson 1994), and (2) following induction of the entire denitrification pathway when environmental conditions inhibit Nos activity to a greater extent than they inhibit Nor activity (Betlach and Tiedje 1981). Temperature, oxygen, pH, available organic carbon, and nitrate and/or nitrite influence N_2O production and consumption rates via their influence on both denitrification enzyme induction and activity (Tiedje 1988, Firestone and Davidson 1989).

The rate of denitrifier N_2O accumulation is thus a product of both denitrification rate ($\Delta[N_2O + N_2]$) and the relative rate of N_2O production,

$$\frac{\Delta N_2O}{\Delta(N_2O + N_2)}$$

or, for simplicity, rN_2O. Within the small range of low oxygen concentrations under which denitrification usually occurs, denitrification rate generally decreases with increasing oxygen and decreasing pH. The quotient rN_2O, on the other hand, generally increases with increasing oxygen, decreasing pH, and decreasing carbon : nitrate ratio (e.g., Firestone et al. 1979, 1980, Firestone and Davidson 1989).

The major environmental controls on denitrification rate and rN_2O have been incorporated into mechanistic models, but these models are, in general, poor predictors of in situ N_2O flux rates (e.g., McGill et al. 1981, Rolston et al. 1984, McConnaughey and Bouldin 1985, Johnsson et al. 1987, Parton et al. 1988, Arah and Smith 1989, Li et al. 1992*a, b,* Ojima et al. 1992, Smith et al. 1993, Parton et al. 1995). Modeling difficulties have been attributed to the high degree of spatial and temporal variability of environmental conditions controlling denitrification and N_2O emissions (e.g., Arah and Smith 1990). An additional, untested explanation is that the soil environment harbors denitrifier populations with denitrification enzymes, especially Nor and Nos, that differ in their sensitivity to environmental variables (Dendooven et al. 1996). If this is the case, our ability to predict changes in local and global N_2O fluxes may hinge on an understanding of denitrifier community composition and population dynamics.

We show in a companion paper (Cavigelli 1998) that the composition of the denitrifying community in contrasting soils from an agricultural field and a successional field differ and that there is great diversity in the sensitivity of Nos to oxygen among different denitrifiers cultured from these soils. We test here the hypothesis that denitrifier community composition can affect denitrification rate and rN_2O using a soil enzyme assay designed to evaluate the effect of oxygen concentration and pH on the activity of all denitrification enzymes in the entire denitrifier community, including those populations that may not be culturable.

Materials and Methods

Our overall strategy has been to compare the sensitivity of denitrification enzymes to very low oxygen concentrations in soils from two sites after controlling for all other environmental factors that regulate the denitrification rate and rN_2O. These factors include temperature, pH, carbon : nitrate ratio, substrate diffusion, and denitrification enzyme induction status. We are aware of no other important environmental factors that can affect denitrification rate and rN_2O in soils.

TABLE 1. Soil properties for the conventionally tilled agricultural field and the never-tilled successional field at the KBS LTER site.

Soil property	Agricultural field	Successional field	*t* test
Texture	sandy loam	sandy loam	
pH†	6.59 ± 0.04	5.70 ± 0.11	**
Water content [g/(g dry soil)]†	10.49 ± 0.65	11.24 ± 0.56	NS
Bulk density (g/cm^3)‡	1.65 ± 0.05	1.35 ± 0.02	*
Total carbon [g/(100 g dry soil)]§	0.77 ± 0.07	1.97 ± 0.12	**
Total nitrogen [g/(100 g dry soil)]§	0.077 ± 0.006	0.166 ± 0.009	**
Nitrate-N [μg/(g dry soil)]‖	4.57 ± 0.95	0.93 ± 0.37	*
Ammonium-N [μg/(g dry soil)]‖	2.73 ± 0.76	8.75 ± 0.35	**
N_2O-N flux ($g \cdot ha^{-1} \cdot d^{-1}$)	2.29 ± 0.08	1.32 ± 0.08	**

Notes: All values are means ± 1 SE for three field replicates except N_2O flux, which is for four field replicates; significance values are based on *t* tests. All data except pH, water content, and N_2O flux are from the KBS LTER web site ⟨http://lter.kbs.msu.edu⟩. Water content and pH were measured on the same soil samples described in this study. Nitrous oxide fluxes were measured using a static chamber method on 18 different sampling dates in 1994 (G. P. Robertson, *unpublished data*).

* $P < 0.05$; ** $P < 0.01$; NS = not significant ($P > 0.1$).

† Sampled 21 September 1994 to a depth of 10 cm.

‡ Sampled prior to plowing, spring 1996, to a depth of 15 cm.

§ Sampled 29 August 1994 to a depth of 25 cm.

‖ Sampled 21 September 1994 to a depth of 15 cm.

Any observed differences in denitrification rate and rN_2O between soils under these controlled conditions, then, is evidence that soil denitrifying communities respond differently to the same environmental conditions, i.e., that denitrifier community composition influences N_2O production.

Study site and soils

We sampled soil from two fields at the Long Term Ecological Research (LTER) site in agricultural ecology at Michigan State University's W. K. Kellogg Biological Station (KBS). KBS is located in southwest Michigan in the northern part of the U.S. corn belt. The area receives ~860 mm/yr of precipitation, evenly distributed throughout the year. Mean annual temperature is 9.4°C. It is often cloudy, with appreciable precipitation occurring on ~100 d/yr. Soils developed on the pitted outwash plain following the Wisconsin glaciation 10^3 yr ago. Soils at our study site are Typic Hapludalfs (fine-loamy, mixed, mesic) of moderate fertility that developed under forest vegetation.

In the fall of 1994, we collected soil samples from a conventionally tilled agricultural field and a never-tilled successional field. The agricultural field had been in a corn–soybean–wheat rotation since 1988 and in various field crop rotations for the prior century, managed in accordance with regional agronomic practices. The successional field was cleared of trees in 1960 and has since been mowed annually or biennially in November or December. Dominant plant species in the successional field at the time of sampling, expressed as percentage of total live biomass, were *Arrhenatherum elatius* (L.) Beauv. ex J.-BC Presl (tall oatgrass, 15%), *Elaeagnus umbellata* Thunb. (autumn olive, 15%), *Solidago canadensis* L. (common goldenrod, 9%), *Rubus allegheniensis* T. C. Porter (blackberry, 9%), *Monarda fistulosa* L. (beebalm, 8%), *Bromus inermis* Leyss. (smooth brome, 7%), *Poa pratensis* L. (Kentucky bluegrass, 7%), *P. compressa* L. (Canada bluegrass, 6%), and *Dactylis glomerata* L. (orchardgrass, 6%). Management of these sites is more fully described on the World Wide Web.[2]

The two fields have geomorphically similar soils that differ in pH, bulk density, total carbon, total nitrogen, and inorganic nitrogen pools (Table 1)—factors that we judged likely to influence denitrifier community composition. From each of three field replicates of the two sites, we composited thirty 2.5 cm diameter soil cores (10-cm depth) for a total of six soil samples. Samples were stored on ice until returned to the laboratory, at which point they were homogenized through 4-mm sieves and stored at 4°C until subsampled for enzyme assays. These are the same soil samples from which denitrifying bacteria described in Cavigelli (1998) were isolated.

Preincubation: denitrifier community enzyme induction

Prior to measuring denitrifier community enzyme sensitivity to very low oxygen concentrations, we induced denitrification enzymes using an anaerobic slurry and confirmed the activity of Nos, the last enzyme in the pathway. This preincubation was necessary to ensure that enzyme activity measurements are not confounded by the enzyme induction status of the community at the time of sampling (e.g., Smith and Tiedje 1979, Dendooven and Anderson 1994). We measured 3.0 g of each soil (field moist) into two sets of 38-mL serum vials, sealed the vials using butyl rubber septa and crimp seals, and removed oxygen by flushing for ~3 min with ultra high purity (UHP; 99.995%) N_2 gas. We added 3.0 mL of degassed water by syringe and equilibrated the headspace pressure to atmospheric pressure using a needle attached to a syringe barrel

[2] URL = ⟨http://lter.kbs.msu.edu⟩

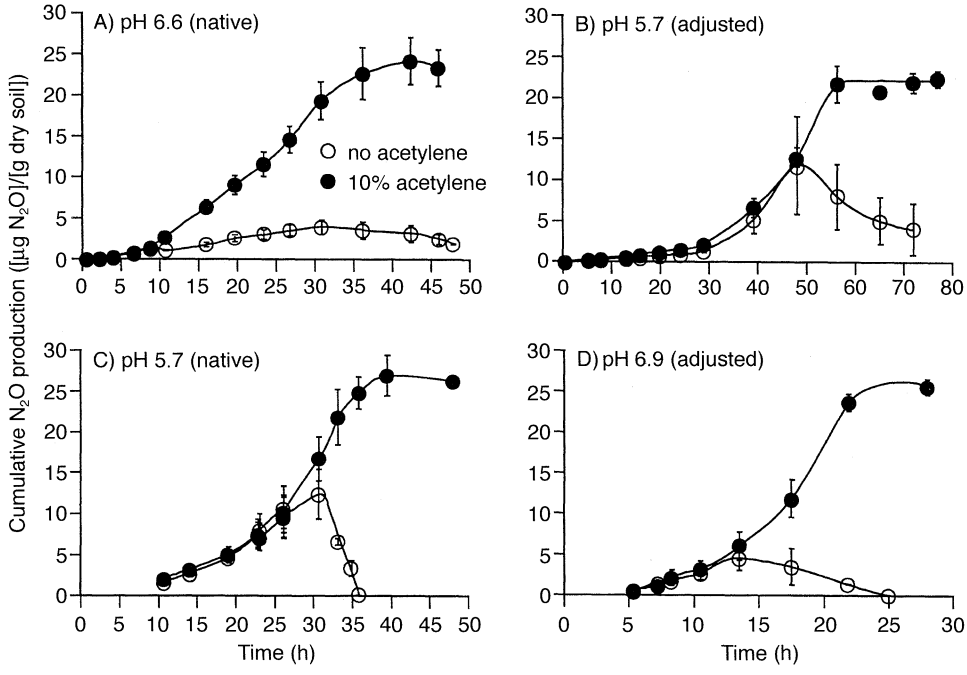

FIG. 1. Denitrifier community enzyme induction curves at two pH levels for soils from (A, B) the conventionally tilled agricultural field and (C, D) the never-tilled successional field at the KBS LTER site. Nitrous oxide reductase activity is indicated by greater N_2O production in the presence than in the absence of acetylene. Values are means, and error bars are ± 1 SE (n = 3 for the agricultural field; n = 2 for the successional field). Note different ranges for x-axes.

containing a small amount of water. To one set of vials, we added 3.6 mL of acetylene, which blocks the reduction of N_2O to N_2 by Nos (Balderston et al. 1976, Yoshinari and Knowles 1976). We made oxygen-free acetylene by adding degassed water to a sealed serum vial containing calcium carbide in an evacuated N_2 atmosphere. After adding acetylene to vials we re-equilibrated the headspace pressure and then monitored headspace gases during the following 30–80 h by sampling with a gas-tight needle and syringe (0.1 mL sample volume using a 1-mL syringe). Comparing N_2O accumulation in the two sets of vials (with and without acetylene) allowed us to monitor Nos activity. Nos activity occurs when the two N_2O accumulation curves diverge (Smith et al. 1978).

We measured both N_2O and O_2 using an HP 5890II gas chromatograph (GC; Hewlett Packard, Rolling Meadows, Illinois) equipped with dual Poropak Q columns and a ^{63}Ni electron capture detector (ECD) operated at 350°C. The column temperature was 50°C and the carrier gas was 10% methane and 90% argon (30 mL/min). Gas concentrations in the headspace were corrected for dissolution in the slurry solution using Bunsen coefficients (Tiedje 1994). Separate syringes (and standard curves) were used for those vials that received acetylene to avoid acetylene cross-contamination.

To assess whether Nos induction regulation by nitrate or nitrite was different in these two communities, we calculated the nitrate concentration in the two soils at the approximate time of Nos induction. We first calculated the amount of nitrate present in each soil at the beginning of the induction assays by converting the amount of N_2O that accumulated in the presence of acetylene to the amount of NO_3^--N required to produce that amount of N_2O (Lensi et al. 1985, Christensen and Tiedje 1988, Binnerup and Sørensen 1992, Højberg et al. 1994). We then subtracted from initial NO_3^--N concentrations the amount of NO_3^--N converted to N_2O at the time of Nos induction (estimated from Fig. 1). These calculations assume that N_2O accumulation in the presence of acetylene is nitrate limited. To test this assumption, we added slurry solution containing 17, 33, 50, and 67 μg NO_3^--N/(g soil) to a separate set of soil subsamples. After denitrification had peaked in vials to which 67 μg NO_3^--N/(g soil) had been added, we added an additional dose of 67 μg NO_3^--N/(g soil). We monitored denitrification in all vials every 2–10 h for 100 h.

Denitrifier community enzyme activity assay

We measured the activity of Nos under very low oxygen concentrations by conducting short-term slurry incubations in which all environmental factors controlling denitrification rate and rN_2O were controlled or provided in nonlimiting amounts. This method is a modification of the denitrification enzyme assay (Smith et al. 1978, Smith and Tiedje 1979, Tiedje 1994).

After confirming Nos activity under preincubation conditions, we flushed preincubation vials that had not been exposed to acetylene with UHP N_2 while shaking until N_2O in the headspace was below the GC detection limit. We randomly assigned each vial to one of two sets. To one set we added 3.6 mL acetylene (≈10% headspace concentration) and equilibrated headspace pressure to atmospheric pressure. We then added 0, 0.05, 0.10, or 0.15 mL of 100% oxygen to all vials together with 0.1 mL of a degassed solution of succinate and nitrate, to a final concentration of 1 mmol/L each. These levels are generally considered nonlimiting for soil denitrifying communities (Tiedje 1994). Vials were shaken at 400 rpm using an orbit shaker (Model 3520, Lab-Line Instruments, Melrose Park, Illinois) immediately after addition of the degassed solution.

We took vial headspace samples every 7–10 min after the start of the incubation and measured N_2O using the GC. Rates of net N_2O production ($\triangle N_2O$; measured in the absence of acetylene) and denitrification ($\triangle[N_2O + N_2]$; measured in the presence of 10% acetylene) were calculated from linear regressions of at least four points sampled within the first 60 min of incubation. In all of our assays, regression coefficients were >0.90, indicating that there was no de novo enzyme synthesis during these short-term incubations. We did not use chloramphenicol, an inhibitor of protein synthesis that is sometimes necessary for similar incubations (Tiedje 1994).

Denitrification rates measured at the four oxygen levels provide a measure of the sensitivity to oxygen of the three enzymes leading to the production of N_2O (Nar, Nir, and Nor). Note that this assay provides a measure of the sensitivity of these three enzymes in combination; it does not measure the sensitivity of each enzyme individually. Net N_2O production rates measured at the four oxygen levels provide an integrated measure of both N_2O production and consumption at low oxygen concentrations. The sensitivity of N_2O consumption, or Nos activity, to oxygen can therefore be measured as rN_2O, the ratio of net N_2O production to denitrification rate ($\triangle N_2O/\triangle[N_2O + N_2]$) at different oxygen levels.

We maintained oxygen concentrations in the slurry solutions at 0.17, 0.27, and 0.45 μmol/L for the 0.05, 0.10, and 0.15 mL oxygen additions, respectively. These levels are well below the estimated 10 μmol/L threshold for denitrification activity (Tiedje 1988). We found no evidence for significant oxygen consumption during these short term incubations, i.e., oxygen concentrations, corrected for GC fluctuations, did not vary during incubations. For convenience we report oxygen concentrations as mL of 100% oxygen added per vial.

We had earlier tested the effect of shaking speed on denitrification rate in similar vials to which 0.3 mL oxygen had been added. Results from this test showed that denitrification rate decreased with increasing shaking speed up to 300 rpm (Table 2), suggesting that oxygen diffusion from gas to water phase was limited at low shaking speeds. Shaking vials at 400 rpm alleviated this problem, i.e., oxygen concentrations in the soil slurry solution were in equilibrium with the gas phase at sufficiently high shaking speeds.

TABLE 2. Denitrification rate at low oxygen concentration (equilibrium slurry concentration, 0.27 μmol/L) with increasing shaking speed for soil from one field replicate of the agricultural field at the KBS LTER site.

Shaking speed (rpm)	Denitrification rate (ng N_2O·[g dry soil]$^{-1}$·min^{-1})
0	1.93[a] ± 0.03
200	1.19[b] ± 0.02
300	0.96[c] ± 0.03
350	0.95[c] ± 0.04
400	0.99[c] ± 0.05

Notes: Values are means ± 1 SE (n = 3 soil subsamples). Above 300 rpm the rate of oxygen diffusion from gas to water phase does not influence denitrification rate. Data were analyzed by ANOVA ($F_{4,10}$ = 140.1, $P < 0.0001$). Rates with different letters after symbols are significantly different, based on Tukey's hsd test (minimum significant difference: 0.16).

To test whether vigorous shaking affected bacterial viability, we compared bacterial plate counts from unamended aerobic slurries of one field replicate from the agricultural field after 4 h of vigorous (400 rpm on orbit shaker) or gentle (using a hematology chemistry mixer, Model 346, Fisher Scientific) shaking. We followed standard plate count techniques (Zuberer 1994) using a modified R2A medium (R2A*; Cavigelli 1998) and conducted the experiment using three replicate vials. We counted all colonies on plates with 30–300 colonies after 24 h of growth at room temperature. Mean plate counts were log-transformed and compared using Student's t test (Ott 1984).

pH of enzyme induction and activity assays

We conducted all enzyme induction and activity assays at both native pH and at a pH level adjusted to that of the other soil. These treatments were necessary to control for effects of pH on denitrification rate and rN_2O (e.g., Firestone et al. 1980). We used phosphate buffer (pH 5.5) to decrease the pH of the soil from the agricultural field to 5.74 ± 0.03 (mean ± 1 SE) and calcium carbonate (0.033g/[g soil]) to increase the pH of the soil from the successional field to 6.89 ± 0.12. To test whether there were any chemical effects of phosphate buffer or calcium carbonate on enzyme induction or activity, we also included control treatments in which phosphate buffer was added to soil from the successional field (pH 5.92 ± 0.17) and calcium carbonate was added to soil from the agricultural field (pH 7.22 ± 0.02). We measured pH in three separate, replicate vials for all soils after inducing Nos, which is the same point at which we measured enzyme activity in the assay vials.

Nitrifier N_2O production

Nitrifying bacteria, which are obligately aerobic, are also an important source of N_2O in many soils (e.g., Bremner and Blackmer 1978, Firestone and Davidson 1989). Nitrifiers tend to produce N_2O at higher oxygen partial pressures than do denitrifiers (e.g., Davidson et al. 1986, Klemmedtsson et al. 1988b) but the oxygen threshold for nitrifier N_2O production is not well characterized (e.g., Goreau et al. 1980).

We investigated whether nitrifiers contributed to N_2O production at the very low oxygen concentrations used in the enzyme activity assay by conducting incubations similar to those described for enzyme induction. We incubated two sets of vials under anaerobic conditions, one set with 10% acetylene and one set with no acetylene in the headspace. We measured N_2O production for ~8 h and then added 0.3 mL oxygen (equilibrium solution concentration: 0.86 μmol/L) to both sets of incubation vials to create microaerobic conditions. We continued to measure N_2O production for an additional 8 h.

We were able to assess whether there was any nitrifier activity under the microaerobic conditions by first calculating the ratio of N_2O production rates under anaerobic vs. microaerobic conditions for both sets of vials. In a 10% acetylene atmosphere, nitrifiers are inhibited (Berg et al. 1982, Klemmedtsson et al. 1988*a*) regardless of the oxygen level. Under an acetylene atmosphere, then, the ratio between N_2O production under anaerobic and microaerobic conditions is a measure of denitrifier inhibition by oxygen. When no acetylene is present, nitrifiers produce N_2O only under microaerobic conditions, if at all. If nitrifiers contribute to N_2O production, the anaerobic : microaerobic N_2O production ratio would be smaller than that in vials containing acetylene. If, on the other hand, only denitrifiers produce N_2O under microaerobic conditions with no acetylene, the anaerobic : microaerobic N_2O production ratio should be of similar magnitude in the presence or absence of acetylene. By comparing ratios in the presence and absence of acetylene, then, we were able to determine whether there was nitrifier activity in these incubations. We compared ratios using Student's *t* test (Ott 1984).

Statistical analyses

We used three separate three-way ANOVAs to determine the effects of site, pH, and oxygen on net N_2O production rate, denitrification rate, and rN_2O. These analyses were conducted using the GLM procedure of SAS version 6.09 (SAS Institute 1996). We used the Type III sum of squares due to our unbalanced design (Potvin 1993) and used the LSMEANS/PDIFF (least squares means) procedure to make a priori pairwise comparisons when main effects were significant. For rN_2O we subjected data to an arcsine square-root transformation since values were constrained between 0 and 1 (Hinkelman and Kempthorne 1994).

TABLE 3. Nitrous oxide production rates with and without acetylene under anaerobic and microaerobic conditions, and the ratio between anaerobic and microaerobic rates for soils from the conventionally tilled agricultural field and the never-tilled successional field at the KBS LTER site.

Condition	Agricultural field	Successional field
N_2O production rate (ng·[g soil]$^{-1}$·h^{-1})		
Without acetylene		
Anaerobic	110 ± 24	268 ± 92
Microaerobic	46 ± 12	127 ± 47
With acetylene		
Anaerobic	441 ± 90	536 ± 120
Microaerobic	170 ± 36	248 ± 63
Anaerobic:microaerobic N_2O production rate ratio		
Without acetylene	2.43 ± 0.10†	2.13 ± 0.06‡
With acetylene	2.64 ± 0.19†	2.18 ± 0.07‡

Notes: Microaerobic conditions were created by adding 0.3 mL oxygen (equilibrium solution concentration of 0.86 μmol O_2/L) to previously anaerobic vials. Results show that nitrifiers do not produce N_2O at low oxygen concentrations in these soils. Values are means ± 1 SE (n = 3 soil subsamples for the agricultural field; n = 2 for the successional field).

† NS, t test, t = 0.96, df = 3, $P > 0.1$.

‡ NS, t test, t = 0.54, df = 2, $P > 0.1$.

RESULTS

Calcium carbonate did not have any influence on rN_2O for the soil from the agricultural field (ANOVA, $F_{1,22} = 0.33$, $P = 0.57$) and phosphate buffer had no influence on rN_2O for the soil from the successional field (ANOVA, $F_{1,14} = 0.19$, $P = 0.67$). For clarity, we therefore present only results for soils incubated at native pH and at a pH level adjusted to that of the other soil. We also present data from only two replicates of the soil from the successional field since one replicate soil sample from this field was lost prior to completing all enzyme assays.

Nitrifier N_2O production

The anaerobic : microaerobic N_2O production rate ratios used to test for nitrifier N_2O production are provided in Table 3; there were no significant differences in ratios between vials with and without acetylene.

Preincubation: denitrifier community enzyme induction assay

We assessed nitrous oxide reductase (Nos) activity during induction assays by comparing N_2O accumulation curves for soils in the presence and absence of acetylene (Fig. 1). N_2O production and consumption patterns similar to these have been observed in a variety of soils (e.g., Klemmedtsson et al. 1988*b*, Dendooven and Anderson 1994) and previous studies, using both chloramphenicol and ^{13}N, have established that these patterns are due to the sequential induction of denitrification enzymes (Smith et al. 1978, Firestone and Tiedje 1979, Firestone et al. 1979, Smith and Tiedje 1979). In general, the sequence of events is as follows:

TABLE 4. Total denitrification for soil from the agricultural field at the KBS LTER site with increasing rate of nitrate addition.

NO_3-N added (μg/[g dry soil])	Total N_2O production ([μg N_2O]/[g dry soil])
0	12.7 ± 0.04
16.7	38.4 ± 2.05
33.3	50.1
50.0	62.1 ± 2.19
66.7	87.2 ± 0.57

Notes: Soil was incubated anaerobically with 10% acetylene in the headspace. Values are means ± 1 SE (n = 2 soil subsamples, except for when 33 μg NO_3-N was added per g soil, in which case n = 1). The regression equation of N_2O production vs. nitrate is $y = 1.04x + 15.6$, $r = 0.99$. Results show that denitrification was nitrate limited in unamended slurries.

(1) N_2O appears, indicating Nar, Nir, and Nor activity, (2) N_2O production rate increases, indicating de novo synthesis of additional Nar, Nir, and Nor, (3) similar N_2O accumulation rates in the presence and absence of acetylene indicate that Nos either has not yet been induced or is not active, and (4) greater N_2O accumulation in the presence of acetylene than in its absence indicates that Nos has been induced and is active since N_2O is being consumed.

In the soil from the agricultural field N_2O accumulation was greater in the presence of acetylene than in its absence, indicating that there was Nos activity <11 h after the beginning of the incubations (Fig. 1A). When the pH of this soil was adjusted to 5.7, N_2O production in the presence of acetylene (Nar, Nir, and Nor induction) and N_2O consumption in the absence of acetylene (Nos induction) were both greatly inhibited (Fig. 1B). Significant denitrification did not occur before ~37 h and N_2O consumption in the absence of acetylene was inhibited for ~48 h. At both pH levels, total denitrification was 23–25 μg N_2O/(g soil).

In the soil from the successional field, there was no Nos activity at the beginning of the incubations at either pH level (Fig. 1C, D). Increasing soil pH to 6.9 resulted in earlier production and consumption of N_2O, indicating that low pH had an inhibitory effect on Nos activity in this soil too. Total denitrification was ~27 μg N_2O/(g soil) at both pH levels.

In a separate set of preincubation vials, we observed a positive, linear relationship between the amount of nitrate added to slurries and total denitrification (Table 4), suggesting that total denitrification was limited by nitrate depletion in these soils. In addition, vigorous denitrification resumed when additional nitrate was added to slurries after N_2O accumulation had ceased (data not shown). Since N_2O production in the presence of acetylene is stoichiometrically related to nitrate consumption during denitrification, we were able to calculate the approximate amount of nitrate present in soil slurries at each point along the induction curves based on headspace N_2O concentrations (Fig. 1). These calculations showed that nitrate concentration at the approximate time of initial Nos activity (the point where the two curves diverge) was lower in the soil from the agricultural field than in the soil from the successional field at all pH values (Table 5), and that nitrate concentration at the time of Nos induction increased with increased pH.

Denitrifier community enzyme activity assay

Shaking soil slurries at 400 rpm did not kill cells: aerobic heterotrophic bacterial counts were 2.9 ± 0.7 × 10^6 colony-forming units (CFU)/(g soil) after gentle shaking and 3.5 ± 0.5 × 10^6 CFU/(g soil) (means ± 1 SE) following vigorous shaking (t test, $t_{0.05,1} = -1.09$, $n = 3$, $P > 0.1$).

Site, pH, and oxygen each affected denitrification rate (Fig. 2, Table 6). Denitrification rate was higher in the soil from the successional field than in the soil from the agricultural field at either pH level ($P \leq 0.0001$, pairwise comparisons between two soils at each oxygen level). A pH effect was observed only for the soil from the successional field (Fig. 2; $P \leq 0.0001$ for soil from the successional field; $P > 0.1$ for soil from the agricultural field). Denitrification rates decreased exponentially with increasing oxygen concentration for both sites at both pH levels (Fig. 2), but the rate of decrease was different for the two sites. For the soil from the agricultural field, there was a significant decrease in denitrification rate at both pH levels only between 0 and 0.05 mL oxygen ($P < 0.005$). For the soil from the successional field, there was a significant decrease in denitrification rate at both pH levels between 0 and 0.05 mL oxygen ($P < 0.0005$), and also between 0.05 and 0.10 mL oxygen at pH 5.7 ($P < 0.01$).

Site, pH, and oxygen each affected net N_2O production rate (Fig. 3, Table 6), but the relationships were sometimes complicated. At pH 5.7, net N_2O production rate was higher in the soil from the successional field than in the soil from the agricultural field ($P \leq 0.0001$) at all oxygen levels except under anaerobic conditions ($P > 0.1$). Net N_2O production rate in the soil from the successional field at pH 6.9 was not different than

TABLE 5. Calculated slurry nitrate concentrations at two pH levels at the point of initial Nos activity during preincubations for soils from the conventionally tilled agricultural field and the never-tilled successional field at the KBS LTER site.

Field	pH	Nitrate concentration ([μg NO_3-N]/[g dry soil])
Agricultural	5.7	1.61 ± 0.53
	6.6 (native)	3.47 ± 0.42
Successional	5.7 (native)	6.90 ± 0.77
	6.9	10.80 ± 1.10

Note: Values are means ± 1 SE (n = 3 replicates for the agricultural field; n = 2 for the successional field).

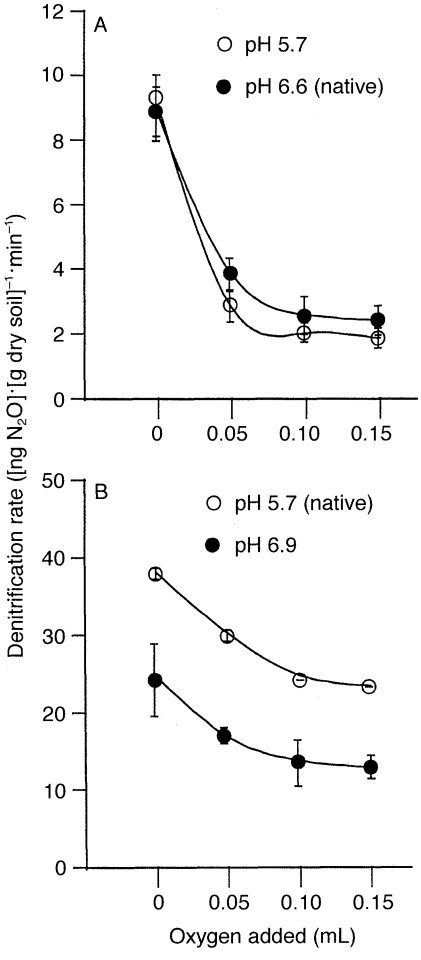

FIG. 2. Denitrification rates at four oxygen concentrations and two pH levels for soils from (A) the conventionally tilled agricultural field and (B) the never-tilled successional field at the KBS LTER site. Values are means, and error bars are ± 1 SE (n = 3 for the agricultural field; n = 2 for the successional field).

for the soil from the agricultural field at either pH level ($P > 0.1$). For the soil from the agricultural field net N_2O production rate was similar at both pH levels ($P > 0.1$) except under anaerobic conditions, in which case the rate was much higher at pH 5.7 than pH 6.6 ($P \leq 0.0001$). Net N_2O production rate also did not change with oxygen level at this site ($P > 0.1$) except for the decrease between 0 and 0.05 mL oxygen at pH 5.7 ($P < 0.0005$). For the soil from the successional field net N_2O production rate was higher at native pH than at pH 6.9 at each oxygen level ($P \leq 0.0001$). At both pH levels, net N_2O production rate increased with oxygen ($P \leq 0.0001$, pairwise comparison between 0 and 0.15 mL oxygen).

The relative rate of N_2O accumulation, rN_2O, increased with increasing oxygen for both sites at both pH levels (Fig. 4, Table 6; $P \leq 0.0001$, pairwise comparison between 0 and 0.15 mL oxygen). The increase tended to be exponential for soil from the successional field and logistic for the soil from the agricultural field regardless of pH level. There were no significant interactions among oxygen and site, oxygen and pH, nor oxygen, site, and pH (Table 6). A significant interaction between site and pH (Table 6), however, is illustrated in Fig. 4: there was a larger change in rN_2O when slurry pH was adjusted for the soil from the agricultural field than for the soil from the successional field ($P \leq 0.0001$). In addition, the two sites differed in rN_2O whether compared at pH 5.7 ($P \leq 0.0001$) or pH 6.6–6.9 ($P < 0.011$), and this difference was independent of oxygen level. At native pH, there was no significant difference in rN_2O under anaerobic conditions ($P > 0.1$), a slightly significant difference at 0.05 mL oxygen ($P < 0.05$) and barely nonsignificant differences at 0.10 mL ($P < 0.056$) and 0.15 mL oxygen ($P < 0.064$).

DISCUSSION

Enzyme activity assays

By controlling all known environmental regulators of denitrification rate and rN_2O (temperature, moisture, enzyme induction status, oxygen, pH), or by providing them in nonlimiting amounts and without diffusion limitation (carbon, nitrate), we created incubation conditions in which the only variable influencing denitrification rate (Fig. 2) and rN_2O (Fig. 4) at a given oxygen concentration and pH level was the composition of the denitrifying community.

The denitrifying community in the soil from the agricultural field had enzymes involved in N_2O production (Nar, Nir, Nor) that were more sensitive to oxygen than that from the successional field, as indicated by the greater rate of decrease in denitrification rate with increasing oxygen in the soil from the agricultural field (Fig. 2). The denitrifying community in the soil from the successional field, on the other hand, had enzymes involved in N_2O production that were more sensitive to pH than were those from the community from the agricultural field, since denitrification rate was greater in this community at native than elevated pH (Fig. 2B). This pH sensitivity is consistent with the concept that the pH optima of soil denitrifier communities (Parkin et al. 1985) and isolates (Burth and Ottow 1983) reflect the pH of their native environment. In contrast, the denitrifier community in the soil from the agricultural field seems to have a broader pH optimum, since total denitrification was the same at both low and high pH levels (Fig. 2A).

The sensitivity of Nos activity to oxygen was different in these two soil denitrifying communities as indicated by differences in the shape of their rN_2O curves, regardless of pH (Fig. 4). In addition, rN_2O was different for the two soils at their native pH (Fig. 4). This difference in rN_2O was not due to pH; if it had been a pH effect, the soil from the successional field would have had a higher rN_2O than the soil from the

TABLE 6. *F* values of three-way ANOVAs to determine effects of site, pH, and oxygen on denitrification rate, net N_2O production rate, and rN_2O (the relative rate of N_2O production) for soils from the conventionally tilled agricultural field and the never-tilled successional field at the KBS LTER site (n = 3 replicates for the agricultural field; n = 2 for the successional field).

Variable	Source of variation	df	MS	*F*	*P*
Denitrification rate	Site	1	3450.1	924.4	0.0001
	pH	1	323.8	86.8	0.0001
	Oxygen	3	200.4	53.7	0.0001
	Soil × pH	1	378.5	101.4	0.0001
	Oxygen × soil	3	20.7	5.6	0.0049
	Oxygen × pH	3	1.7	0.5	0.71
	Oxygen × soil × pH	3	1.4	0.4	0.78
Net N_2O production rate	Site	1	104.1	163.8	0.0001
	pH	1	87.9	138.3	0.0001
	Oxygen	3	3.4	5.4	0.0057
	Soil × pH	1	38.2	60.2	0.0001
	Oxygen × soil	3	13.5	21.2	0.0001
	Oxygen × pH	3	0.1	0.2	0.91
	Oxygen × soil × pH	3	3.5	5.5	0.0054
rN_2O†	Site	1	1.162	244.5	0.0001
	pH	1	0.550	115.7	0.0001
	Oxygen	3	0.235	49.4	0.0001
	Soil × pH	1	0.117	24.6	0.0001
	Oxygen × soil	3	0.007	1.4	0.26
	Oxygen × pH	3	0.002	0.4	0.73
	Oxygen × soil × pH	3	0.001	0.3	0.86

† Data were subjected to an arcsine square-root transformation since values were constrained to be between 0 and 1.

agricultural field since Nos activity is generally inhibited by low pH (e.g., Nommik 1956, Focht 1974, Blackmer and Bremner 1978, Firestone et al. 1979, 1980, Koskinen and Keeney 1982, Weier and Gilliam 1986, Christensen and Tiedje 1988, Christensen et al. 1990). That the soil from the successional field always had a significantly lower rN_2O than the soil from the agricultural field when incubated at similar pH (Fig. 4) confirms that differences in rN_2O were not due to pH. Since all environmental regulators of rN_2O are controlled or provided in nonlimiting amounts in these incubations, differences in rN_2O must be due to differences between the two denitrifier communities in Nos enzyme sensitivity to oxygen.

Since the two denitrifier communities responded differently to the same environmental variables we conclude that they must be composed of different denitrifier taxa. Indeed, the denitrifier community structure, based on denitrifying bacteria isolated from these same soil samples, was very different between soils (Cavigelli 1998). It follows, then, that in situ N_2O production is influenced by denitrifier community composition in these two soils. Under identical environmental conditions a greater proportion of total denitrification flux ($N_2O + N_2$) remained as N_2O in the soil from the agricultural field than in the soil from the successional field. These results suggest a significant functional role for soil denitrifier community composition in these two soils.

Nitrifier N_2O production

The lack of significant difference between anaerobic : microaerobic N_2O production rate ratios (Table 3) indicates that nitrifiers were not a significant source of N_2O in our incubations. Denitrification, then, is the source of all N_2O production in our assays.

Preincubation: enzyme induction assays

Although not the primary focus of this study, we found evidence that the regulation of denitrification enzyme induction may also differ between these two denitrifying communities. Nitrate and/or nitrite concentrations have been shown to be important regulators of Nos induction (Blackmer and Bremner 1978, 1979, Firestone et al. 1979, 1980). In the preincubations, nitrate concentration at the time of initial Nos activity was different for the two denitrifying communities (Table 5). This difference was not a pH effect, since the difference in nitrate concentration at the time of initial Nos activity was greater between the two soils when preincubated at similar pH than when preincubated at native pH (Table 5). These differences may also be due to differences in denitrifier community composition, but since we did not control all environmental variables important in regulating denitrification enzyme induction in the preincubations, further work is required to test this hypothesis.

The denitrifying communities in the two soils were similar in some N_2O production characteristics. Nitrous oxide reductase activity appeared after 13–55 h in all soils regardless of pH level (Fig. 1), a period consistent with the 16–33 h threshold observed by others (Firestone and Tiedje 1979, Dendooven and Anderson 1994, Dendooven et al. 1996). Also, initial denitrifier enzyme

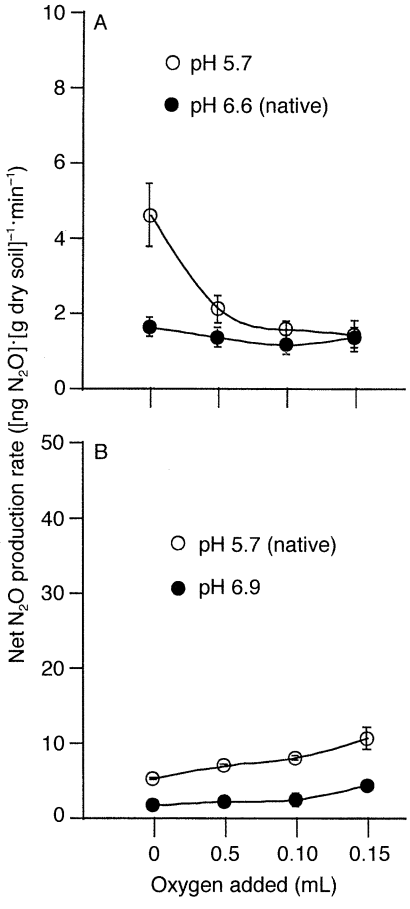

FIG. 3. Net N_2O production rates at four oxygen concentrations and two pH levels for soils from (A) the conventionally tilled agricultural field and (B) the never-tilled successional field at the KBS LTER site. Values are means, and error bars are ± 1 SE (n = 3 for the agricultural field; n = 2 for the successional field).

activity seemed to be inhibited at pH 5.7 in both communities (Fig. 1).

The presence of Nos activity in soil from the agricultural field at the beginning of induction incubations (Fig. 1) suggests that there were anaerobic microsites in this soil despite very dry conditions at the time of sampling (Table 1). Or, it may be that denitrification enzymes were maintained in the field under nondenitrifying conditions (Smith and Parsons 1985).

Significance

Our findings provide evidence that microbial diversity is not necessarily functionally redundant, as often assumed. Though redundancy may be the case within such broad functional groups as carbon mineralizers (e.g., Schimel 1995), our results support the developing view that diversity is likely to be functionally significant within more narrowly defined functional groups such as the denitrifiers (Conrad 1996, Schimel 1995). These results also have important practical implications. Efforts to model N_2O flux from soils to the atmosphere may need to incorporate some measure of denitrifier community composition in order to accurately predict N_2O fluxes. Also, it could be that management-level differences between these sites (agricultural practices such as fertilization, tillage, and/or plant community manipulation) select for denitrifying organisms with lower Nos activity than in adjacent, less intensively managed fields. If so, land use policy changes that are likely to emerge from recent climate conventions (Bolin 1998) may need to consider, for example, the effect of agricultural practices on the selection of denitrifier communities.

CONCLUSIONS

Our results support the hypothesis that denitrifier community composition influences N_2O production in soils. We collected samples from two geomorphically

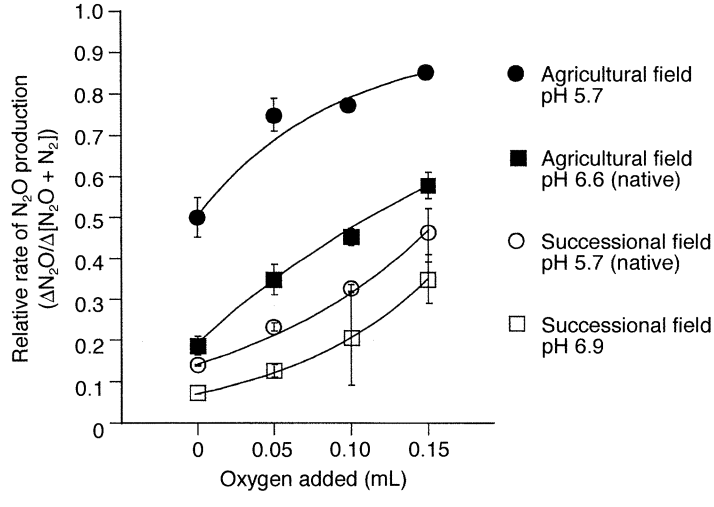

FIG. 4. Relative rate of N_2O accumulation, rN_2O, at four oxygen and two pH levels for soil from the conventionally tilled agricultural field and the never-tilled successional field at the KBS LTER site. Values are means, and error bars are ± 1 SE (n = 3 for the agricultural field; n = 2 for the successional field).

similar soils that differed in plant community composition and disturbance regime: an agricultural field and a successional field. By controlling, or providing in nonlimiting amounts, all known environmental regulators of denitrifier N_2O production and consumption, we created conditions in which the only variable contributing to differences in denitrification rate and rN_2O in the two soils was the denitrifier community. Both denitrification rate and rN_2O differed for the two soils under these controlled incubation conditions. Oxygen inhibited the activity of enzymes involved in N_2O production (Nar, Nir, Nor) to a greater extent in the denitrifying community from the agricultural field than that from the successional field. The Nar, Nir, and Nor enzymes of the denitrifying community from the successional field, on the other hand, were more sensitive to pH than were those in the denitrifying community from the agricultural field. Under identical environmental conditions, the denitrifying community in the soil from the successional field had relatively more active Nos enzymes, which reduce N_2O to N_2, than the denitrifying community in the agricultural field. Also, the shape of the rN_2O curve with increasing oxygen was different for each denitrifying community. Each of these differences suggests that the denitrifying communities in these two soils are different and that they do not respond to environmental regulators in the same manner.

We are not aware of other studies showing that native microbial community composition can regulate an important ecosystem function. Models of N_2O flux from soils may need to include the influence of microbial community composition on N_2O flux.

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