Role of denitrifier diversity in rates of nitrous oxide consumption in a terrestrial ecosystem

M.A. Cavigelli*, G.P. Robertson

W.K. Kellogg Biological Station, Center for Microbial Ecology, Department of Crop and Soil Sciences, Michigan State University, Hickory Corners, MI 49060, USA

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Abstract

The ecosystem consequences of microbial diversity are largely unknown. We tested the hypothesis that soil microbial diversity influences ecosystem function by quantifying denitrification enzyme activity among denitrifying bacteria isolated from two geomorphically similar soils with significantly different in situ nitrous oxide (N2O) emission rates. We sampled soil from two southwest Michigan sites on the same soil series that differed in plant community composition and disturbance regime — a conventionally-tilled agricultural field and a never-tilled successional field. We isolated denitrifying bacteria from these soils, characterized them based on their fatty acid profiles, and compared denitrifier community composition for the two fields. For 31 representative isolates, we measured the sensitivity of nitrous oxide reductase (Nos) — which catalyzes the reduction of N2O to N2 — to low oxygen concentrations. Of the 93 denitrifying bacteria isolated from the agricultural field and 63 from the successional field, fatty acid profiles suggested the presence of 27 denitrifying taxa with only 12 common to both soils. In each field type the four numerically dominant taxa were either rare or absent in the other field. In addition, we found substantial diversity in the sensitivity of isolate Nos enzymes to oxygen, indicating that the taxonomic diversity present among denitrifiers in these two soils is functionally significant. These results demonstrate a clear physiological basis for differences in denitrifier community function previously described (Cavigelli and Robertson, 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. Ecology 81, 229–241.) and indicate that differences in denitrifier community composition alone can potentially influence in situ N2O production. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Denitrification; Denitrifiers; Microbial community function; Microbial community structure; Nitrous oxide

1. Introduction

Microbial diversity is often assumed to be functionally redundant, which implies that ecosystem functions performed by microbes are controlled solely by abiotic factors and trophic-level interactions such as predation (e.g. Meyer, 1993; Beare et al., 1995; Heal et al., 1996, but see Schimel, 1995; Wall and Moore, 1999). In both mathematical and schematic models of biogeochemical processes microbial communities are usually treated as black boxes that transform inputs to outputs at rates defined by environmental factors empirically calibrated. For general processes such as carbon turnover these models appear to work well.

Predicting in situ biogeochemical fluxes other than carbon, however, has proven difficult. Modeling soil N2O fluxes, for example, has received considerable attention because the atmospheric concentration of this important greenhouse gas and natural catalyst of stratospheric ozone degradation is increasing, and soil is a major global source of N2O (e.g. Houghton et al., 1996). However, in situ N2O fluxes are very difficult to predict despite the incorporation of substantial environmental detail into N2O models (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., 1992a; Li et al., 1992b; Ojima et al., 1992; Smith et al., 1993; Parton et al., 1995). This difficulty may be due in part to a failure to also include functional differences in microbial community composition into models. If, for example, the microbial communities responsible for producing and consuming N2O vary in their taxonomic composition, and if different taxa produce or consume N2O at different rates under the same environmental conditions, then prediction of N2O flux will be difficult without incorporating community differences into models.
Denitrifying and nitrifying bacteria are the primary biological sources, and denitrifiers are the only biological sink, of N\textsubscript{2}O (Conrad, 1996). Aside from their role in N\textsubscript{2}O production and consumption, denitrifiers provide a good model for studying the influence of microbial diversity on ecosystem function because they are one of the best-studied and most phylogenetically diverse groups of soil microorganisms (Zumft, 1992; Zumft, 1997; Tiedje, 1994). The denitrification pathway involves four enzymes that are usually induced sequentially under anaerobic conditions (Tiedje, 1994):

\[
\text{NO}_3^- \xrightarrow{\text{Nar}} \text{NO}_2^- \xrightarrow{\text{Nir}} \text{NO} \xrightarrow{\text{Nor}} \text{N}_2 \xrightarrow{\text{Nos}} \text{N}_2\text{O}.
\]

Nitrous oxide accumulates during denitrification when the first three denitrification enzymes, nitrate reductase (Nar), nitrite reductase (Nir), and nitric oxide reductase (Nor) are more active than Nos (Betlach and Tiedje, 1981). Nitrous oxide reductase seems to be more sensitive than the other three enzymes to oxygen, low carbon-to-nitrate ratio, and low pH — the primary environmental regulators of these enzymes’ synthesis and activity (e.g. Tiedje, 1988). Thus, these environmental regulators are often considered the prime determinants of in situ N\textsubscript{2}O emission rates (e.g. Firestone and Davidson, 1989). If, however, denitrifier communities are composed of denitrifying taxa whose Nos enzymes are differentially sensitive to oxygen, carbon-to-nitrate ratio or pH, then denitrifier community composition may also influence in situ N\textsubscript{2}O fluxes.

In Cavigelli and Robertson (2000), we used a soil enzyme assay to evaluate the effect of oxygen concentration and pH on the activity of denitrification enzymes responsible for the production and consumption of N\textsubscript{2}O. We sampled soil from two sites — an agricultural field and a successional field — that have geomorphically similar soils but different plant community composition, disturbance regime and annual in situ N\textsubscript{2}O fluxes (Robertson et al., 2000). We found that oxygen inhibited the activity of enzymes involved in N\textsubscript{2}O production (Nar, Nir and Nor) to a greater extent in the denitrifying community from the agricultural field than in the denitrifying community from the successional field. On the other hand, Nar, Nir and Nor were more sensitive to pH in the denitrifying community from the successional field than in the denitrifying community from the agricultural field. Nitrous oxide reductase activity, which catalyzes N\textsubscript{2}O reduction to N\textsubscript{2}, was greater in the denitrifying community from the successional field than in the denitrifying community from the agricultural field at almost all pH values and oxygen concentrations tested. These results supported our hypothesis that soil denitrifier communities in these two fields are different and that this difference is functionally significant.

We investigated whether these functionally significant community-level differences indicate differences in denitrifier physiology at the population level. Thus, we isolated denitrifying bacteria from these same two soils (same samples) and quantified, under tightly controlled laboratory conditions, the sensitivity of their Nos enzyme activity to low oxygen concentrations. We hypothesize that different denitrifier taxa are present in the two sites and that isolate N\textsubscript{2}O consumption rates exhibit a range of sensitivity to oxygen concentration, thus demonstrating that population-level differences among denitrifying bacteria influence N\textsubscript{2}O consumption rates in, and thus N\textsubscript{2}O fluxes from, soils.

2. Materials and methods

2.1. 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). On 21 September 1994, we collected soil samples from a conventionally-tilled agricultural field and a never-tilled successional field. The agricultural field was planted to soybeans \textit{(Glycine max (L.) Merr.} as part of a corn \textit{(Zea mays L.)–soybean–wheat \textit{(Triticum aestivum L.)} rotation; the successional field contained a diverse plant community dominated by herbaceous perennials. This site, its soils and management are more fully described on the world wide web (http://kbs.msu.edu/lter/). The two fields we sampled have geomorphically similar soils that differ in pH, bulk density, total C, total N, and inorganic N pools (Cavigelli and Robertson, 2000) — factors that we judged likely to influence denitrifier community composition (Tiedje, 1988). In addition, N\textsubscript{2}O flux from the agricultural field is consistently more than double that from the successional field (mean values based on data collected weekly or biweekly from 1991 to 1999: 3.22 ± 0.45 and 1.13 ± 0.11 g N\textsubscript{2}O–N ha\textsuperscript{-1} d\textsuperscript{-1} for the agricultural and successional fields, respectively; Robertson et al., 2000).

We sampled three replicates of each field type, for a total of six samples. Each sample was a composite of 30 individual 2.5 cm dia soil cores taken to a depth of 10 cm. Samples were stored on ice until returned to the lab, where they were homogenized by sieving (4 mm) and stored at 4°C for less than 10 d before we began plate counts and isolations.

2.2. Media and growth conditions

We used a modified R2A medium (R2A\textsuperscript{−}) for both liquid and solid media and grew all isolates in an anaerobic glove box (Coy, Ann Arbor, MI) with an atmosphere of 5% CO\textsubscript{2}, 10% H\textsubscript{2} and the balance N\textsubscript{2}. The R2A\textsuperscript{−} contained (l\textsuperscript{-1}): 1.7 g NH\textsubscript{4}Cl, 4.0 g KNO\textsubscript{3}, 2.8 g sodium succinate, 1.5 g sodium pyruvate, 2.0 g peptone, 2.0 g casamino acids, 2.0 g yeast extract, 1.0 g KH\textsubscript{2}PO\textsubscript{4}, 1.6 g K\textsubscript{2}HPO\textsubscript{4}, 60 mg CaCl\textsubscript{2}\cdot2H\textsubscript{2}O, 80 mg MgCl\textsubscript{2}\cdot6H\textsubscript{2}O, 28 mg FeSO\textsubscript{4}\cdot7H\textsubscript{2}O, 20 mg Na\textsubscript{2}SO\textsubscript{4}, 40 mg MnCl\textsubscript{2}\cdot4H\textsubscript{2}O, 4 mg H\textsubscript{3}BO\textsubscript{3}, 4 mg ZnCl\textsubscript{2}, 4 mg NiCl\textsubscript{2}\cdot6H\textsubscript{2}O, 1.2 mg CuCl\textsubscript{2}\cdot2H\textsubscript{2}O, and 400 μg Na\textsubscript{3}MoO\textsubscript{4} (Fries et al., 1994). No vitamins were included. The final
solution was adjusted to pH 7.2 using NaOH. Solid medium was made by adding 15 g Bacto agar (Difco, Detroit, MI) per liter. We monitored anaerobic conditions in the glove box using a bottle of sterile medium containing resazurin dye adjusted to $-210$ Eh using cysteine-HCl (Costilow, 1981), and by periodically sampling the glove box atmosphere for oxygen using a gas-tight needle and syringe. Gas samples were measured using the gas chromatograph described under gas measurements. Bacterial transfers were made under aerobic conditions inside a laminar flow hood since denitrifying bacteria seem to grow best when initially exposed to air (R.E. Murray, personal communication).

2.3. Enumeration and isolation

We enumerated and isolated denitrifying bacteria using
techniques slightly modified from those of Gamble et al. (1977). We prepared a 10-fold dilution series by adding 10 g soil (fresh weight) and Tween 80 (2–3 drops) to 90 ml phosphate buffer (pH 7.0). We used a Waring blender to mix the soil (speed 5) for 2 min and then pipetted, while stirring, 1 ml of solution into 9 ml of phosphate buffer (pH 7.0). R2A agar plates were inoculated with 0.1 ml of solution from the 10⁻²–10⁻⁵ dilution tubes. After 3–6 d growth in the anaerobic glove box we counted all colonies on plates containing fewer than 300 colonies (10⁻⁴–10⁻⁶ dilution plates). We then transferred all colonies from one replicate of each of these plates to test tubes containing R2A broth and a small inverted tube to capture gases formed during growth. We disposed of those colonies that did not produce gas. We used characterized denitrifying isolates collected from the laboratory of J.M. Tiedje (Michigan State University) as reference strains (Table 1). These strains represent nine of the 14 denitrifying species commonly recovered from soils, and two of four genera recovered with moderate to low frequency (Tiedje, 1994). Six fluorescent pseudomonads that do not denitrify — four strains of Pseudomonas putida and two biovars (bv.) of P. fluorescens (A and G) — were also included as reference strains. We used the FAME profiles of reference strains grown under identical conditions as test organisms to help identify the unknown isolates and to determine the Euclidean distance, following cluster analysis, that defined taxa. We also analyzed two reference strains (Alcaligenes xylosoxidans subsp. xylosoxidans NCIB 11015 and P. stutzeri JM300) as positive controls with each set of unknowns. These positive controls provided a test of FAME procedure consistency. The FAME profiles of each set of positive controls grouped tightly following cluster analysis, indicating that the FAME methodology was consistent over time.

2.5. Statistical analyses of FAME data

Since the number of FAME peaks identified increases with the amount of biomass extracted, we standardized GC peak sizes to that of the isolate with the lowest biomass (as estimated by total FAMEs extracted). Individual FAME peaks that fell below the GC detection limit (set at 500 peak height units) after standardization were dropped from the data set. This precaution ensured that differences found among isolates are due to inherent differences in fatty acid content, and not to differences in the amount of biomass harvested and extracted.

FAME profiles of known and unknown isolates were then subjected to hierarchical cluster analysis using NTSYS (Applied Biostatistics, Inc., Setauket, NY). We ran a preliminary cluster analysis using all isolate replicates as separate samples to assess replicate similarity. In the few cases where replicates did not cluster together, we regrew isolates and extracted and analyzed their FAME profiles to identify outliers in initial runs. Average FAME profiles were then calculated for each isolate and these data were subjected to cluster analysis. We constructed dendrograms using the Euclidean distance metric and unweighted pair-groups method using arithmetic averages (UPGMA) linkage. Cluster analyses of FAME data had shown that a number of combinations of distance metrics and linkage methods with biases toward the formation of different types of clusters (Everitt, 1980; Milligan and Cooper, 1987) gave essentially identical dendrograms (Cavigelli et al., 1995).

2.6. Nitrous oxide reductase activity assay

We quantified Nos sensitivity to oxygen for select isolates by measuring N₂O consumption rates under anaerobic conditions and at three separate, very low oxygen concentrations. We selected one or two isolates from each taxa identified by cluster analysis and all isolates from one taxa
containing seven unknown isolates (taxon 30). We first confirmed Nos activity in the selected isolates by testing for N$_2$O consumption. For each isolate we transferred three drops of cell culture at late log phase (approximately 40% transmittance at 560 nm when grown aerobically) to six 38 ml vials containing 3 ml of degassed R2A$^-$ and an anaerobic headspace. We added acetylene (3.6 ml) aseptically to three of these vials and shook all vials at 100 rpm on an orbital shaker (Model 3520, Lab-Line Instruments, Melrose Park, IL). After N$_2$O production had appeared in vials containing acetylene (evidence that the enzymes leading to the production of N$_2$O are active), we injected N$_2$O (5 ml of 1.01% standard, which resulted in 70 nmol N$_2$O l$^{-1}$ in solution) into the vials containing no acetylene and monitored headspace N$_2$O. Nos activity was confirmed by N$_2$O consumption.

We used a second set of incubations to measure the effect of oxygen on isolate N$_2$O consumption rates. We transferred 2 ml aliquots of cell cultures actively consuming N$_2$O to four autoclaved 38 ml serum vials containing an anaerobic atmosphere. We equilibrated headspace pressure with atmospheric pressure and then added 0, 0.25, 0.50, or 0.75 ml of laboratory atmosphere (filtered through a 0.22 µm filter) and 5 ml of a 1.01% N$_2$O standard to each vial. Vials were shaken at 400 rpm, a speed we had determined to be sufficient for eliminating oxygen diffusion effects on denitrification rate when the same vials were used for soil slurry incubations (Cavigelli and Robertson, 2000). We then measured N$_2$O and oxygen every 7–10 min for a period not longer than 120 min to determine isolate N$_2$O consumption rates. We replicated this procedure three to five times for each test isolate.

We tested for differences in N$_2$O consumption rates at each oxygen concentration for each isolate using analysis of covariance (oxygen as main effect and time as covariate) with the GLM procedure in SAS, version 6.09 (SAS Institute, 1996) and we compared consumption rates at each oxygen concentration using the SAS ‘estimate’ statement. We quantified the effect of oxygen on Nos activity for each test isolate by plotting the natural log of N$_2$O consumption rate against oxygen concentration. These slopes, which are equivalent to the exponential decay constant $k$ for untransformed data, represent the sensitivity of Nos activity to oxygen. We subjected these data to analysis of covariance (isolate as main effect and oxygen as covariate) using the GLM procedure in SAS and made a priori comparisons using the ‘estimate’ statement (SAS Institute, 1996).

### 2.7. Gas measurements

We measured both N$_2$O and O$_2$ using an HP 5890II gas chromatograph (GC; Hewlett Packard, Rolling Meadows, IL) equipped with dual Porapak 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$^{-1}$). Gas concentrations in the headspace were corrected for dissolution in solution using Bunsen coefficients (Tiedje, 1994).

Mean calculated oxygen concentrations in solution, after adding 0.25, 0.50, or 0.75 ml atmosphere, were 53 ± 7, 100 ± 13, and 141 ± 20 nmol l$^{-1}$, respectively. These oxygen concentrations are well below the postulated 10 µmol oxygen l$^{-1}$ 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 simplicity we report oxygen concentrations as ml of atmosphere added per vial.

### Table 2

Mean number of anaerobic heterotrophic bacteria viable on R2A$^-$ agar, gas-producers in R2A$^-$ broth, and confirmed denitrifiers for soils from the conventionally-tilled agricultural field and the never-tilled successional field at the W.K. Kellogg Biological Station Long-Term Ecological Research site (Note: Values are means ±1 SE (n = 3), total number of bacteria counted or isolated from all plates with less than 300 colonies (sum of counts from 10$^{-1}$, 10$^{-2}$, and 10$^{-3}$ dilution plates) from three field replicates, and percentages of these totals; *P > 0.10)

<table>
<thead>
<tr>
<th></th>
<th>Agricultural field</th>
<th>Successional field</th>
<th>t test</th>
</tr>
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<tbody>
<tr>
<td><strong>Means</strong></td>
<td></td>
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</tr>
<tr>
<td>Anaerobic heterotrophs ($\times 10^5$)</td>
<td>1.2 ± 0.3</td>
<td>0.9 ± 0.6</td>
<td>0.13 **</td>
</tr>
<tr>
<td>Gas producers ($\times 10^5$)</td>
<td>3.8 ± 0.7</td>
<td>1.8 ± 2.0</td>
<td>1.24 **</td>
</tr>
<tr>
<td>Confirmed denitrifiers ($\times 10^5$)</td>
<td>2.2 ± 0.3</td>
<td>1.2 ± 1.8</td>
<td>0.74 **</td>
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<tr>
<td><strong>Total number</strong></td>
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<tr>
<td>Anaerobic heterotrophic colonies</td>
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<td>400</td>
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<td>Gas producers</td>
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<td></td>
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<tr>
<td>Denitrifiers</td>
<td>93</td>
<td>63</td>
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</tr>
<tr>
<td><strong>Percentage</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Heterotrophic colonies that produced gas</td>
<td>38</td>
<td>21</td>
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<tr>
<td>Heterotrophic colonies that denitrified</td>
<td>23</td>
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<tr>
<td>Gas-producers that denitrified</td>
<td>59</td>
<td>75</td>
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</table>
3. Results

3.1. Enumeration and isolation

We isolated a total of 156 denitrifiers: 93 from the agricultural field and 63 from the successional field. From the agricultural field, we isolated 67, 21 and 5 denitrifiers from the $10^{-2}$, $10^{-3}$ and $10^{-6}$ dilution plates, respectively. From the successional field, we isolated 58, 5 and 0 denitrifiers from the $10^{-2}$, $10^{-3}$ and $10^{-6}$ dilution plates, respectively. There were no differences in the number of viable heterotrophic colonies growing anaerobically on R2A plates inoculated with the two soils, in gas producers growing in R2A broth, nor in confirmed denitrifiers (Table 2). When results from all dilution series were combined, a greater percentage of the heterotrophic colonies on plates from the agricultural field than from the successional field were confirmed as denitrifiers.

3.2. Denitrifier community composition

Results of the cluster analysis are presented as a dendrogram in Fig. 1 and taxa membership is provided in Table 3. Three broad groups are defined at a Euclidean distance of 0.53 units (A, B and C in Fig. 1). The only reference strains in Group A are from the alpha and beta subdivisions of the proteobacteria (Table 3). Group B, the largest group, contains the majority of field isolates but only four reference strains, two of which are Bacillus species, the only Gram-positive reference strains. The other two reference strains within Group B are of uncertain classification (Tables 1 and 3). Group C contains only 26 field isolates, but many reference strains; all but three of these reference strains belong to the gamma subdivision of the proteobacteria (Tables 1 and 3). One third (34%) of isolates from the agricultural field, but only 14% from the successional field, clustered in Group A. Half (54%) of the isolates from the agricultural field and almost two-thirds (63%) of the isolates from the successional field clustered into Group B. Group C contained 12% of the isolates from the agricultural field and 23% of the isolates from the successional field.

We defined the threshold for taxon delineation at a Euclidean distance of 0.13 since (1) all replicates of each control organism clustered at a distance <0.13, (2) all reference strains (except P. putida and some P.fluorescens biovars) clustered within the same group at this distance, and (3) most reference isolates clustered only with reference strains of the same or very similar species at this distance (Fig. 1, Table 3). In addition, of the 14 taxa defined at this distance that contained reference strains, only four contained more than one “species” (taxa 1, 26, 29, 30).

Thirty-three taxa were identified at a Euclidean distance of 0.13 units. The 156 denitrifying isolates from the two fields represented 27 taxa (six of the 33 taxa contained only reference strains; Table 3). Only eight of the 27 clusters containing field isolates also contained reference strains (Table 3). The 93 isolates from the agricultural field represented 22 different taxa; the 63 isolates from the successional field represented 17 different taxa. Twelve taxa were common to both soils, but very few of these represented similar proportions of their respective communities. The four numerically dominant taxa in the agricultural field (taxa 1, 7, 14 and 20) were not common, if even present, in the successional field. For example, isolates clustering with the Bacillus reference strains (taxa 7) were very common in the agricultural field — they were isolated from the $10^{-2}$, $10^{-3}$ and $10^{-6}$ dilution plates — but no isolates from the successional field clustered with Bacillus reference strains. Likewise, the four numerical dominants in the successional field (taxa 2, 5, 6 and 23) were isolated infrequently from the agricultural field. Taxa of intermediate abundance also differed in the frequency of isolation between soils.
Table 3

Denitrifying bacteria isolated from the conventionally-tilled agricultural field and the never-tilled successional field at the W.K. Kellogg Biological Station Long-Term Ecological Research site, and reference strains, grouped into taxa defined by cluster analysis (Fig. 1). (Note: Numbers following reference strains are ATCC accession numbers or other strain identification numbers as noted in Table 1. Isolates in bold were used as test isolates for Nos assays. Isolates in italics lost the ability to reduce nitrous oxide. Isolates underlined did not grow after storage in glycerol.)

<table>
<thead>
<tr>
<th>Groups defined at Euclidean distance</th>
<th>Soil isolates</th>
<th>Reference strains</th>
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<td>Agricultural field</td>
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</tr>
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<td>1</td>
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</table>
None of our isolates clustered with *P. putida* or *P. fluorescens* bv. *G*, five of six non-denitrifier reference strains.

We calculated denitrifier taxonomic diversity based on the 27 taxa that contained field isolates and using the Shannon–Weaver diversity index (Shannon and Weaver, 1949). The diversity index was 2.39 for the soil from the agricultural field and 2.34 for the soil from the successional field. We dissected this diversity measurement by calculating taxonomic richness (Margalef, 1958) and evenness (Pielou, 1966). The denitrifier community from the agricultural field had slightly higher species richness (4.63 vs. 3.86) and slightly lower evenness (0.77 vs. 0.83) than that from the successional field.

### 3.3. Nitrous oxide reductase activity

We used 31 isolates, representing 20 taxa, as test organisms (Table 3). For taxa containing more than one isolate we chose test isolates randomly. Under our test conditions, all denitrifiers produced large amounts of N\textsubscript{2}O in vials with 10% acetylene in the headspace and negligible amounts of N\textsubscript{2}O in vials containing no acetylene (Fig. 2). Since acetylene blocks Nos activity, this combination of results suggests that Nos is active in these cultures. We confirmed Nos activity by observing rapid N\textsubscript{2}O consumption following its addition to separate vials not containing acetylene (Fig. 2A). The shape of N\textsubscript{2}O production curves in the presence of acetylene was similar for all isolates (Fig. 2A) but four (Fig. 2B–D). These four isolates belonged to four different taxa that clustered near each other within Group B (taxa 6, 7, 8 and 10; Table 3).

We were not able to measure Nos activity of seven taxa that contained field isolates because all isolates within these groups were either not viable after glycerol storage or had lost the ability to reduce N\textsubscript{2}O (taxa 2, 4, 9, 16, 17, 18 and 21; Table 3), a common phenomenon when denitrifiers are repeatedly cultured (e.g. Gamble et al., 1977; Tiedje, 1994).

When cultures shown to have active Nos were transferred to vials containing fresh media, rapid N\textsubscript{2}O consumption (i.e. Nos activity) under anaerobic conditions was inhibited by oxygen in all test isolates (Table 4). For 25 of 31 isolates, the effect of oxygen was apparent with the addition of only 0.25 ml of air (53 ± 7 nmol oxygen l\(^{-1}\)). For four isolates [77 (taxon 7); 89 (taxon 10); 119 (taxon 5); and 120 (taxon 12)], the influence of oxygen became evident only at the higher oxygen concentrations (Table 4). Isolates 46 and 184 showed no significant differences in N\textsubscript{2}O consumption rates with increasing oxygen according to ANCOVA, but a priori comparisons of N\textsubscript{2}O consumption rates at each oxygen concentration showed significantly lower N\textsubscript{2}O consumption at 0.75 ml atmosphere added compared to the rate under anaerobic conditions (Table 4).

We measured exponential decay constants at two different times after N\textsubscript{2}O consumption began for seven of the isolates (33, 47, 65, 69, 85, 89 and 119) to test whether variables that were likely to covary with time of sampling (e.g. population size, nitrate concentration) affected the influence of oxygen on N\textsubscript{2}O consumption rate. In all cases, while rates of N\textsubscript{2}O consumption at each oxygen concentration varied with time of sampling (data not shown), \(k\) values were not different between sampling times (Table 5), i.e. the effect of oxygen on relative rates did not differ with sampling time. We used only the data from the first sampling time in analyses described below.

We also compared Nos sensitivity to oxygen for isolates belonging to the same taxon to test whether our taxonomic divisions were physiologically meaningful. In all cases, according to \(t\) tests conducted using the SAS ‘estimate’ statement following an analysis of covariance, there were no differences in mean Nos sensitivity to oxygen between any two isolates belonging to the same taxon (Table 6). Results from taxon 30 are presented separately (Table 7) to account for the large number of pairwise comparisons necessary to compare means among the seven test isolates.

ANCOVA showed that there were significant differences among the 31 test isolates’ \(k\) values (Table 8), which ranged from 0.87 to 3.37 (Fig. 3). As a rough estimate of the deni-
trifier community $k$ value for each soil, we multiplied the number of isolates in each group by the mean $k$ value for that group and then summed values for all groups within each soil. The calculated $k$ values for the denitrifying communities from the two soils were not different: the mean $k$ value for the denitrifying community from the agricultural field was 1.95 (95% confidence interval = 0.12) and that for the successional field was 1.86 (95% confidence interval = 0.20).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Taxa number & Isolate number & Field of origin$^a$ & Nitrous oxide consumption rate$^b$ (ng N$_2$O min$^{-1}$) & $F$ statistic$^c$ \\
& & & ml air added & \\
& & & 0 & 0.25 & 0.50 & 0.75 & \\
\hline
1 & 65 & A & 0.79a & 0.31b & 0.20c & 0.18c & 78.9*** \\
1 & 175 & S & 0.64a & 0.18b & 0.14c & 0.11d & 68.5*** \\
3 & 101 & A & 0.88a & 0.30b & 0.14c & 0.09c & 134*** \\
5 & 119 & S & 0.57a & 0.51ab & 0.40bc & 0.29c & 10.8*** \\
6 & 85 & A & 0.20a & 0.11b & 0.04c & 0.04c & 23.5*** \\
7 & 77 & A & 0.84a & 0.87a & 0.76a & 0.37b & 12.5*** \\
8 & 46 & A & 0.29a & 0.18ab & 0.19ab & 0.15b & 3.5*** \\
10 & 89 & A & 0.25a & 0.22ab & 0.11b & 0.11b & 3.5* \\
11 & 184 & S & 0.23a & 0.27a & 0.13b & 0.07b & 2.49*** \\
12 & 120 & S & 0.39a & 0.29ab & 0.18bc & 0.09c & 8.09*** \\
13 & 134 & S & 0.23a & 0.13b & ND & 0.11b & 5.33* \\
14 & 47 & A & 0.50a & 0.28b & 0.14c & 0.11c & 11.3*** \\
14 & 168 & S & 0.58a & 0.31b & 0.17c & 0.13c & 65.0*** \\
15 & 140 & S & 0.70a & 0.28b & 0.19bc & 0.06c & 18.5*** \\
20 & 69 & A & 0.16a & 0.10b & 0.07b & 0.05b & 7.24** \\
22 & 106 & A & 0.50a & 0.20b & 0.11b & 0.12b & 14.2*** \\
23 & 33 & A & 1.37a & 0.88b & 0.59c & 0.39d & 78.0*** \\
25 & 161 & S & 1.22a & 0.76b & 0.44c & 0.42c & 6.66** \\
24 & 120 & S & 0.39a & 0.29ab & 0.18bc & 0.09c & 8.09*** \\
25 & 64 & A & 0.96a & 0.84b & 0.60c & 0.39d & 49.0*** \\
24 & 165 & A & 0.85a & 0.66b & 0.44c & 0.32d & 47.6*** \\
25 & 165 & A & 0.96a & 0.84b & 0.60c & 0.39d & 49.0*** \\
25 & 165 & A & 0.85a & 0.66b & 0.44c & 0.32d & 47.6*** \\
30 & 70 & A & 0.28a & 0.16b & 0.07c & 0.06c & 55.8*** \\
30 & 78 & A & 0.36a & 0.15b & 0.12b & 0.05c & 23.4*** \\
30 & 90 & A & 0.49a & 0.19b & 0.14b & ND & 27.1*** \\
30 & 98 & A & 0.33a & 0.11b & 0.06b & 0.05b & 5.30* \\
30 & 99 & A & 0.57a & 0.27b & 0.28b & 0.14c & 40.8*** \\
30 & 191 & S & 0.49a & 0.31b & 0.23b & 0.09c & 24.4*** \\
30 & 194 & S & 0.37a & 0.23b & 0.12c & 0.06d & 36.0*** \\
31 & 114 & S & 1.30a & 0.99b & 0.72c & 0.52d & 99.7*** \\
33 & 100 & A & 0.73a & 0.28b & 0.24b & 0.25b & 148*** \\
\hline
\end{tabular}
\caption{Exponential decay constants, $k$, used to measure the sensitivity of Nos to oxygen for seven isolates measured at two different times after N$_2$O consumption began. These experiments test whether variables that are likely to covary with time of sampling affect the influence of oxygen on N$_2$O consumption rate. Results show that sampling time did not influence $k$ values (Note: $t$ tests and their $P$ values were calculated using the estimate statement in SAS $(n \geq 2)$).}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Isolate number & Exponential decay constant, $k$ & $t$ test & $P$ \\
& First sample & Second sample & \\
\hline
33 & $-1.77 \pm 0.25$ & $-1.69 \pm 0.36$ & 0.20 & 0.89 \\
47 & $-2.69 \pm 0.31$ & $-2.03 \pm 0.29$ & 0.12 & 0.11 \\
65 & $-1.98 \pm 0.25$ & $-1.87 \pm 0.35$ & 0.28 & 0.78 \\
69 & $-1.62 \pm 0.25$ & $-1.69 \pm 0.23$ & 0.21 & 0.83 \\
85 & $-2.77 \pm 0.25$ & $-2.68 \pm 0.44$ & 0.19 & 0.85 \\
89 & $-1.50 \pm 0.24$ & $-1.47 \pm 0.29$ & 0.11 & 0.91 \\
119 & $-0.97 \pm 0.31$ & $-1.03 \pm 0.31$ & 0.14 & 0.89 \\
\hline
\end{tabular}
\caption{Exponential decay constants, $k$, used to measure the sensitivity of Nos to oxygen for seven isolates measured at two different times after N$_2$O consumption began. These experiments test whether variables that are likely to covary with time of sampling affect the influence of oxygen on N$_2$O consumption rate. Results show that sampling time did not influence $k$ values (Note: $t$ tests and their $P$ values were calculated using the estimate statement in SAS $(n \geq 2)$).}
\end{table}

4. Discussion

Denitrifier community composition was markedly different between soils at two levels of taxonomic resolution. At a coarse scale, a greater proportion of isolates from the agricultural field than the successional field clustered with refer-
ence strains belonging to the alpha and beta subdivisions of the proteobacteria. Conversely, a greater proportion of isolates from the successional field than from the agricultural field clustered with reference strains belonging to the gamma subdivision of the proteobacteria (Table 3). At a finer scale of resolution, the distribution of isolates among 27 taxa also differed significantly between the two soils. Only 12 of these taxa were isolated from both soils. The four most abundant taxa in each soil were not commonly isolated from the other soil and taxa of intermediate abundance also differed in frequency of isolation between soils. In addition, two components of diversity, taxonomic richness and evenness, differed slightly between sites.

Differences in denitrifier community composition between fields were likely due, at least in part, to those soil characteristics that differed between fields: total C, total N, inorganic N pools, pH and bulk density. In general, soil organic C seems to be the most important factor selecting for soil denitrifier populations (Tiedje, 1988). Soil pH (Parkin et al., 1985) and moisture — largely through its effect on oxygen (Tiedje et al., 1982) — also influence denitrifier community composition. Soil nitrate, on the other hand, may have a potential effect on denitrifier community composition in agricultural soils only (Tiedje, 1988). Although soil moisture did not differ between fields when samples were taken (Cavigelli and Robertson, 2000), soil moisture dynamics at our two sites certainly differ in response to differences in plant communities, disturbance regimes, soil bulk densities, soil organic C and other factors. More direct effects of plant community structure on denitrifier community structure, such as effects of root exudates and residues on relative competitive abilities of denitrifier taxa, may also need to be considered. Our study was not designed to determine the factors that regulate denitrifier community composition, but our results suggest that we need to better understand the factors that select for denitrifier populations, especially if denitrifier community structure influences denitrifier community function.

While community composition differed between sites, population diversity did not: the Shannon–Weaver index was similar for the two communities. This similarity indicates an important distinction between population diversity and community composition: the same level of diversity may represent significantly different community composition. This distinction may have important implications for identifying factors influencing community function since community function may have more to do with community composition than diversity per se (e.g. Grime, 1997; Hooper and Vitousek, 1997; Huston, 1997, but see Tilman et al., 1997).

Table 6
Exponential decay constants, k, used to measure the sensitivity of Nos to oxygen for isolates belonging to taxa with two test isolates. Results show that there were no differences in the k values between isolates belonging to the same taxa (Note: t tests and their P values were calculated using the estimate statement in SAS (n ≥ 3))

<table>
<thead>
<tr>
<th>Taxon number</th>
<th>Isolate number</th>
<th>Exponential decay constant, k</th>
<th>t test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65</td>
<td>$-1.98 \pm 0.25$</td>
<td>0.13</td>
<td>0.89</td>
</tr>
<tr>
<td>13</td>
<td>47</td>
<td>$-2.69 \pm 0.31$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>168</td>
<td>$-2.67 \pm 0.24$</td>
<td>0.05</td>
<td>0.96</td>
</tr>
<tr>
<td>23</td>
<td>33</td>
<td>$-1.78 \pm 0.25$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>182</td>
<td>$-1.69 \pm 0.26$</td>
<td>0.23</td>
<td>0.82</td>
</tr>
<tr>
<td>24</td>
<td>161</td>
<td>$-1.84 \pm 0.27$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>P. flu. F ATCC 17513</td>
<td>$-2.03 \pm 0.23$</td>
<td>0.50</td>
<td>0.61</td>
</tr>
<tr>
<td>25</td>
<td>64</td>
<td>$-1.23 \pm 0.25$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>165</td>
<td>$-1.32 \pm 0.25$</td>
<td>0.26</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 7
Exponential decay constants, k, for the seven isolates belonging to taxon 30 and t test results to compare k values among the seven isolates. Results show that there were no differences among k values for the seven isolates belonging to taxon 30 (Note: Values in parentheses are P values associated with each t test; t tests and their P values were calculated using the estimate statement in SAS (n ≥ 3))

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>Exponential decay constant, k</th>
<th>Isolate number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>70</td>
<td>78</td>
</tr>
<tr>
<td>70</td>
<td>$-2.24 \pm 0.31$</td>
<td></td>
</tr>
<tr>
<td>78</td>
<td>$-2.36 \pm 0.25$</td>
<td>0.30 (.77)</td>
</tr>
<tr>
<td>90</td>
<td>$-2.56 \pm 0.49$</td>
<td>0.46 (.65)</td>
</tr>
<tr>
<td>98</td>
<td>$-2.16 \pm 0.26$</td>
<td>$-0.18$ (.86)</td>
</tr>
<tr>
<td>99</td>
<td>$-1.75 \pm 0.35$</td>
<td>$-1.03$ (.31)</td>
</tr>
<tr>
<td>191</td>
<td>$-2.11 \pm 0.31$</td>
<td>0.28 (.78)</td>
</tr>
<tr>
<td>194</td>
<td>$-2.42 \pm 0.25$</td>
<td>$-0.44$ (.66)</td>
</tr>
</tbody>
</table>
Our denitrifier isolates exhibited a wide range in the sensitivity of Nos enzyme activity to oxygen. Our measure of Nos sensitivity to oxygen, the exponential decay constant for N₂O consumption with increasing oxygen concentration (k), varied significantly from 0.87 to 3.37 (Fig. 3). Others have shown that regulation of denitrification enzymes by environmental variables seems to vary considerably among denitrifying isolates. For example, in a series of studies comparing regulation of N₂O production among select denitrifiers, N₂O production and the N₂O-to-N₂ ratio were influenced more by bacterial species than by oxygen partial pressure (Abou Seada and Ottow, 1985; Anderson and Levine, 1986; Munch, 1991), soil texture (and its effect on oxygen diffusion; Munch and Ottow, 1986), nitrate concentration (Munch, 1989), or pH (Burth and Ottow, 1983). Also, three denitrifiers grown under the same conditions differed in growth rate, cell yield, the ability to utilize exogenous denitrification intermediates, and in the relative rates of various steps in the denitrification pathway (Carlson and Ingraham, 1983). Nitrite reductase regulation by oxygen, nitrate and nitrite has also been shown to differ, sometimes considerably, among seven denitrifier strains commonly found in soils (Ka et al., 1997).

While these examples indicate that there exists physiological diversity among select denitrifying isolates, they do not indicate an ecologically relevant pattern to diversity since each of these studies compared denitrification enzyme activity among small numbers of denitrifying isolates, usually chosen from culture collections. The few studies that include some measure of both native denitrifier community composition and denitrification enzyme activity among small numbers of denitrifying isolates, usually chosen from culture collections. The few studies that include some measure of both native denitrifier community composition and denitrification enzyme activity did not compare the isolates’ denitrification enzyme sensitivities to environmental regulators (Gamble et al., 1977; Christensen and Bonde, 1985; Weier and MacRae, 1992). If physiological differences among isolates are to be related to community-level N₂O production potentials, then such differences must be demonstrated among organisms belonging to the same native communities, as in this study.

Complicating the interpretation of denitrifier enzyme studies is the possibility that differences in enzyme sensitivities among isolates are attributable not to differences in denitrifier physiology but to differences in isolates’ enzyme status at the beginning of experiments (Burth and Ottow, 1983; Abou Seada and Ottow, 1985; Munch and Ottow, 1986; Anderson and Levine, 1986; Munch, 1989; Munch, 1991). Denitrification enzyme status has been shown to be an important regulator of N₂O production potential (e.g. Dendooven and Anderson, 1994). We avoided this complication by ensuring that all denitrification enzymes were fully induced prior to measuring Nos sensitivity to oxygen. We are thus confident that the diversity in Nos sensitivity to oxygen among denitrifier isolates from our sites is due to physiological diversity and not an artifact of our experimental approach.

Since Nos activity is also regulated by nitrate, nitrite and nitric oxide (e.g. Hochstein and Tomlinson, 1988), we tested the likelihood that there were interactions between the influence of oxygen and nitrogen substrates in our incubations. We measured the sensitivity of Nos activity to oxygen at two different times for a subset of isolates. An interaction between oxygen concentration and nitrogen substrate.
isolates had unusual N2O consumption curves, suggesting curves in the presence of acetylene (Fig. 2B–D). Two may be other forms of physiological diversity that influence enzymes (Nar, Nir and Nor) to environmental factors, there and pH) and to differential sensitivity of other denitrification other environmental factors (such as the C-to-nitrate ratio also depend on differential sensitivity of Nos enzymes to conditions.

Despite differences in oxygen sensitivity of Nos activity among isolates, we did not find any differences in Nos activity regulation at the community level based on calculating a weighted average of $k$. There may, however, be some limitations to this means of estimating community function. For example: (1) isolates may not represent all functionally significant denitrifying species in situ; (2) the taxa containing field isolates that were not viable or had lost Nos activity during culturing were not included in community-level measurements; and (3) interactions among organisms — even if all numerically and functionally dominant species are isolated — are not considered when studying organisms in isolation. Such interactions include those among denitrifiers and those between denitrifiers and non-denitrifier members of the soil community, including plant roots. Some, or all, of these factors may have been important in this study, especially in light of the differences we found in Nos sensitivity to oxygen between these same two communities using a soil enzyme assay, a method that does not rely on culturing organisms (Cavigelli and Robertson, 2000). In that study, we found that, under identical environmental conditions, the Nos enzymes of the denitrifying community in the soil from the successional field were more active than those of the denitrifying community in the soil from the agricultural field. The population-level differences we describe here, nonetheless, are in agreement with the community-level differences described in Cavigelli and Robertson (2000) since the differences shown there are dependent on there being physiological diversity among the denitrifier populations that comprise those communities.

In addition to the physiological differences in Nos sensitivity to oxygen among isolates, we found evidence for physiological differences among these isolates that is probably not captured using the exponential decay constants. Four isolates exhibited uncharacteristic N2O production curves in the presence of acetylene (Fig. 2B–D). Two isolates had unusual N2O consumption curves, suggesting that Nos activity relative to Nar activity was delayed or inhibited in these isolates. Since in situ N2O fluxes may also depend on differential sensitivity of Nos enzymes to other environmental factors (such as the C-to-nitrate ratio and pH) and to differential sensitivity of other denitrification enzymes (Nar, Nir and Nor) to environmental factors, there may be other forms of physiological diversity that influence in situ N2O fluxes.

Although it is becoming increasingly clear that isolates may not represent numerically dominant bacteria in environmental samples (e.g. Torsvik et al., 1990; Richaume et al., 1993; Tsuji et al., 1995), there is no reason to doubt that the physiological diversity we found in this study would not also be found within any other subsample of these two denitrifier communities. In fact, our isolate selection methods would, if anything, tend to select for organisms with similar characteristics (e.g. Gorlach et al., 1994), making our estimate of in situ physiological diversity conservative.

We have thus shown that there is taxonomic diversity among denitrifying bacteria isolated from our two soils and that this taxonomic diversity is functionally significant. Different denitrifying taxa consumed N2O at different rates when grown under identical conditions, supporting our hypothesis that denitrifier community composition has the potential to influence in situ N2O fluxes. This level of regulatory control may, therefore, need to be incorporated into N2O flux models.

5. Conclusion

Our study indicates that microbial taxonomic diversity may have functional significance. Denitrifier community composition was different in two geomorphically similar soils sampled from an agricultural and a successional field. Since culturing bacterial isolates under one set of conditions tends to select for organisms with similar characteristics, the physiological diversity shown here probably represents a conservative estimate of that which exists in situ. We found significant diversity in the sensitivity of these isolates’ Nos enzymes to oxygen. When we calculated a mean, weighted measure of Nos sensitivity to oxygen for each denitrifier community, however, there was no difference between the two fields. The discrepancy between community-level measurements extrapolated from individual isolates versus those measured for the entire community in a soil enzyme assay (Cavigelli and Robertson, 2000) emphasizes that differences in isolate physiology must be placed in the context of the entire community in order to assess their importance to ecosystem-level function. A complete understanding of the effect of denitrifier community composition on N2O fluxes will depend on further study of the effect of environmental regulators, including those other than oxygen, on the activity of Nos and the other enzymes in the denitrification pathway. Our results, nonetheless, show that considering the effect of even a single regulator — oxygen — on a single denitrification enzyme — Nos — demonstrates that denitrifier taxonomic diversity has clear potential to influence an important ecosystem function in these soils.

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