AN ABSTRACT OF THE DISSERTATION OF

Jeremy J. Rich for the degree of Doctor of Philosophy in Soil Science presented on June 27, 2003.

Title: Community Composition and Activities of Denitrifying Bacteria in Soils

Abstract approved:

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David D. Myrold

Few studies have directly compared denitrifying community composition and activities in soils by coupling molecular-genetic techniques and traditional measures of denitrification. I investigated communities of denitrifying bacteria from adjacent meadow and forest soils in the Cascade Mountains, Oregon. A key gene in the denitrification pathway, $N_2O$ reductase (*nosZ*), served as a marker for denitrifying bacteria. Denitrifying enzyme activity (DEA) was an order of magnitude higher in meadow than in forest soils. Denitrifying community composition differed between vegetation types based on multivariate analyses of *nosZ* T-RFLPs. Screening 225 *nosZ* clones yielded 47 unique denitrifier genotypes. The majority of *nosZ* fragments sequenced from meadow or forest soils were most similar to *nosZ* from *Rhizobiaceae* in *α-Proteobacteria*.

In a second study, I examined denitrifying bacteria from three adjacent habitats in Oregon: agricultural soil that received N-fertilizer inputs, naturally vegetated riparian soil, and creek sediment. The ratio of $N_2O$ produced as a result of

denitrification in the presence of glucose (10 mM) and $NO_3^-$ (5 mM) was higher for riparian soil (0.64 ± 0.02; mean ± standard error, n = 12) compared to agricultural soil (0.19 ± 0.02) or creek sediment (0.32 ± 0.03). Mean DEA was similar among habitats, but mean $N_2O$-reductase activity was about 70% higher in agricultural soil than in riparian soil or creek sediment. Denitrifying community composition differed among habitats based on *nosZ* T-RFLPs. The creek sediment community was unique. Communities in agricultural and riparian soil were more closely related but distinct. Sequences of *nosZ* obtained from riparian soil were closely related to *nosZ* from *Rhizobiaceae* or distantly related to *nosZ* from *Ralstonia* or *Azospirillum* spp.

Both studies indicated that denitrifying community composition differed among adjacent habitats. The same dominant denitrifying genotypes were found in all soils, but relative abundances of these genotypes differed among habitats. Less dominant genotypes were consistently only found in certain habitats. Denitrifying community composition and activities were correlated, but relationships between composition and activities differed between studies. Previously overlooked denitrifiers related to *Rhizobiaceae* may dominate in these soils.

# Community Composition and Activities of Denitrifying Bacteria in Soils

by

Jeremy J. Rich

A DISSERTATION

submitted to

Oregon State University

In partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented June 27, 2003

Commencement June 2004

Doctor of Philosophy dissertation of Jeremy J. Rich presented on June 27, 2003.

APPROVED:

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Major Professor, representing Soil Science

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Head of the Department of Crop and Soil Science

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Jeremy J. Rich, Author

# ACKNOWLEDGEMENTS

I acknowledge my major advisor David Myrold for his support, availability, encouragement of independent thinking and pursuing of new ideas, ability to listen, his suggestion for studying the agricultural-riparian system, and quick turn-around time on manuscripts; also his lead-authorship on the National Science Foundation-Microbial Observatory (NSF-MO) grant, which funded the bulk of my research, in addition to the grant from the Oregon State University (OSU)-Agricultural Research Foundation (ARF). Thank you to Peter Bottomley and Kermit Cromack, Jr., co-PIs on the NSF-MO, for their input, patience, and collaborative spirit. Thank you to all the PIs on the NSF-MO for suggesting to examine adjacent meadow and forest soils in the H. J Andrews. Thank you to Peter Bottomley for helpful discussions and suggestions about specific aspects of my research. Thank you to my other committee members: Stephen Giovannoni for giving career advice and influencing my Ph.D. instruction through two courses, and Dan Arp for giving research advice and loaning equipment.

Thank you to student collaborators on the NSF-MO: Anne Mintie, Rachel Heichen, Kirk Waterstripe, and Kim Lamothe for sharing data, field work, and sample processing. I am grateful to Nancy Posavatz for her aid in PCR techniques, organization of NSF-MO work, PCR data in the OSU-ARF grant application, assistance in analyzing T-RFLP data, and making inorganic N measurements. Thank you to other colleagues that helped with NSF-MO field sampling.

I acknowledge Bruce McCune for his invaluable instruction in using multivariate statistical techniques to analyze community data, through his course, co-authored book and software.

Thank you to colleagues at the Center for Gene Research and Biotechnology at OSU, particular Caprice Rosato for running fragment analyses, Mark Dasenko for DNA sequencing, Scott Givan for bioinformatics, and Doug Borngasser for computer support.

I acknowledge the H. J. Andrews Experimental Forest and the Long Term Ecological Research network for facilitating the work in Chapter 2.

Thank you to Jenny Davis and Steve Griffith for advice about measuring denitrifying enzyme activities and information on the agricultural-riparian field site. Thank you to Don Wirth of Cala Farms, Inc. for his interest and cooperation in the agricultural-riparian study.

Thank you to my office-mates Shawna McMahon, Stacie Kageyama, and Stephanie Boyle for use of the office computer during my thesis writing. Also thank you to Shawna McMahon for help with soil profile descriptions and Stacie Kageyama for plant identifications. Thank you to all the participants of "journal club" over the years and Mark Williams for stimulating discussions. I am grateful to the Crop and Soil Science staff, particularly Jayne Smith and Tracy Mitzel for their office help, Marcia Brett for computer support, and Sara Griffith for posting web pages.

Thank you to friends and family for their support and friendship. In particular, I am grateful to my wife Heather Leslie for her understanding, scientific and personal advice, support, help, and critique of presentations.

# CONTRIBUTION OF AUTHORS

David Myrold was my major advisor, and so he aided in most aspects of the thesis, from carrying out research to commenting on manuscripts. Peter Bottomley and Kermit Cromack, Jr. were co-PIs on the study described in Chapter 2 and they gave useful comments on the manuscript. For Chapter 2, Peter Bottomley aided in the discussion and Rachel Heichen provided nitrogen mineralization data.

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**Community Composition and Activities of Denitrifying Bacteria in Soils**

## Chapter 1: Introduction to the Dissertation

Over the last 15 years, DNA-based studies have revealed tremendous prokaryotic diversity in soils (Curtis et al. 2002, Dunbar et al. 2002, Torsvik et al. 1990, Zhou et al. 2002). Most of this diversity was previously unknown because about 99% of bacterial taxa fail to grow under conditions of traditional culture-based surveys (Amann et al. 1995, Torsvik et al. 1990). As a result of finding high prokaryotic diversity in soils, the question has emerged: Does prokaryotic diversity influence ecosystem processes (Torsvik and Øvreås 2002)? A parallel debate regarding macroscopic communities has received considerable attention (Hector et al. 1999, Hooper and Vitousek 1997, McGrady-Steed et al. 1997, Naeem et al. 1994, Tilman and Downing 1994). However, despite tremendous advances in characterizing phylogenetic diversity of prokaryotes (Amann et al. 1995, Giovannoni et al. 1990, Hugenholtz et al. 1998, Pace 1997, Woese 1987), quantifying total numbers of bacterial taxa in natural systems remains a great challenge (Curtis et al. 2002, Torsvik and Øvreås 2002). A more tractable approach has been to examine community composition and activities of prokaryotic functional groups (Becker et al. 2001, Eller and Frenzel 2001, Fey and Conrad 2000, Gieske et al. 2001, King et al. 2001, Kleikemper et al. 2002, Mintie et al. 2003, Ramakrishnan et al. 2001, Ravenschlag et al. 2000). Qualitative relationships between community composition and process rates have been found for methanogens (Lueders and Friedrich 2002), sulfate reducers (Castro et al. 2002), and nitrifiers (McCaig et al. 1999), but not always (Phillips et al. 2000).

In contrast to other functional groups, little is known about the distribution of denitrifying bacteria in natural environments, and how their community structure relates to denitrification rates. The process of denitrification can limit primary productivity and contribute to global warming through both the production and consumption of nitrous oxide ($N_2O$). An essential component in the global nitrogen (N) cycle, denitrification is the reduction of nitrate ($NO_3^-$) or nitrite ($NO_2^-$) to $N_2O$ or $N_2$ in the absence of $O_2$, in the pathway:

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

with abbreviations for the individual N-oxide reductases shown above arrows, e.g., Nos for $N_2O$ reductase. Denitrifying bacteria are defined solely by the facultative ability to denitrify. Most denitrifiers respire $O_2$ preferentially.

Denitrifying bacteria are metabolically and phylogenetically diverse (Zumft 1999). Over 130 species of denitrifiers in 50 genera are known (Zumft 1999). Most species belong in the *α*-, *β*-, and *γ-Proteobacteria*, but representatives of Gram-positive bacteria and halophilic *Archaea* have been isolated (Zumft 1999). The metabolic diversity found among denitrifiers includes lithotrophs that couple denitrification with S (e.g., *Thiobacillus*) or $H_2$ oxidation (e.g., *Paracoccus*), diazotrophs (e.g., *Azospirillum* or *Bradyrhizobium*), and phototrophs (e.g., *Rhodopseudomonas*) (Zumft 1997). Denitrifiers that mineralize monoaromotic compounds, such as toluene, have received more attention recently (Song et al. 1999, Zumft 1997). Of the denitrifiers, *Pseudomonas* spp. tend to be the most readily isolated organisms from soils or aquatic systems (Gamble et al. 1977, Tiedje 1988), leading to the notion that *Pseudomonas* spp. are dominant denitrifiers in soils. However, limited reports indicate that *Rhizobium*

spp. may be prevalent in some soils (Tiedje 1988), and *Rhizobium* spp. do not usually grow on the general complex medium used in typical denitrifier surveys (Tiedje 1988).

Because denitrification is scattered among different phylogenetic groups, functional genes in the denitrification pathway, such as $NO_2^-$ reductase (*nirK* and *nirS*) and $N_2O$ reductase (*nosZ*), have been used as markers for denitrifying communities. The first studies utilizing PCR to amplify denitrification genes from environmental samples focused on denitrifying communities in marine sediments (Braker et al. 2001, Braker et al. 1998, Braker et al. 2000, Michotey et al. 2000, Scala and Kerkhof 1998, Scala and Kerkhof 1999, Scala and Kerkhof 2000).

Few researchers have examined denitrifier community structure and functioning in soils. Avrahami et al. (2002) recently found that denitrifier community structure was related to ammonium ($NH_4^+$) addition and enhanced $N_2O$ emissions from soil. Cavigelli and Robertson (2000) hypothesized that soils with different physiological responses under denitrifying conditions also had different community composition. The phylogenetic diversity of denitrifiers has been examined in a few soils (Priemé et al. 2002, Rösch et al. 2002). Philippot et al. (2002) found that communities of $NO_3^-$-reducing bacteria differed between planted and unplanted soil.

Although many individual denitrifying bacteria reduce $NO_3^-$ to $N_2$, denitrification, in principle, consists of three modules of reactions that can occur independently (Zumft 1999), i.e.:

$$(1)\ NO_3^- \rightarrow NO_2^-,\ (2)\ NO_2^- \rightarrow NO \rightarrow N_2O, \text{ and } (3)\ N_2O \rightarrow N_2.$$

For example, some denitrifiers have a truncated pathway, lacking Nos, whereas other strains grow with $N_2O$ as the sole electron acceptor (Carlson and Ingraham 1983,

Zumft 1999). *Wolinella succinogenes* is an intriguing organism that reduces $NO_3^-$ to $NH_4^+$, and $N_2O$ to $N_2$, but not $NO_2^-$ to $N_2O$ (Wolin et al. 1961, Yoshinari 1980, Zumft 1997). In organisms that carryout complete denitrification, Nos synthesis and activity can be repressed and inhibited to a greater extant by $O_2$ compared to the other reductases (Betlach and Tiedje 1981, Otte et al. 1996), depending on the denitrifying strain (Zumft 1999). Similarly in soils, $O_2$ appears to inhibit Nos activity more than the other N-oxide reductases (Firestone et al. 1980, Firestone and Tiedje 1979).

Because denitrifier distribution in soils remains largely unknown and because of the important environmental role of denitrifiers, I investigated the community composition and functioning of denitrifying bacteria in soils and sediments. Examining relationships between denitrifier distribution and activities may improve understanding of denitrification, and provide insights into the functional role of microbial diversity.

My objectives were to:

1. Determine if and how denitrifying communities differed between adjacent habitats.
2. Examine relationships between denitrifying community composition and activities.
3. Analyze phylogenetic relationships among denitrifiers.

Habitats consisted of different vegetation types or different agricultural management regimes. To pursue these objectives, I conducted two separate studies in Oregon, at sites with contrasting levels of denitrification. I compared denitrifying communities from relatively pristine meadow and forest soils in the H. J. Andrews Experimental Forest in the Western Cascade Mountains. This work, referred to as the H. J. Andrews study, was done in collaboration with researchers from microbiology,

soil science, and forest ecology. A major goal of the H. J. Andrews study was to examine spatial variation in denitrifying communities from meadow to forest soils.

In an agroecosystem study, I focused on denitrifiers in the Willamette Valley, where the use of N fertilizer may influence water quality and $N_2O$ emissions. The agroecosystem site consisted of three adjacent habitats: a fertilized agricultural field, naturally vegetated riparian area, and creek sediment. Environmental conditions at the sites favored low to moderate denitrification activities in the H. J. Andrews soils and high activities in the agroecosystem soils and sediments.

To examine denitrifier distribution, I analyzed a key gene in the denitrification pathway, $N_2O$ reductase (*nosZ*), and generated community profiles of *nosZ* using the PCR and terminal-restriction fragment length polymorphisms (T-RFLPs). I applied multivariate statistics to determine community differences and correlations between community composition and activities. I used standard activity assays to quantify rates of denitrification. To identify putative denitrifiers from the soils and further elucidate T-RFLP profiles, I analyzed phylogenetic relationships among denitrifiers based on *nosZ* sequences. Nitrous oxide-reductase activity was examined in greater detail in the agroecosystem study, whereas more extensive *nosZ* cloning, screening, and sequencing was done in the H. J. Andrews study. The same *nosZ* T-RFLP measurements were made in both studies.

# Chapter 2:

# Community Composition and Functioning of Denitrifying Bacteria from Adjacent Meadow and Forest Soils

Jeremy J. Rich, Rachel S. Heichen, Peter J. Bottomley, Kermit Cromack, Jr., and David D. Myrold

Applied and Environmental Microbiology (in review)

American Society for Microbiology, Washington D. C.

## ABSTRACT

We investigated communities of denitrifying bacteria from adjacent meadow and forest soils. Our objectives were to explore spatial gradients in denitrifier communities from meadow to forest, examine if community composition was related to ecological properties, such as vegetation type and process rates, and determine phylogenetic relationships among denitrifiers. A key gene in the denitrification pathway, nitrous oxide reductase (*nosZ*), served as a marker for denitrifying bacteria. Denitrifying enzyme activity (DEA) was measured as a proxy for function. Other variables, such as nitrification potential and soil C:N ratio were also measured. Soil samples were taken along transects that spanned meadow-forest boundaries at two sites in the H. J. Andrews Experimental Forest in the Western Cascade Mountains of Oregon, USA. Results indicated strong functional and community differences between the meadow and forest soils. Levels of DEA were an order of magnitude higher in the meadow soils. Denitrifier community composition was related to process rates and vegetation type based on multivariate analyses of *nosZ* T-RFLP profiles. Denitrifier communities formed distinct groups according to vegetation type and site. Screening 225 *nosZ* clones yielded 47 unique denitrifier genotypes; the most dominant genotype occurred 31 times and half the genotypes occurred once. Several dominant and less-dominant denitrifying genotypes were more characteristic of either meadow or forest soils. The majority of *nosZ* fragments sequenced from meadow or forest soils were most similar to *nosZ* from the *Rhizobiaceae* group in *α-Proteobacteria*. Denitrifier community composition, as well as environmental factors, may contribute to denitrification rates in these systems.

## INTRODUCTION

In addition to ameliorating eutrophication and removing excess nitrate ($NO_3^-$) from drinking water, the process of denitrification can limit primary productivity and contribute to global warming through the formation of nitrous oxide ($N_2O$). Despite the essential role of denitrification in the global nitrogen (N) cycle, little is known about how denitrifier community structure relates to denitrification rates in natural environments. Examining this relationship may improve understanding of denitrification, and provide insights into the functional role of microbial diversity.

Denitrifying bacteria are defined solely by the ability to reduce $NO_3^-$ or nitrite ($NO_2^-$) to $N_2O$ or $N_2$ in the absence of oxygen. Denitrification is not specific to any one phylogenetic group; the trait is found in about 50 genera, mostly in the *Proteobacteria* (Zumft 1999). Therefore, to analyze denitrifier diversity, functional genes in the denitrification pathway, such as nitrite reductase (*nirK* and *nirS*) and nitrous oxide reductase (*nosZ*), have been retrieved from environmental samples using PCR and cloning and sequencing (Braker et al. 2000, Rösch et al. 2002, Scala and Kerkhof 1999). Denitrifier community profiles have been generated using PCR-coupled terminal restriction fragment length polymorphisms (T-RFLPs) (Avrahami et al. 2002, Braker et al. 2001, Scala and Kerkhof 2000). Avrahami et al. (2002) recently found that denitrifier community composition was related to $NH_4^+$ addition and enhanced $N_2O$ emissions from soil.

Plant communities presumably have strong effects on belowground microbial community structure (Kowalchuk et al. 2002, Philippot et al. 2002). Therefore, we investigated denitrifier communities in two broadly contrasting plant communities of

meadow vegetation and adjacent coniferous forest in the Western Cascade Mountains of Oregon, USA. We hypothesized that because these vegetation types likely differed in N cycling processes, denitrifier communities would also differ. Differences in N cycling were assumed because net production of $NO_3^-$ is higher in analogous meadow soils compared to coniferous forests (Davidson et al. 1992, Ingham et al. 1989). Although factors leading to vegetation differences are complex (Magee and Antos 1992, Miller and Halpern 1998), annual turnover and higher concentrations of N in meadow plant tissue likely contribute to differences in N cycling (Hunt et al. 1988, Ollinger et al. 2002).

The objective of this study was to examine spatial variation in denitrifier communities and N cycling processes from adjacent meadow and forest soils. We confirmed that N cycling processes differed between meadow and forest using standard activity assays. Community composition was measured with T-RFLP profiles of the *nosZ* fragment analyzed by Rösch et al. (2002). We assessed relationships between denitrifier communities and functioning with multivariate techniques, and we investigated phylogenetic relationships among *nosZ* clones. Recently, Mintie et al. (2003) examined nitrifier populations from the same soils.

## METHODS

**Site characteristics and sampling.** Study sites were located at the H. J. Andrews Experimental Forest (44.2°N, 122.2°W) in the Western Cascade Mountains of Oregon, USA. The Western Cascades consist of deeply eroded mountains formed by volcanic activity primarily five to forty million years ago (Orr et al. 1992). The dominant vegetation type is moist temperate coniferous forest. Adjacent meadow and

forest vegetation exist on well-drained soils at higher elevations with south facing slopes (Miller and Halpern 1998). We examined two sites, referred to as Carpenter and Lookout, with adjacent meadow and forest vegetation and similar soils. Aspect was 210°SW at Carpenter and 180°S at Lookout. Slope was approximately 50% at Carpenter and 35% at Lookout. Elevation at both sites was 1500 m. The mean air temperature range during the years of 1970 to 2000 was 10°C to 22°C for summer and −2°C to 4°C for winter (Smith 2002). Mean annual precipitation from 1980 to 1989 was approximately 3000 mm, falling mainly between November and March (http://www.fsl.orst.edu/lter/data/spatial/gislist.cfm). Basic characteristics of the surface soil are shown in Table 2.1. The soils at both sites were relatively young, poorly developed, well-drained, and rich in organic matter. The meadow soils at both sites were Lithic Cryandepts, Lucky Boy soil series (U.S. taxonomic designation), with 15% surface rock at Lookout and amorphous-very dark brown surface (A) horizons underlain with continuous, unconsolidated rocky material at a depth of about 100 cm at Carpenter and 40 cm at Lookout. The forest soils were Pachic Haplumbrepts, Blue River soil series with dark brown surface (A) horizons, 20 to 40 cm in depth, and poorly developed subsurface (B) horizons, to a depth of 70 to 80 cm. Meadow vegetation consisted of a variety of herbaceous plants, including the most dominant species at Carpenter of *Rudbeckia occidentalis,* bracken fern (*Pteridium aquilinium*), and pearly everlasting (*Anaphalis margaritacea*). At Lookout the dominant species were lupine (*Lupinus polyphyllus*), purple lovage (*Ligusticum grayi*), Oregon sunshine (*Eriophyllum lanatum*), and Indian paintbrush (*Castilleja hispida*). There is no record of human influence at the Lookout site, but a logging operation took place in the

**Table 2.1.** Properties of the H. J. Andrews mineral soil used in this study (mean of 0 to 10 cm depth).

| | Carpenter site | | Lookout site | | |
|---|---|---|---|---|---|
| Measurement† | Meadow | Forest | Meadow | Forest | Reference |
| pH | 5.9 | 5.8 | 5.8 | 5.7 | Mintie et al. (2003) |
| Total C (g C $kg^{-1}$ soil) | 94.7 | 131.8 | 115.9 | 142.3 | Mintie et al. (2003) |
| Total N (g N $kg^{-1}$ soil) | 6.8 | 5.8 | 9.3 | 9.2 | Mintie et al. (2003) |
| C:N | 13.8 | 22.8 | 12.7 | 15.7 | Mintie et al. (2003) |
| Nitrate (mg N $kg^{-1}$ soil)‡ | 2.9 | 1.5 | 3.5 | 1.9 | This study |
| Ammonium (mg N $kg^{-1}$ soil) | 4.6 | 1.4 | 2.6 | 1.7 | This study |
| Net Nitrification (mg N $kg^{-1}$ soil $day^{-1}$)* | 2.60 | 0.04 | 2.15 | 0.20 | This study |
| Net Ammonification (mg N $kg^{-1}$ soil $day^{-1}$) | -0.42 | 0.43 | -0.20 | 0.79 | This study |
| Net N mineralization (mg N $kg^{-1}$ soil $day^{-1}$) | 2.18 | 0.47 | 1.96 | 0.99 | Mintie et al. (2003) |
| Nitrification potential (mg N $kg^{-1}$ soil $day^{-1}$) | 8.90 | 0.26 | 8.67 | 0.74 | Mintie et al. (2003) |
| DEA (mg N $kg^{-1}$ soil $day^{-1}$)** | 0.78 | 0.04 | 1.58 | 0.17 | This study |

†Units are normalized by dry soil.
‡Nitrate and ammonium were measured with an autoanalyzer, as reported for N mineralization (Mintie et al. 2003).
*Net rates were measured over a 10-day laboratory incubation (Mintie et al. 2003).
**Denitrifying enzyme activity.

vicinity of the Carpenter site in 1962 (H. J. Andrews on-site archive). Dominant tree species in the forests at both sites were Douglas-fir (*Pseudotsuga menziesii*), noble fir (*Abies procera*), Pacific silver fir (*Abies amabilis*), and grand fir (*Abies grandis*). Mean tree age in the forest was 92 yr at Carpenter and 48 yr at Lookout.

Soil samples were taken along meadow-to-forest transects at the two sites. Three replicate transects were established at each site. The transects were distributed throughout the site at a distance of at least 20 m between transects. Each transect ran perpendicular to the meadow-forest boundary and consisted of eight, evenly spaced sampling points, four in the forest and four in the meadow. Sampling points were spaced at 20-m intervals at Carpenter and 10-m intervals at Lookout, with greater spacing at Carpenter to account for the wider meadow-to-forest transition at this site. We sampled the mineral soil (0 to 10 cm), under the organic-humus layer, in the meadow and forest by compositing five replicate soil cores (inner diameter, 6 cm) at each sampling point within a 0.5-m radius. The sample unit consisted of the composite soil sample; 48 soil samples were taken, and thus 48 sample units were analyzed in this study. Samples were taken 26 June 2000, corresponding to the early part of the growing season. The soil was brought back to the laboratory on ice, stored at 4°C, and sieved (4.75-mm mesh) about 24 h after sampling. Soil for DNA extractions was immediately frozen at -20°C after sieving. Soil for the denitrification potential measurement was kept at 4°C until analyzed within 72 h after sampling.

**Denitrification potentials.** Denitrification potentials, or denitrifying enzyme activity (DEA), measurements were carried out following Tiedje (1994). Fresh soil (10 g) was added to 125-ml Erlenmeyer flasks with 25 ml of solution containing glucose

(10 mM), $NO_3^-$ (5 mM), and phosphate buffer (50 mM, pH 7.0). The flasks were sealed with neoprene stoppers and made anaerobic by repeated evacuation and flushing with Ar. Standard-grade acetylene was purified through an acid trap (Hyman and Arp 1987) and added to the headspace (10% v/v). The flasks were incubated on a rotary shaker (250 rpm, 21°C). Gas samples (4 ml) were taken at 15 and 75 min and stored in Vacutainers® (3 ml) until analyzed for $N_2O$ by gas chromatography using a $^{63}Ni$ electron capture detector (Myrold 1988). Production of $N_2O$ was linear during the 15-to-75-min interval, based on a subset of flasks sampled every 15 min (data not shown). Statistical differences in DEA were assessed using a two-factor ANOVA for site and vegetation type (SAS v. 7.0).

***nosZ* PCR and T-RFLPs.** PCR was used to generate 700-bp *nosZ* fragments from the soil denitrifying community. Designed in 1999 based on available sequence data and Scala and Kerkhof (1998), our *nosZ* primers are almost identical to those independently developed by Rösch et al. (2002). Our primer sequences were: 5'-CG**C**TGTTC**I**TCGACAG**Y**CAG-3' [bold nucleotides differ from (Rösch et al. 2002)] for the forward primer (*nosZ*-F-1181) and 5'-ATGTGCA**K**IGCRTGGCAGAA-3' for the reverse primer (*nosZ*-R-1880; I=inosine, Y=T+C, K=T+G, R=A+G). Numbers in parentheses indicate nucleotide positions at the ends of the *Pseudomonas stutzeri* 700-bp *nosZ* fragment (Genbank accession no. M22628).

DNA was extracted from soil samples (0.3 g fresh weight) or pure cultures using the FastDNA® kit for bacteria cells or soil (Bio101, Inc.), according to the manufacturer's instructions. DNA extracts were checked on agarose gels (1.1%) stained with ethidium bromide (0.5 μg $ml^{-1}$). The DNA was quantified using a DNA

fluorometer TKO 100 (Hoefer Scientific) with calf thymus DNA standard. Reaction mixtures (50 µl) contained soil DNA (100 ng), AmpliTaq® DNA polymerase (2.5 U), GeneAmp® PCR buffer (1x), $MgCl_2$ (2 mM), dNTPs (0.2 mM each), forward and reverse primers (0.2 µM each), and bovine serum albumin (0.064 g $ml^{-1}$). The forward primer was fluorescently labeled with 6-Fam. For PCR amplification of soil DNA, the temperature profile on a PTC-100 hot bonnet thermocycler (MJ Research, Inc.) was 94°C for 3 min; 25 cycles of 94°C (45 sec), 56°C (1 min), and 72°C (2 min); followed by a final extension of 72°C for 7 min. For each sample, the PCR products of three reactions were pooled and purified using a QIAquick PCR purification kit (Qiagen), according to the manufacturer's instructions. PCR products were checked using agarose gels stained with ethidium bromide. Fluorescently labeled PCR products were quantified on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Inc.) with a known concentration of a 6-Fam labeled *nosZ* fragment obtained from the amplification of *P. stutzeri* DNA.

Samples for T-RFLP analysis consisted of approximately 1 ng of fluorescently labeled *nosZ* PCR products, digested in 25 µl aliquots with 6.25 U of restriction endonuclease (37°C, 3 h), followed by heat inactivation (65°C, 15 min). Three T-RFLP profiles were generated per sample unit in separate reactions with the endonucleases *Cfo*I (isoschisimer of *Hha*I), *Msp*I, or *Rsa*I (Roche, Co.). Restriction digests (20 µl) were purified through Sephadex™ G-50 (Amersham Bioscience) and dried as a pellet in a speed-vac centrifuge. Dried pellets were rehydrated with Hi-Di® formamide (9.92 µl) and X-Rhodamine MapMarker™ 1000 (0.08 µl) internal lane size standard. The mixture was heated (96°C, 3 min) and cooled on ice (5 min) prior to running the

samples in 96-well microtiter plates on an ABI Prism® 3100 Genetic Analyzer, with an injection time of 22 s, run time of 54 min, and a 36-cm capillary containing 3100 POP-4® polymer.

**Analysis of T-RFLP data.** Size and relative abundance of terminal-restriction fragments (T-RFs) was quantified using GeneScan® v3.5 software (Applied Biosystems, Inc.). Fragment peak heights above a detection limit of 10 relative fluorescent units were summed for each profile; only fragments with a signal above 1.5% of the sum were included in the analyses. The area of T-RFs that differed in size by ≤1.5 bp in an individual profile were summed and considered as one fragment. Sequencing of clones indicated that almost all individual T-RFs with this variation were the same size in base pairs. Each fragment was expressed as a percent of the total fragment abundance in each sample profile or scored as either present or absent. The T-RFLP profile data are available upon request.

PC-ORD v4.01 (McCune and Mefford 1999) was used for all multivariate statistical community analyses of the *nosZ* T-RFLP profiles. Differences in community composition were assessed graphically using the ordination method of Non-metric Multidimensional Scaling (NMS), based on Sørensen's distance (Kruskal 1964, McCune and Grace 2002). NMS approximates community relatedness among samples (based on the distance measure) with synthetic axes (McCune and Grace 2002). NMS was constrained to two ordination axes with a random starting configuration, 300 iterations, instability criterion of 0.0001, 40 runs with the real data and 50 runs with randomized data; Monte Carlo tests of the real data vs. the randomized data were used to assess axes significance. Functional parameters (e.g., DEA and nitrification

potential) and individual T-RFs were correlated with the NMS axes to evaluate relationships between overall community composition, function, and individual T-RFs. Nitrification potential and DEA values were log transformed for the correlation analysis because the rates varied by an order of magnitude. Correlation coefficients between process rates (or environmental variables) and NMS axes were displayed as vectors radiating from the center of the plot. Each vector was calculated as the hypotenuse of a right triangle with each side of the triangle being the correlation coefficient of the process rate with axis one and two (McCune and Grace 2002). The vector angle was a function of the relative proportion between the correlation coefficients (McCune and Grace 2002). A Multi-response Permutation Procedure (MRPP), with Sørensen's distance and rank transformation, was used to test for significant differences in community composition between groups defined by vegetation type and site (McCune and Grace 2002, Mielke 1984). The MRPP *A*-statistic describes the effect size or with-in group relatedness relative to that expected by chance alone, and is somewhat analogous to an $r^2$-value (McCune and Grace 2002). To identify T-RFs that differentiated denitrifier communities by vegetation type and site, Indicator Species Analysis (Dufrêne and Legendre 1997) was run with 1000 randomizations in the Monte Carlo test.

***nosZ* cloning, screening, and sequencing.** *nosZ* fragments generated from the PCR of soil DNA were cloned using the pGEM®-T Easy Vector System (Promega), according to the manufacturer's instructions. Template DNA for the PCR was pooled from two representative samples of each vegetation type. The PCR was run as described above, except with 30 cycles. Prior to cloning, the 700-bp *nosZ* fragments

were excised from an agarose gel and purified (Qiagen). Clones were screened using *Cfo*I, *Msp*I, and *Rsa*I T-RFLPs as described for direct soil profiles, defined here as T-RFLP profiling. Genotypic diversity was analyzed using species area curve analysis, treating a unique *nosZ* clone as a species, as implemented in PC-ORD v4.01 (McCune and Mefford 1999). Species area curve analysis consisted of randomly sub-sampling the clone library dataset 500 times at each level of clone screening, which is analogous to generating rarefaction curves.

Plasmids were prepared using QIAprep Minipreps (Qiagen) according to the manufacturer's instructions. Both strands of the *nosZ* fragment were sequenced for 34 clones, using the SP6 and T7-promoter primers flanking the PCR insert in the plasmid. Sequencing reactions were done with ABI Prism® BigDye™ Terminator Cycle Sequencing and the samples were analyzed on an ABI Prism® 3100 Genetic Analyzer.

***nosZ* phylogenetic analyses.** Analyses of *nosZ* sequences were carried out for the translated amino acid sequences using Phylip v3.573 (Felsenstein 1993) and TREE-PUZZLE v5.0 (Schmidt et al. 2002). Following the approach of Friedrich (2002), with the exception of *Ralstonia eutropha* ATCC 17699 (Goris et al. 2001), *nosZ* sequences were selected from cultured strains if their phylogenetic position could be confirmed using publicly available strain-specific SSU rRNA sequence data and the Ribosomal Database Project's (RDP) hierarchy browser and sequence match tool (Maidak et al. 2001). Amino acid sequences of *nosZ* were aligned using ClustalX (Thompson et al. 1997), excluding residues corresponding to the primer sequences. Distance, parsimony, and maximum likelihood methods were used to infer phylogenetic relationships. Dayhoff's 001 substitution matrix (Dayhoff et al. 1979) was used to measure

evolutionary distance in PROTDIST (Phylip), and trees were constructed using the neighbor-joining algorithm (Phylip). For parsimony, PROTPARS (Phylip) was used, and for maximum likelihood analysis, TREE-PUZZLE was applied, using the JTT (Jones et al. 1992) amino acid substitution matrix for evolutionary distance. Bootstrap analysis of 1000 replicates was carried out for neighbor-joining trees and 500 replicates for parsimony trees (Phylip).

The Genbank accession numbers of the H. J. Andrews *nosZ* sequences are AY259180 to AY259212.

## RESULTS

**Denitrification potentials.** Strong functional differences were found between meadow and forest soils at the two sites (Table 2.1, Fig. 2.1). Denitrifying enzyme activity was generally an order of magnitude higher in the meadow compared to the forest. This difference was highly significant at both sites (p-value<0.0001 for vegetation type, two-factor ANOVA). DEA, in units of ng $N_2O$-N $g^{-1}$ dry soil $h^{-1}$ (mean ± 1 standard error, n = 12), of the meadow soils were 32.4 ± 7.1 for Carpenter and 66.0 ± 19.4 for Lookout; the forest soils were 1.6 ± 0.5 for Carpenter and 7.0 ± 1.4 for Lookout. DEA showed a weak tendency to be higher at Lookout (p-value=0.07 for site, two-factor ANOVA). The mean activity along the transects only changed significantly after crossing the meadow-forest boundary (Fig. 2.1), within the sampling interval of 10 m at Lookout or 20 m at Carpenter.

**Amplification of *nosZ* from pure cultures and soil DNA.** The *nosZ* primers amplified the expected fragment from five positive controls possessing *nosZ*, including

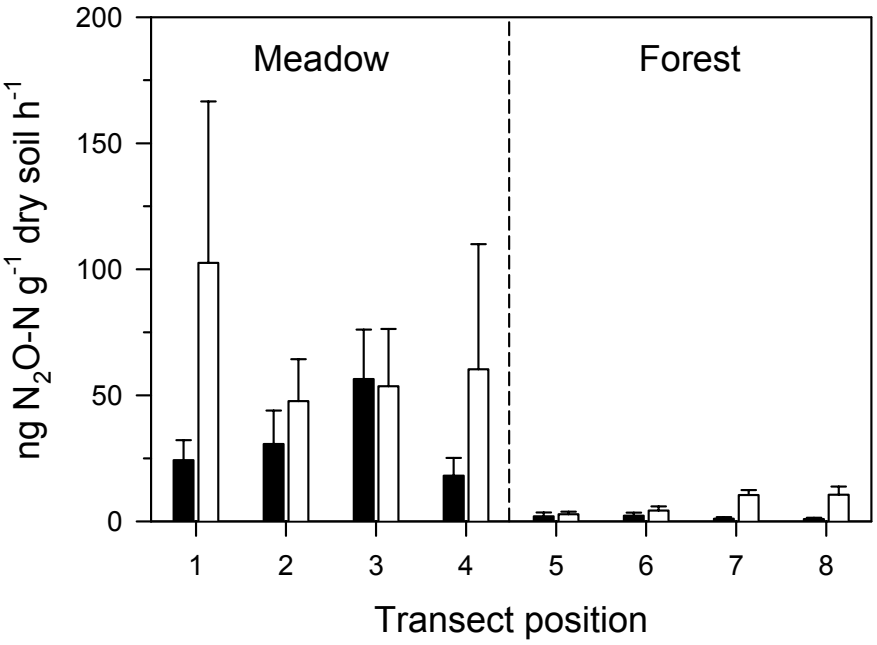



**Fig. 2.1.** Spatial variation of denitrifying enzyme activity (DEA) from adjacent meadow and forest soils in the H. J. Andrews. Bars are the mean ± 1 standard error for each position along replicate transects at the Carpenter site (closed) and Lookout site (open) (n = 3). Distance between transect positions was 20 m at Carpenter and 10 m at Lookout (see methods).

*Paracoccus denitrificans* ATCC 17741, *Ralstonia eutropha* ATCC 17699, "*Achromobacter cycloclastes*" ATCC 21921, *Pseudomonas stutzeri* ATCC 14405, and *Pseudomonas aeruginosa*. The primers did not amplify DNA from two negative controls lacking *nosZ* (*Serratia marcescens* and *Burkholderia cepacia* cep31$^{T}$groupI). Yields of DNA extracted from the H. J. Andrews soil ranged from 7 to 35 μg DNA $g^{-1}$ dry soil. The PCR products that were amplified from the soil DNA were of the expected size (700 bp). Three of the 48 field replicates were excluded from subsequent analyses because of poor PCR amplification.

***nosZ* T-RFLP profiles.** Each *nosZ* T-RFLP profile from the H. J. Andrews soil consisted of one or two dominant T-RFs and several less dominant T-RFs (Fig. 2.2). Overall, a total of 61 T-RFs were detected with 14 to 24 T-RFs for each endonuclease (Table 2.2). Four of the 61 T-RFs were dominant (i.e., *Cfo*I 357 bp, *Msp*I 111-112 bp, *Rsa*I 666 bp and 700 bp), comprising 58% of the mean total fragment abundance of each profile. These four were the only T-RFs that occurred in all samples. The rest of the T-RFs comprised a mean of 2-12% of the total fragment abundance of each profile and occurred in 2-98% of the samples; of these, 21 T-RFs occurred only once. Each endonuclease produced a mean of 2.7 to 7.1 T-RFs per profile, depending on the endonuclease, vegetation type, and site (Table 2.2). *Cfo*I and *Rsa*I tended to produce more fragments than *Msp*I (Table 2.2). The meadow samples tended to have slightly more fragments in each profile on average, but this result was inconsistent among endonucleases and sites (Table 2.2).

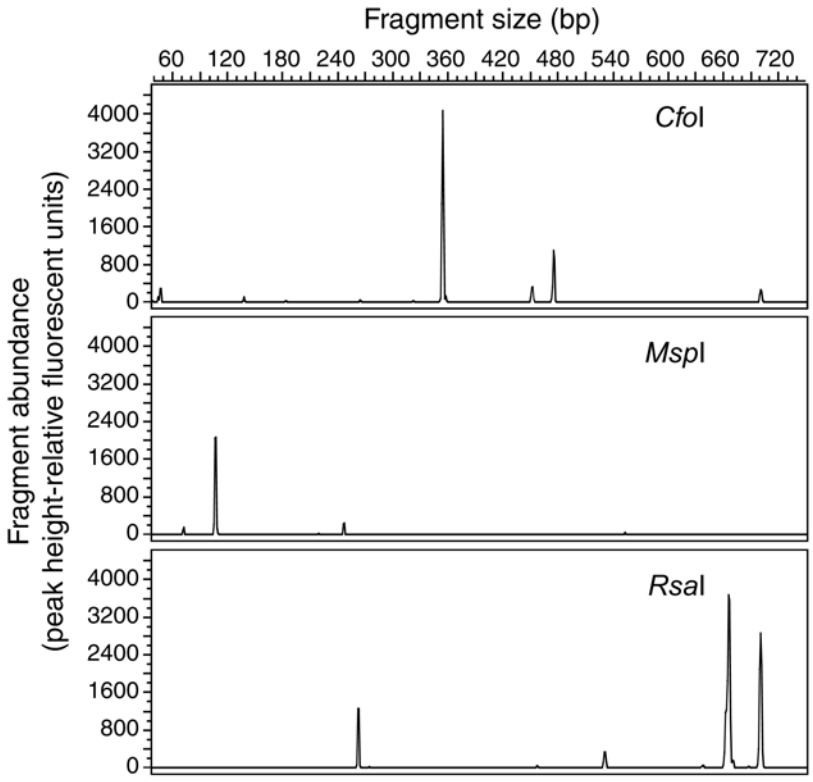



**Fig. 2.2.** Example electropherograms of *nosZ* T-RFLP profiles for an H. J. Andrews soil sample from Carpenter meadow. Each panel is identified by the restriction endonuclease used to generate the profile.

**Table 2.2**. Number of terminal-restriction fragments (T-RFs) in the *nosZ* T-RFLP profiles of H. J. Andrews soils.

| Site | Vegetation type | n† | *Cfo*I | | *Msp*I | | *Rsa*I | | Combined T-RFLPs | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total | Mean (SE) | Total | Mean (SE) | Total | Mean (SE) | Total | Mean (SE) |
| Carpenter | Meadow | 12 | 12 | 7.1 (0.4) | 6 | 4.2 (0.2) | 13 | 4.9 (0.3) | 31 | 16.2 (0.7) |
| | Forest | 10 | 11 | 5.7 (0.5) | 10 | 4.2 (0.5) | 10 | 5.0 (0.2) | 31 | 14.9 (1.1) |
| Lookout | Meadow | 12 | 15 | 6.9 (0.6) | 8 | 3.3 (0.4) | 15 | 6.0 (0.7) | 38 | 16.2 (1.4) |
| | Forest | 11 | 11 | 5.1 (0.2) | 5 | 2.7 (0.2) | 7 | 4.5 (0.2) | 23 | 12.4 (0.4) |
| All samples | | 45 | 24 | 6.2 (0.3) | 14 | 3.6 (0.2) | 23 | 5.1 (0.2) | 61 | 15.0 (0.5) |

†Number of field replicates analyzed.

***nosZ* community composition, functional relationships, and individual T-RFs.** Denitrifier community composition differed significantly between vegetation type and site, based on multivariate analyses of combined *Cfo*I, *Msp*I, and *Rsa*I *nosZ* T-RFLP profiles (Fig. 2.3; Table 2.3). MRPP *A*-statistics of about 0.3 in Table 2.3 reflect strong differences between groups (McCune and Grace 2002). Differences were evident based on proportional abundance or presence/absence of *nosZ* T-RFs (Fig. 2.3A and 2.3B, Table 2.3). Four groups corresponding to vegetation type and site were observed based on proportional abundance (Fig. 2.3A, Table 2.3). Differences by site were less apparent for the forest based on presence/absence (Fig. 2.3B, Table 2.3). NMS axes one and two were statistically significant (p-value=0.02), and the axes explained 91% (Fig. 2.3A) and 63% (Fig. 2.3B) of the sample variation. Differences between vegetation type and site were also found by analyzing separate *Cfo*I, *Msp*I, or *Rsa*I T-RFLP profiles, but these differences were not as strong as for the combined profiles, and relationships between vegetation type and site varied somewhat depending on the endonuclease (data not shown). Ordination plots of *Cfo*I profiles appeared most similar to the combined profile plots.

Functional variables were related to denitrifier community composition, as demonstrated by plotting the relative magnitude and direction of correlation coefficients ($r^2$) between NMS axes and functional variables (Fig. 2.3). Examples of the strongest correlates were selected for Fig. 2.3. Nitrification potential and DEA rates tended to increase from forest to meadow along axis one (Fig. 2.3). DEA also tended to increase towards Lookout meadow in Fig. 2.3A. Net nitrification gave almost identical correlation coefficients as for nitrification potentials (data not shown). Accumulation of

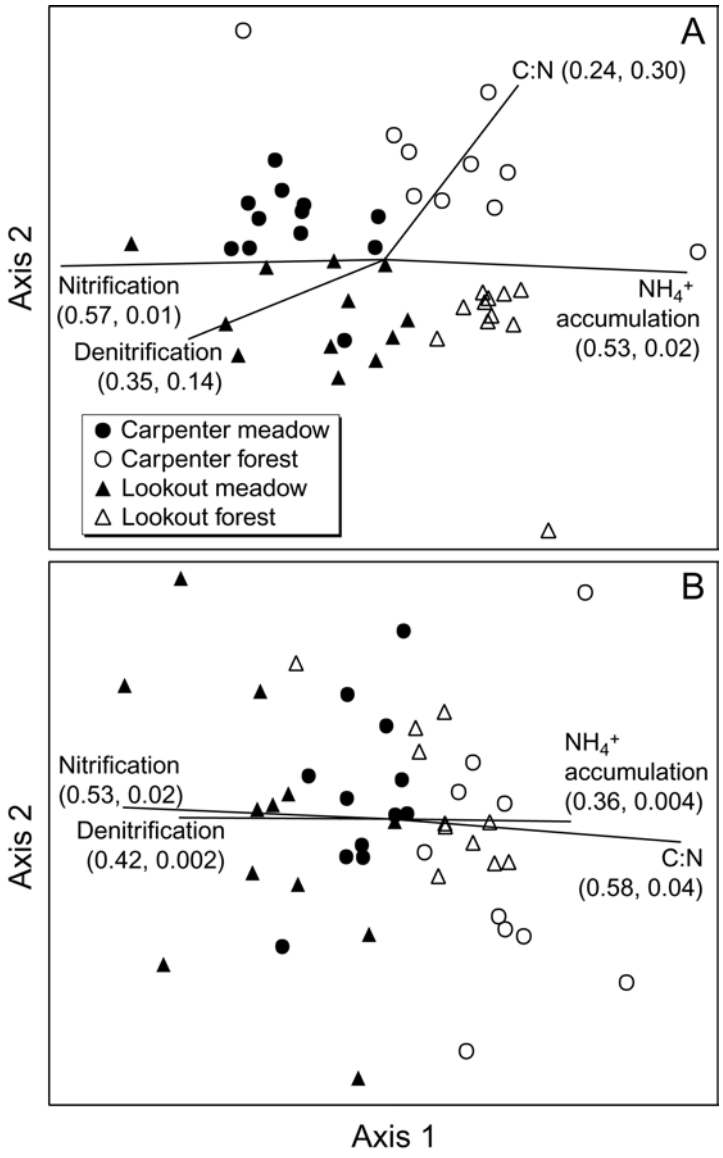



**Fig. 2.3.** Non-metric multidimensional scaling (NMS) of denitrifier community composition based on combined *nosZ* T-RFLP profiles (*Cfo*I, *Msp*I, and *Rsa*I) of H. J. Andrews transect soil samples. Panel (A) is based on proportional abundance of *nosZ* T-RFs, and panel (B) on presence/absence of *nosZ* T-RFs. Vectors show the direction and relative magnitude of correlation coefficients ($r^2$) between NMS axes and functional variables; $r^2$-values are shown in parentheses for the correlation between the functional variable and axis one or two, in the order of ($r^2$ axis 1, $r^2$ axis 2). Abbreviations of functional variables are nitrification for $\log_{10}$ of nitrification potential, denitrification for $\log_{10}$ of denitrifying enzyme activity (DEA), C:N for the soil carbon to nitrogen ratio, and $NH_4^+$ accumulation for net $NH_4^+$ production (i.e., ammonification).

**Table 2.3.** Multi-response Permutation Procedure (MRPP), testing for significant differences between pre-defined groups, based on *nosZ* T-RFLP profiles (*Cfo*I, *Msp*I, and *Rsa*I combined).

| | *A*-statistic† | |
|---|---|---|
| Test | Proportional abundance | Presence/ absence |
| *Difference by vegetation type:* | | |
| Carpenter meadow vs. Carpenter forest | 0.29 ($p<10^{-5}$) | 0.23 ($p<10^{-5}$) |
| Lookout meadow vs. Lookout forest | 0.26 ($p<10^{-5}$) | 0.34 ($p<10^{-5}$) |
| *Difference by site:* | | |
| Carpenter meadow vs. Lookout meadow | 0.18 ($p<0.001$) | 0.17 ($p<0.001$) |
| Carpenter forest vs. Lookout forest | 0.24 ($p<10^{-5}$) | 0.05 ($p=0.02$) |
| *Randomized data*:* | | |
| Carpenter meadow vs. Carpenter forest | −0.04 ($p=1.0$) | −0.01 ($p=0.7$) |

†The *A*-statistic measures within group relatedness compared to that expected by chance; *A*-statistics of about 0.3 reflect strong differences between groups (McCune and Grace 2002).
*T-RFLP profiles from the Carpenter site were randomized among sample units.

$NH_4^+$ (i.e., net ammonification) tended to increase from meadow to forest along axis one (Fig. 2.3). Soil C:N ratios increased from meadow to forest, particularly in the direction of Carpenter forest, where C:N ratios were highest (Fig. 2.3; Table 2.1).

Although denitrifier communities differed significantly based either on proportional abundance or presence/absence, different T-RFs contributed to separating the communities in each analysis (Table 2.4). This was determined by ranking correlation coefficients between individual T-RFs and NMS axes (Table 2.4). Only two T-RFs (*Cfo*I 454 bp and *Rsa*I 451 bp) were strongly correlated with NMS axes in both analyses (Table 2.4). Dominant T-RFs tended to contribute more to separating communities based on proportional abundance, whereas less dominant T-RFs were more significant based on presence/absence. Note that fragments present in every sample show no difference based on presence/absence. Relative abundances of *Rsa*I 666 bp and *Rsa*I 700 bp were most dominant for differentiating the communities along NMS axis one based on proportional abundance (Table 2.4). *Rsa*I 666 bp declined from meadow to forest, whereas *Rsa*I 700 bp increased from meadow to forest (Fig. 2.4). Based on presence/absence, *Rsa*I 451 bp and *Cfo*I 454 bp and 361 bp were most dominant for differentiating the communities along axis one (Table 2.4). *Cfo*I 454 bp was found in every meadow sample but in none of the forest samples, except near the boundary at Lookout (Fig. 2.4). Conversely, *Rsa*I 451 bp was found preferentially in the forest (Fig. 2.4).

Indicator species analysis was used to further identify T-RFs more prevalent in either vegetation type or site (Fig. 2.5). This procedure examines the relative abundance and presence/absence of individual T-RFs in a predefined group compared

**Table 2.4.** Top-ten correlation coefficients (Pearson's *r*-value) between *nosZ* T-RFs and NMS axes one or two, based either on proportional abundance or presence/absence.

| Proportional abundance | | | Presence/Absence | | |
|---|---|---|---|---|---|
| T-RF | axis 1‡ | axis 2* | T-RF | axis 1‡ | axis 2* |
| (1) *Rsa*I 666 bp | **−0.90**† | 0.24 | (1) ***Rsa*I 451 bp** | **0.77** | 0.11 |
| (2) *Msp*I 111-112 bp | 0.28 | **−0.84** | (2) ***Cfo*I 454 bp** | **−0.76** | 0.12 |
| (3) *Rsa*I 700 bp | **0.82** | 0.03 | (3) *Cfo*I 361 bp | **−0.72** | −0.04 |
| (4) *Cfo*I 476 bp | −0.40 | **0.81** | (4) *Rsa*I 672 bp | **−0.71** | 0.00 |
| (5) *Msp*I 250 bp | **−0.78** | 0.16 | (5) *Rsa*I 457 bp | **−0.64** | −0.12 |
| (6) *Msp*I 78 bp | −0.08 | **0.73** | (6) *Cfo*I 282 bp | **0.61** | −0.21 |
| (7) ***Cfo*I 454 bp** | **−0.64** | −0.13 | (7) *Rsa*I 103 bp | **0.57** | −0.32 |
| (8) *Cfo*I 357 bp | **0.60** | −0.37 | (8) *Rsa*I 660 bp | **−0.55** | 0.09 |
| (9) ***Rsa*I 451 bp** | **0.53** | −0.39 | (9) *Rsa*I 57 bp | **−0.48** | −0.20 |
| (10) *Msp*I 222 bp | **−0.44** | 0.11 | (10) *Msp*I 340 bp | **0.47** | −0.21 |

‡Negative *r*-values indicate that T-RFs decreased from meadow to forest along axis 1 (i.e., greater prevalence of T-RFs in meadow), whereas positive *r*-values indicate that T-RFs increased from meadow to forest along axis 1 (i.e., greater prevalence of T-RFs in forest).

*Negative *r*-values indicate that T-RFs decreased from Lookout to Carpenter along axis 2 (i.e., greater prevalence of T-RFs at Lookout), whereas positive *r*-values indicate that T-RFs increased from Lookout to Carpenter along axis 2 (i.e., greater prevalence of T-RFs at Carpenter).

†$r^2$-values ≥0.20 and T-RFs found in each list are highlighted in bold.

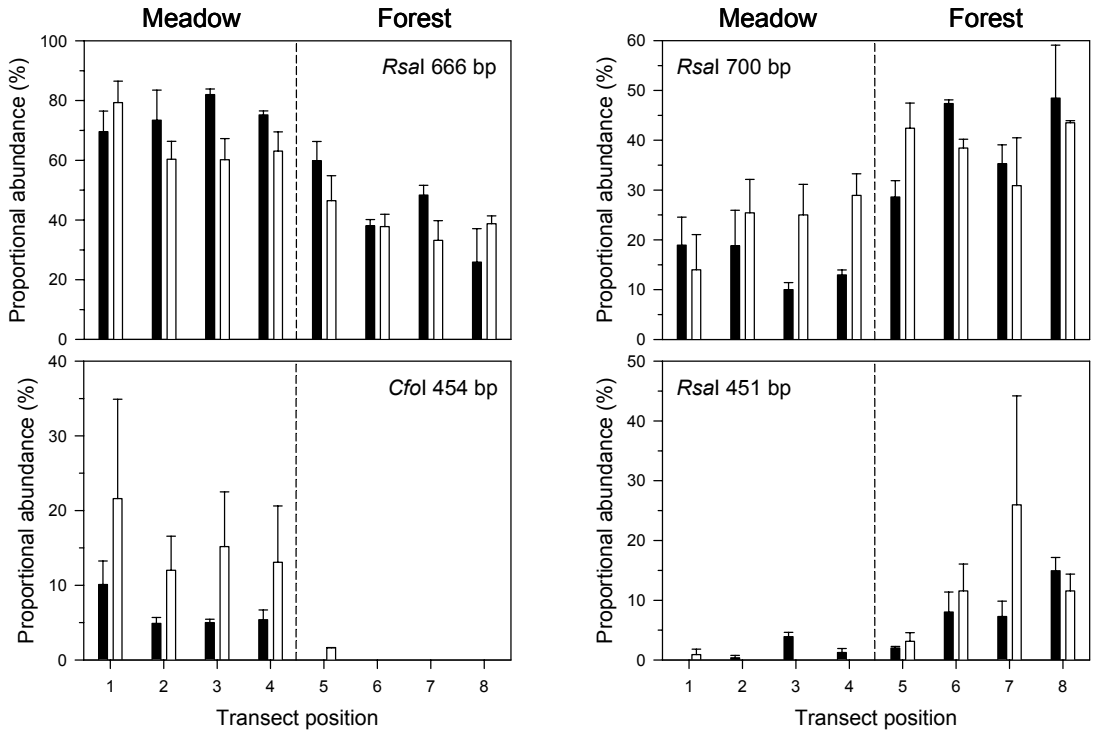



**Fig. 2.4.** Spatial variation of some key *nosZ* T-RFs from adjacent meadow and forest soils in the H. J. Andrews. Bars are the mean ± 1 standard error for each position along replicate transects at Carpenter site (closed) and Lookout site (open) (n = 3 for all positions, except n = 2 for position 5 at Lookout and positions 6 and 8 at Carpenter). Each panel shows an individual T-RF, identified by the corresponding restriction endonuclease and fragment size. Distance between transect positions was 20 m at Carpenter and 10 m at Lookout (see methods).

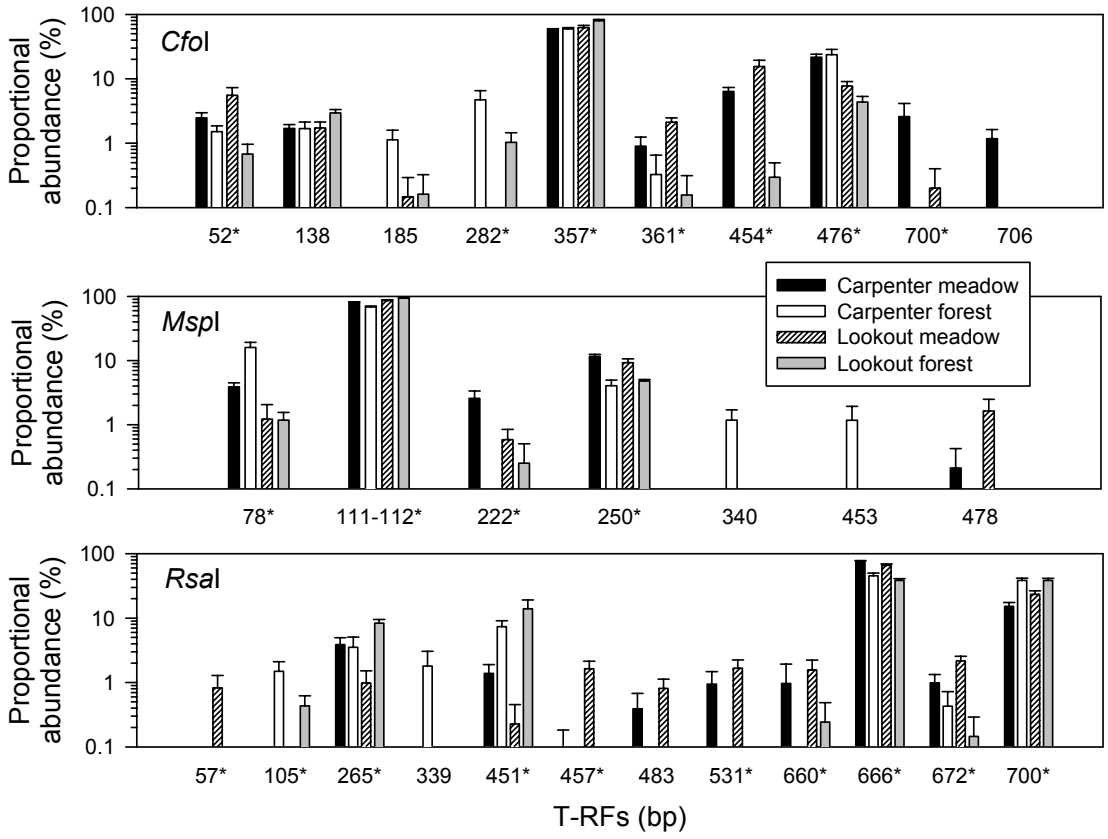



**Fig. 2.5.** *nosZ* T-RFs that differed significantly between vegetation type or site, based on Indicator Species Analysis ($p$-value <0.05). Each panel shows the restriction endonuclease used to generate the T-RFs. T-RFs marked with an asterisk (*) were present in sequenced *nosZ* clones.

to that expected by chance (McCune and Grace 2002). Twenty-nine significant indicator T-RFs ($p$-value <0.05) were found. Recognizing the several-orders-in-magnitude range in proportional abundance, indicator T-RFs are shown in Fig. 2.5 on a $\log_{10}$ scale to allow for inspection of less abundant T-RFs. On this scale, relatively large differences in the proportional abundance of more dominant fragments appear small (Fig. 2.5).

***nosZ* clones.** Forty-seven *nosZ* genotypes were identified among 225 clones based on *Cfo*I, *Msp*I, and *Rsa*I T-RFLP profiles of each clone. Two-thirds of the library consisted of clones from meadow soils and one-third from forest soils. Twenty-four genotypes occurred once and eight occurred twice; the most dominant genotype occurred 31 times. Based on species area curve analysis, a mean of 21 genotypes were found after screening 50 *nosZ* clones, 31 genotypes after screening 100 clones, and 44 genotypes after screening 200 clones. Twenty-one of the 29 indicator T-RFs shown in Fig. 4 were present in sequenced *nosZ* clones. One out of the 34 sequenced clones was non-specific for *nosZ* (i.e., not identifiable using Genbank's BLAST search), but the T-RFs for this clone were not detected in direct soil profiles.

***nosZ* fragment size.** Actual T-RF size was determined by sequencing *nosZ* clones. Fragment size based on T-RFLP profiling of the same clones was less precise and less accurate, but within a few base pairs or less (Table 2.5). In terms of accuracy, individual T-RFs >100 bp differed by <1 to 3 bp from sequencing (absolute mean difference 0.9 bp). T-RFs <100 bp were 3 to 7 bp smaller than expected (mean 5.5 bp; Table 2.5). In terms of precision, individual T-RFs varied in size by an average of 1.5 bp and a range of 0.3 to 5.1 bp. The dominant T-RF *Rsa*I 666 bp was identified as that

**Table 2.5.** Size of *nosZ* terminal restriction fragments (T-RFs) or PCR products of *nosZ* clones based on sequencing or T-RFLP profiling.

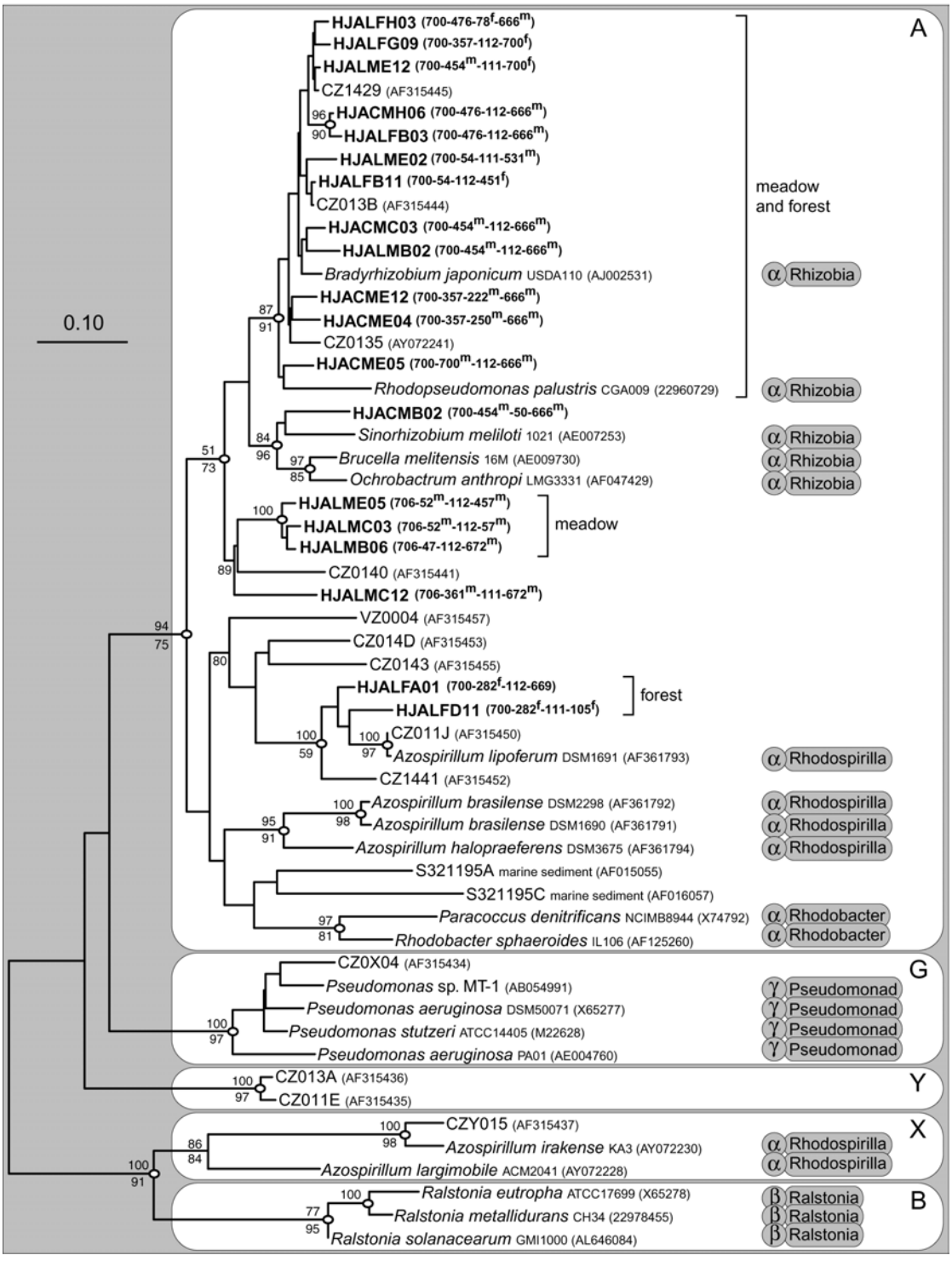
| | | Fragment size (bp) | | |
|---|---|---|---|---|
| | | | T-RFLP profiling | |
| Nuclease | n† | Sequencing | Mean | Range |
| *Cfo*I | 1 | 47 | 40.2 | NA‡ |
| *Cfo*I | 4 | 52 | 45.5 | 44.4-46.1 |
| *Cfo*I | 4 | 54 | 47.5 | 47.3-47.9 |
| *Cfo*I | 2 | 282 | 279.9 | 279.8-280.1 |
| *Cfo*I | 10 | 357 | 355.0 | 354.5-355.3 |
| *Cfo*I | 1 | 361 | 358.4 | NA |
| *Cfo*I | 7 | 454 | 452.6 | 451.5-453.1 |
| *Cfo*I | 3 | 476 | 475.7 | 474.8-476.3 |
| *Cfo*I | 1 | 700 | 697.9 | NA |
| *Msp*I | 1 | 46 | 41.3 | NA |
| *Msp*I | 1 | 50 | 45.1 | NA |
| *Msp*I | 2 | 78 | 74.6 | 74.4-74.8 |
| *Msp*I | 4 | 111 | 108.9 | 108.7-109.1 |
| *Msp*I | 21 | 112 | 109.5 | 107.9-110.1 |
| *Msp*I | 2 | 222 | 222.3 | 222.0-222.5 |
| *Msp*I | 2 | 250 | 249.4 | 249.4 |
| *Rsa*I | 1 | 57 | 51.5 | NA |
| *Rsa*I | 1 | 105 | 102.5 | NA |
| *Rsa*I | 1 | 265 | 263.8 | NA |
| *Rsa*I | 1 | 295 | 294.0 | NA |
| *Rsa*I | 4 | 451 | 450.8 | 450.4-451.0 |
| *Rsa*I | 1 | 457 | 454.4 | NA |
| *Rsa*I | 1 | 531 | 531.6 | NA |
| *Rsa*I | 1 | 660 | 659.3 | NA |
| *Rsa*I | 15 | 666 | 666.7 | 664.0-669.1 |
| *Rsa*I | 1 | 669 | 671.7 | NA |
| *Rsa*I | 2 | 672 | 671.6 | 671.3-672.0 |
| *Rsa*I | 4 | 700 | 700.5 | 700.4-701.6 |
| none | 29 | 700 | 700.8 | 697.9-703.2 |
| none | 4 | 706 | 705.3 | 704.4-706.4 |

†Number of clones with the T-RF; ‡NA, not applicable.

exact size in all fifteen sequencing cases, whereas with T-RFLP profiling, the T-RF varied by a range of 5.1 bp, from 664.0 to 669.1 bp (Table 2.5). Six T-RFs and the two undigested PCR products consistently varied by a range greater than 1 bp in T-RFLP profiling (Table 2.5). T-RFs of soil clones and direct soil T-RFLP profiles were in close agreement based on T-RFLP profiling, differing by an absolute mean of 0.4 bp (data not shown).

***nosZ* phylogeny.** Phylogenetic analysis of *nosZ* sequences revealed five major clusters of denitrifying bacteria (Fig. 2.6). Phylogenetic relationships among the cultured strains were similar based either on *nosZ* or SSU rRNA, with the exception of cluster X (Fig. 2.6). Tree topology was supported by all the phylogenetic analyses, with some differences in bootstrap support (Fig. 2.6). All the H. J. Andrews *nosZ* sequences grouped in cluster A, along with *α-Proteobacteria* (except cluster X) and most clones from a German forest soil and representatives of uncultured marine bacteria (Fig. 2.6, cluster A). The majority of the H. J. Andrews clones were most closely related to *nosZ* sequences from the *Rhizobiaceae* group and the German forest soil (Fig. 2.6). Separate from the *Rhizobiaceae nosZ*, two minor branches of H. J. Andrews clones formed meadow or forest specific groups (Fig. 2.6). These clones had sequences with T-RFs found preferentially in meadow or forest in the direct soil T-RFLP profiles (Fig. 2.6). Two clones from the German forest soil formed a major cluster without any pure culture sequences (cluster Y, Fig. 2.6), and another clone grouped with the sequence of *Azospirillum irakense* (cluster X, Fig. 2.6). A number of meadow and forest *nosZ* clones from the H. J. Andrews grouped with *Bradyrhizobium japonicum* USDA110 and

**Fig. 2.6.** Phylogenetic tree based on evolutionary distances among *nosZ* amino acid sequences (208 positions) from soils, marine sediment, and cultured bacteria. Clones from the H. J. Andrews soils are shown in bold, and CZ or VZ identify clones from a German forest soil (Rösch et al. 2002). Except for the H. J. Andrews clones, accession numbers are shown in parentheses. Phylogenetically defined taxonomic groups of cultured strains are identified to the subdivision and family level of *Proteobacteria*, respectively, based on SSU rRNA sequence analysis in the Ribosomal Database Project (RDP). Note that the suffix "-aceae" was dropped from the family name. Nodes with open ovals had >90% distance bootstrap support. Numbers above the branches are parsimony bootstrap values and below the branches are maximum likelihood quartet-puzzling support values (analogous to bootstrap values). Size of the PCR products and T-RFs of the H. J. Andrews *nosZ* clones are shown in parentheses in the order of (PCR product-*Cfo*I-*Msp*I-*Rsa*I). T-RFs from direct soil T-RFLP profiles that were more prevalent in the meadow or forest are identified with a superscript m for meadow and f for forest. Scale bar represents an evolutionary distance of 0.1.


0.10
HJALFH03 (700-476-78f-666m)
HJALFG09 (700-357-112-700f)
HJALME12 (700-454m-111-700f)
CZ1429 (AF315445)
HJACMH06 (700-476-112-666m)
HJALFB03 (700-476-112-666m)
HJALME02 (700-54-111-531m)
HJALFB11 (700-54-112-451f)
CZ013B (AF315444)
HJACMC03 (700-454m-112-666m)
HJALMB02 (700-454m-112-666m)
Bradyrhizobium japonicum USDA110 (AJ002531)
HJACME12 (700-357-222m-666m)
HJACME04 (700-357-250m-666m)
CZ0135 (AY072241)
HJACME05 (700-700m-112-666m)
Rhodopseudomonas palustris CGA009 (22960729)
HJACMB02 (700-454m-50-666m)
Sinorhizobium meliloti 1021 (AE007253)
Brucella melitensis 16M (AE009730)
Ochrobactrum anthropi LMG3331 (AF047429)
HJALME05 (706-52m-112-457m)
HJALMC03 (706-52m-112-57m)
HJALMB06 (706-47-112-672m)
CZ0140 (AF315441)
HJALMC12 (706-361m-111-672m)
VZ0004 (AF315457)
CZ014D (AF315453)
CZ0143 (AF315455)
HJALFA01 (700-282f-112-669)
HJALFD11 (700-282f-111-105f)
CZ011J (AF315450)
Azospirillum lipoferum DSM1691 (AF361793)
CZ1441 (AF315452)
Azospirillum brasilense DSM2298 (AF361792)
Azospirillum brasilense DSM1690 (AF361791)
Azospirillum halopraeferens DSM3675 (AF361794)
S321195A marine sediment (AF015055)
S321195C marine sediment (AF016057)
Paracoccus denitrificans NCIMB8944 (X74792)
Rhodobacter sphaeroides IL106 (AF125260)
CZ0X04 (AF315434)
Pseudomonas sp. MT-1 (AB054991)
Pseudomonas aeruginosa DSM50071 (X65277)
Pseudomonas stutzeri ATCC14405 (M22628)
Pseudomonas aeruginosa PA01 (AE004760)
CZ013A (AF315436)
CZ011E (AF315435)
CZY015 (AF315437)
Azospirillum irakense KA3 (AY072230)
Azospirillum largimobile ACM2041 (AY072228)
Ralstonia eutropha ATCC17699 (X65278)
Ralstonia metallidurans CH34 (22978455)
Ralstonia solanacearum GMI1000 (AL646084)
meadow and forest
meadow
forest
α Rhizobia
α Rhizobia
α Rhizobia
α Rhizobia
α Rhodospirilla
α Rhodospirilla
α Rhodospirilla
α Rhodospirilla
α Rhodobacter
α Rhodobacter
γ Pseudomonad
γ Pseudomonad
γ Pseudomonad
γ Pseudomonad
α Rhodospirilla
α Rhodospirilla
β Ralstonia
β Ralstonia
β Ralstonia
A
G
Y
X
B

*Rhodospeudomonas palustris* CGA009. These clones had T-RFs indicative of forest or meadow but were not distinguishable phylogenetically (Fig. 2.6).

## DISCUSSION

Based on *nosZ* distribution in the H. J. Andrews soils, denitrifier community composition appeared linked to differences in vegetation type and process rates. Analogous studies have demonstrated that shifts in the relative abundance of dominant organisms, and not complete turnover in community structure, can be associated with relatively large differences in biogeochemical processes (Eller and Frenzel 2001, Kleikemper et al. 2002, Lueders and Friedrich 2002, Ramakrishnan et al. 2001). Similarly, we found the same dominant denitrifier genotypes in meadow or forest soils, despite an order-of-magnitude greater denitrification activity in the meadow. Denitrifier community composition differed based on changes in relative abundance of genotypes or presence/absence of less dominant genotypes. But to what extent community changes are ecologically significant and how denitrifier communities influence process rates, remains unknown.

Based on a cross section of studies (Bohlen et al. 2001, Griffiths et al. 1998, Parsons et al. 1991, Vermes and Myrold 1992), mean DEA rates for temperate soils vary by four orders of magnitude (i.e., <1 to 3000 ng N $g^{-1}$ $h^{-1}$), with some of the lowest rates in mature coniferous forests in the Western Cascades (Vermes and Myrold 1992) and highest rates in wet agricultural systems receiving N inputs (Parsons et al. 1991). Although greater than forests in the H. J. Andrews, our meadow values are in the low to medium range of DEA (i.e., 10 to 100 ng N $g^{-1}$ $h^{-1}$). Intriguingly, DEA and net nitrification rates were of similar magnitude in meadow and forest soils (Table 2.1).

Although major $NO_3^-$ sinks were not evaluated in this study (e.g., denitrification vs. assimilatory $NO_3^-$ reduction), potential rates suggest that available $NO_3^-$ could be readily denitrified in these soils. In addition, because denitrification is notoriously variable, both spatially and temporally (Parkin 1990), and because these soils are high in organic matter and total N, more active denitrification could occur at anaerobic microsites or during transient conditions that favor anaerobic activity, such as during spring snow-melt or after the onset of autumn rains.

Denitrifier communities in the meadow were much more active under denitrifying conditions compared to the forest (Fig. 2.2). Similar differences between meadow and forest soils were found based on potential $N_2O$-reductase activities (Appendix A). Because $NO_3^-$ or an intermediate in the denitrification pathway is necessary for sustained denitrification activity (Härtig and Zumft 1999, Sabaty et al. 1999), lack of available $NO_3^-$ could explain low denitrification activity in the forest soils. Nitrate availability in the forest appeared limited for several reasons, including very low nitrification potential rates and accumulation of primarily $NH_4^+$ in N mineralization incubations (Table 2.1). Denitrifiers were present in the forest probably because most denitrifiers are facultative aerobic heterotrophs, which do not depend on denitrification activity for growth (Tiedje 1988). Greater $NO_3^-$ availability in the meadow may explain preferential selection of certain denitrifier genotypes in the meadow (e.g. *Cfo*I 454 bp, *Msp*I 250 bp, *Rsa*I 666 bp), but the ability to denitrify may not provide a strong selective advantage in these soils. Differences in substrate quality, as indicated by differences in C:N ratios (Table 1, Fig. 2), may be more important for determining denitrifier community structure.

The DNA-based T-RFLP approach provides a genetic profile of the denitrifier community; it does not measure specific activities or absolute abundances of individual denitrifiers. In addition, T-RFLP profiles may not reflect the actual relative abundance of genotypes in starting DNA templates because of PCR bias associated with degenerative primers that target functional genes (Lueders and Friedrich 2003). This bias has been demonstrated for model methanogenic communities, but for real communities it is more difficult to quantify because these are complex and genotype abundances are not known (Lueders and Friedrich 2003). Therefore, although we cannot rule out the possibility that certain genotypes are artificially over- or underrepresented in our *nosZ* T-RFLP profiles, qualitative trends are valid (Lueders and Friedrich 2003), as are differences between communities because all samples were analyzed identically. In addition, strong differences between vegetation types based either on proportional abundance or presence/absence demonstrate that our T-RFLP results were robust, and not necessarily dependent on proportional abundance (Fig. 2.3, Table 2.3).

To simultaneously analyze the distribution of *nosZ* T-RFs, we systematically characterized the denitrifier communities by applying multivariate statistics. We evaluated differences in overall community composition between habitats, assessed relationships between communities and process rates, and identified potentially key *nosZ* genotypes. By using non-parametric multivariate techniques, such as NMS, we avoided errors associated with assumptions that are often not met with community data, such as whether community members are linearly related or normally distributed (McCune and Grace 2002). NMS allowed us to differentiate communities based on the

overall community composition and MRPP provided a statistical test for these differences. Furthermore, the MRPP statistics can be compared among studies.

We quantified the degree to which denitrifier community composition was related to process rates by correlating process rates to NMS axes. About 50% of the variation in process rates (or C:N ratio) was explained with denitrifier community composition, based on cumulative $r^2$-values between rates and NMS axes (Fig. 2.3). King et al. (2001) constructed a more rigorous model for communities of sulfate-reducing bacteria, but their model did not appear to explain process variation any better. Environmental factors, such as soil water and $NO_3^-$ content often explain less than 50% of DEA variation or *in situ* denitrification (Myrold 1988, Parsons et al. 1991). NMS also allowed us to quantify different trends between sites. For example, when analyzing only forest samples, cumulative $r^2$-values with NMS axes (based on *nosZ* T-RF proportional abundance) were 0.95 for C:N ratios and 0.56 for DEA. Higher C:N ratios and lower DEAs at the Carpenter site likely reflect the older age structure of the forest at Carpenter compared to Lookout.

A potential problem with the PCR, and most methods for measuring diversity, is the inability to detect all the organisms of interest in a system. We amplified *nosZ* from pure cultures that represented a cross spectrum of *nosZ* sequences from *Proteobacteria*. In addition, we found divergent *nosZ* sequences from a poorly drained grassland soil that were most similar to *Azospirillum irakense* (53% similar based on amino acid sequences), *Ralstonia solanacearum* (64%), or *Pseudomonas stutzeri* (88%) (Chapter 3). Analogous to Friedrich (2002), we addressed lateral gene transfer of *nosZ* by comparing phylogenetic relationships of *nosZ* and taxonomic relationships based on

SSU rRNA (Fig. 2.6). Although we detected a major discrepancy for two *Azospirillum* spp. (cluster X, Fig. 2.6), relationships based on *nosZ* and SSU rRNA were in reasonable agreement for the rest of the strains.

Therefore, the majority of denitrifiers detected in the H. J. Andrews soils may share taxonomic and functional similarities to *Rhizobiaceae* in the *α-Proteobacteria*. Other studies have found numerous denitrifier sequences from soils that group closely with representatives of *Rhizobiaceae* (Philippot et al. 2002, Priemé et al. 2002, Rösch et al. 2002). The *Rhizobiaceae* are metabolically diverse. Symbiotic $N_2$-fixing *Rhizobium* and *Bradyrhizobium* spp. are known to denitrify (Tiedje 1988) and legumes are common in the meadow at each site. However, some *Rhizobium* spp. from soil lack symbiotic genes and behave as free-living saprophytes (Sullivan et al. 1996). The *Rhizobiaceae* strains shown in Fig. 2.6 utilize a wide range of organic substrates, such as aromatic constituents present in polyphenols and lignin (Parke and Ornston 1986, Paulsen 2002). Furthermore, 2,4-D degrading and oligotrophic bacteria that are closely phylogenetically related to *Bradyrhizobium* spp. have been isolated from soil (Kitagawa et al. 2002, Saito et al. 1998). None of the classical types of denitrifiers, such as pseudomonads or paracocci, were detected in the H. J. Andrews soils, nor were they prevalent in other soils or marine sediments (Philippot et al. 2002, Rösch et al. 2002, Scala and Kerkhof 1999).

H. J. Andrews *nosZ* clones with T-RFs indicative of meadow or forest (i.e., *Cfo*I 454 bp and *Rsa*I 451, 666, and 700 bp) did not necessarily form separate phylogenetic groups (Fig. 2.6). Other studies comparing different habitats or treatments have found similar results (Philippot et al. 2002, Priemé et al. 2002). In contrast, a few

H. J. Andrews *nosZ* clones with T-RFs indicative of habitat (i.e., *Cfo*I 52 and 282 bp and *Rsa*I 57, 105, 457, and 672 bp) formed monospecific phylogenetic groups according to meadow or forest (Fig. 2.6). The role of these specific groups in differentiating meadow and forest denitrification processes remains unclear.

In conclusion, denitrifier community composition and functioning appeared linked across meadow and forest soils. Denitrification activity was an order of magnitude higher in the meadow compared to the forest and denitrifier communities differed between vegetation types. The use of multivariate statistics enhanced our ability to quantify differences between denitrifier communities and relationships between community composition and process rates. Based on *nosZ* phylogenetic analysis, denitrifiers related to *Rhizobiaceae* were prevalent in the H. J. Andrews soils. This result awaits confirmation by either using genomic techniques that link taxonomic and functional genes or by attempting to culture the strains from soil, and then measuring absolute population size using quantitative techniques (Grüntzig et al. 2001). In addition to environmental factors, denitrifier community composition appears to contribute to denitrification rates, but a cause and effect relationship remains to be determined.

## ACKNOWLEDGMENTS

Financial support for this work was provided by the National Science Foundation Microbial Observatory Program (MCB-9977933) and the Oregon Agricultural Experiment Station.

We are grateful to Caprice Rosato, Mark Dasenko, and Scott Givan at the Center for Gene Research and Biotechnology at Oregon State University for providing DNA fragment analysis, sequencing, and bioinformatics support. We acknowledge the H. J. Andrews staff and the National Science Foundation Long Term Ecological Research program for infrastructure. Thank you to Nancy Ritchie-Posavatz for contributing to study design and analytical measurements, Anne Mintie for nitrification potential data, Shawna McMahon for soil profile descriptions, Stacie Kageyama for plant identification, Kirk Waterstripe for tree ages, and a number of other colleagues for field assistance.

# Chapter 3:

# Community Composition and $N_2O$-reductase Activity of Denitrifying Bacteria from Adjacent Agricultural Soil, Riparian Soil, and Creek Sediment in Oregon, USA

Jeremy J. Rich and David D. Myrold

Journal yet to be determined

## ABSTRACT

We examined denitrifying bacteria in wet soils and creek sediment from an agroecosystem in Oregon, USA that received inputs of synthetic-N fertilizer. Our objective was to determine if and how denitrifier community composition and activity differed in three adjacent habitats: a fertilized agricultural field planted to perennial ryegrass, a naturally vegetated riparian area, and creek sediment. Using $C_2H_2$ inhibition, denitrifying enzyme and $N_2O$-reductase activities were determined in short-term incubations of anaerobic slurries. A key gene in the denitrification pathway, $N_2O$ reductase (*nosZ*), served as a marker for denitrifiers. The ratio of $N_2O$ produced as a result of denitrification in the presence of glucose (10 mM) and $NO_3^-$ (5 mM) was substantially higher for riparian soil (0.64 ± 0.02; mean ± standard error, n = 12) compared to agricultural soil (0.19 ± 0.02) or creek sediment (0.32 ± 0.03). Mean denitrifying enzyme activity (DEA) was similar among habitats, ranging from 0.8 to 1.1 μg N $g^{-1}$ dry soil $h^{-1}$. But in the absence of $C_2H_2$, the riparian soil produced over three times more $N_2O$ than agricultural soil or creek sediment. In slurries that received $N_2O$ (18 μM) but not $NO_3^-$, mean $N_2O$-reductase activity ranged from 1.1 to 2.0 μg N $g^{-1}$ dry soil $h^{-1}$, with about 1.8 times higher activity in agricultural soil than in riparian soil or creek sediment. Community composition of denitrifiers differed significantly among habitats based on *nosZ* T-RFLPs. The creek sediment community was unique. Communities in the agricultural and riparian soil were more closely related but distinct. A number of unique genotypes were detected in creek sediment. Sequences of *nosZ* obtained from riparian soil were closely related to *nosZ* from *Bradyrhizobium* spp. or distantly related to *nosZ* from *Ralstonia* or *Azospirillum* spp. The results indicated that,

although community composition and activity differed among habitats, no simple correlation accurately described the relationship between activity and community composition.

## INTRODUCTION

During the past 40 years, annual inputs of biologically available nitrogen (N) have about doubled, mostly due to increased production and use of synthetic-N fertilizers (Smil 2001). Undesirable runoff and leaching of N from agricultural fields can be ameliorated in some instances by naturally vegetated riparian areas (i.e., zones of land adjacent to bodies of water) between agricultural fields and aquatic ecosystems (Martin et al. 1999). Because denitrification can be a dominant sink for nitrate ($NO_3^-$) and because riparian soils tend to be wetter and have more organic carbon compared to upland soils (i.e., conditions that favor denitrification), denitrification in riparian zones has received considerable attention (Martin et al. 1999). However, little is known about the community composition of denitrifying bacteria in riparian soils or adjacent agricultural and aquatic ecosystems. Whether denitrifier community composition contributes to differences in denitrification processes remains unknown. In addition, because the greenhouse gas nitrous oxide ($N_2O$) is both produced and consumed during denitrification, understanding links between denitrifier communities and $N_2O$ evolution has relevancy to processes that effect global warming.

Denitrification is the reduction of $NO_3^-$ or nitrite ($NO_2^-$) to $N_2O$ or dinitrogen ($N_2$) in the absence of $O_2$, in the pathway:

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

with abbreviations for the individual N-oxide reductases shown above arrows, e.g., Nos for $N_2O$ reductase. Denitrifying bacteria are defined solely by the facultative ability to denitrify. Most denitrifiers respire $O_2$ preferentially. Although many individual denitrifying bacteria reduce $NO_3^-$ to $N_2$, denitrification, in principle, consists of three

modules of reactions [i.e., (1) $NO_3^- \rightarrow NO_2^-$, (2) $NO_2^- \rightarrow NO \rightarrow N_2O$, and (3) $N_2O \rightarrow N_2$] that can occur independently (Zumft 1999). For example, some denitrifiers have a truncated pathway, lacking Nos, whereas other strains grow with $N_2O$ as the sole electron acceptor (Carlson and Ingraham 1983, Zumft 1999). Furthermore, in organisms that carryout complete denitrification, Nos synthesis and activity can be repressed and inhibited to a greater extant by $O_2$ compared to the other reductases (Betlach and Tiedje 1981, Otte et al. 1996), depending on the denitrifying strain (Zumft 1999). Nitrous oxide is a free intermediate in the denitrification pathway (Zumft 1997). If $N_2O$ production exceeds $N_2O$ consumption, $N_2O$ can diffuse away from the site of activity (Firestone et al. 1980). Therefore, the ratio of $N_2O$-to-$N_2$ that is evolved during denitrification can vary depending on the organisms involved and denitrifying conditions (Carlson and Ingraham 1983, Cavigelli and Robertson 2001, Zumft 1999). Generally for denitrifying soil communities, lower $N_2O/N_2$ ratios are associated with lower redox conditions (Firestone et al. 1980, Weier et al. 1993). Higher $N_2O/N_2$ ratios are associated with greater $NO_3^-$ or $NO_2^-$ concentrations, lower pH, or presence of $O_2$ (Firestone et al. 1980, Firestone and Tiedje 1979, Weier et al. 1993).

Denitrification is not specific to any one phylogenetic group; the trait is found in about 50 genera, mostly in the *Proteobacteria* (Zumft 1999). Therefore, to analyze denitrifier communities, functional genes in the denitrification pathway, such as $NO_2^-$ reductase (*nirK* and *nirS*) and $N_2O$ reductase (*nosZ*), have been retrieved from environmental samples using PCR and cloning and sequencing [Ch. 2 and (Braker et al. 2000, Rösch et al. 2002, Scala and Kerkhof 1999)]. Denitrifier community profiles have been generated using PCR-coupled terminal restriction fragment length

polymorphisms (T-RFLPs) (Avrahami et al. 2002, Braker et al. 2001, Scala and Kerkhof 2000). Variation in denitrification processes has been related to denitrifier community composition in some soils [Ch. 2 and (Avrahami et al. 2002)].

The objective of this study was to determine if and how denitrifier community composition and activity differed in soil or sediment from three adjacent habitats: a fertilized agricultural field, naturally vegetated riparian area, and intermittent stream. Previous work at the same site found that little fertilizer N actually reached the riparian soil because of *in situ* denitrification in the agricultural field (Horwath et al. 1998) or because most water flow from the agricultural field to the stream by-passed riparian soils in low-lying swales and secondary-stream channels (Wigington et al. 2003). In this study, we used anaerobic incubations of slurries to assess denitrifying enzyme activities. Nos activity was determined with additions of $NO_3^-$ or $N_2O$ in the presence or absence of $C_2H_2$. The same *nosZ* fragment examined by Rösch et al. (2002) was used as a marker for denitrifier communities. A few *nosZ* sequences were retrieved from riparian soil, and compared to *nosZ* sequences from environmental clones and pure cultures.

## METHODS

**Site and sampling description.** The study site was located in the Willamette Valley, Oregon, USA (44°32'N, 123°04'W, elevation 90 m). With 1100 mm of mean annual precipitation, this location has cool wet winters (mean temperature 6°C) and warm dry summers (mean temperature 19°C) (http://www.ocs.orst.edu). The site consisted of a riparian area (60 m wide by 300 m long) surrounded by extensive agricultural fields (450 hectares) planted to perennial ryegrass. Lake Creek, an

intermittent stream (1 to 3 m wide) that drained the surrounding agricultural fields, ran length-wise through the center of the riparian area. The site was fairly flat but sloped gradually (<1 m change in elevation) from the agricultural field to the riparian area. The riparian area had not been cultivated in 25 yr and was vegetated with native and non-native grasses (Wigington et al. 2003). Soils at the site consisted of poorly drained silt loams of the Dayton (Typic Albaqualfs) and Holcomb (Mollic Albaqualfs) soil series (Wigington et al. 2003). Soil pH was 5.4 for agricultural soils, 4.9 for riparian soils, and 5.6 for creek sediment.

The sample unit consisted of a core (6 cm diameter) of the top 10 cm of mineral soil or creek sediment. Twelve cores were taken in each habitat during the study period from 4 April to 16 May 2001, which coincided with peak growth of grass vegetation and moderate stream flow. The agricultural field received 65 kg N $ha^{-1}$ on 12 March as urea and $(NH_4)_2SO_4$ and 95 kg N $ha^{-1}$ on 10 April as urea. The cores were taken randomly in 1 by 10 m plots (three cores per plot). Plots were placed at 60-m intervals in three positions (east, middle, and west) in the agricultural field and riparian area. The creek sediment had plots in the east and west positions, in addition to plots that were near (<1 m) and far (>20 m) from trees lining the creek. Two plots in the agricultural field and riparian area were sampled on different dates to assess temporal variation. Cores were kept at 4°C until analyzed for denitrification activity 1 to 5 d after sampling. Activity was stable over this period based on analyzing samples in random order (data not shown).

**Denitrifying enzyme and $N_2O$-reductase activities.** The following activities were determined using laboratory incubations of anaerobic slurries: denitrifying

enzyme activity (DEA), the ratio of $N_2O$ formed as an end product of denitrification (abbreviated as $rN_2O$), and maximum potential $N_2O$-reductase activity (abbreviated $Nos_{maxp}$).

Following standard procedures to determine DEA (Tiedje 1994), $N_2O$ formation was measured in slurries that received glucose (10 mM), $NO_3^-$ (5 mM), and acetylene ($C_2H_2$) (10% v/v). Nitrous oxide formation ($N_2O_{den}$) was also measured in duplicate slurries that did not receive $C_2H_2$. Based on previous work (Cavigelli and Robertson 2000, Firestone and Tiedje 1979, Hénault et al. 2001), $rN_2O$ was calculated using the following equation: $rN_2O = N_2O_{den}$ / DEA. $N_2O$-reductase activity was calculated as the difference in $N_2O$ flux rate with and without $C_2H_2$.

Analogous to Hénault et al. (2001), $Nos_{maxp}$ was determined indirectly by measuring $N_2O$ disappearance in slurries that received glucose (10 mM) and $N_2O$ (approximately 18 μM = 780 ppm v/v). Nitrous oxide formation was also measured in duplicate slurries that received $C_2H_2$ (10% v/v). Incubations were kept relatively short (60 to 85 min) to assure that reaction rates remained constant. To calculate $Nos_{maxp}$, we assumed that $N_2O$ concentrations in the absence of $C_2H_2$ were dependent on the reaction: $NO_3^- \xrightarrow{k_1} N_2O \xrightarrow{k_2} N_2$, where $k_1$ = $NO_3^-$ reduction to $N_2O$ and $k_2$ = $N_2O$ reduction to $N_2$. It follows that net $N_2O$ disappearance reflected the difference between $k_1$ and $k_2$, as expressed in the equation: $N_2O_m = k_2 - k_1$; where $N_2O_m$ = measured rate of $N_2O$ disappearance. Based on reported $K_m$ values of 0.1 to 0.4 μM for $N_2O$ reduction in soils (Holtan-Hartwig et al. 2000), we assumed that the concentration of $N_2O$ did not limit $k_2$. Therefore, $k_2$ was equal to $Nos_{maxp}$. Given our assay conditions, $C_2H_2$ probably did not affect the rate of $NO_3^-$ reduction to $N_2O$ (Knowles 1990). Therefore, we

assumed that $N_2O$ formation in the presence of $C_2H_2$ was equal to $k_1$. $Nos_{maxp}$ was calculated by adding the absolute rates of $N_2O$ disappearance in the absence of $C_2H_2$ and $N_2O$ formation in the presence of $C_2H_2$ (i.e., $Nos_{maxp} = N_2O_m + k_1$).

Slurries were prepared by vigorously homogenizing each core on a reciprocal shaker (10 min) in 2-l flasks with two parts (based on fresh soil or sediment weight) phosphate buffer (50 mM, pH 7.0). The resulting pH for slurries was 6.2 for agricultural soil, 6.0 for riparian soil, and 6.3 for creek sediment. For each slurried core, samples (50 ml) were dispensed into four Erlenmeyer flasks (125 ml). Samples for DNA analysis were collected by centrifugation (14,000 × *g*, 10 min, 4°C) of slurry aliquots (1.8 ml), and pellets were immediately frozen (-80°C). All the Erlenmeyer flasks received a concentrated solution of glucose and half the flasks, a concentrated solution of $NO_3^-$. Flasks were immediately sealed with neoprene stoppers and made anaerobic by five cycles of evacuation and flushing with Ar. Nitrous oxide (ultra-high purity, 99.99%) was added to half of the flasks that did not receive $NO_3^-$. Standard grade $C_2H_2$ was added to half of the flasks that received $NO_3^-$ or $N_2O$. Identical results were obtained with $C_2H_2$ synthesized from calcium carbide under anaerobic conditions, or when cores and slurries were processed in an anaerobic glove bag with deoxygenated solutions (data not shown). Incubations were immediately initiated by placing the flasks (1 atm) on a rotary shaker (250 rpm, 25°C). During incubations of 60 or 85 min, gas samples were taken every 15 or 20 min and stored in Vacutainers® (3 ml) until analyzed for $N_2O$ by gas chromatography using a $^{63}Ni$ electron capture detector (Myrold 1988). Rates of $N_2O$ consumption or production were obtained by linear regression (e.g., Fig. 3.1).

Concentrations of inorganic N ($NO_3^-$ and $NH_4^+$) in 2 N KCl extracts of soil cores (0 to 10 cm) were measured by standard colorimetric methods with an autoanalyzer.

***nosZ* PCR, T-RFLPs, and data analyses.** Procedures for analyzing *nosZ* fragments from soil DNA, including PCR amplification and T-RFLP profiling, were performed as previously described (Chapter 2). Briefly, DNA was extracted from thawed and resuspended slurry pellets using the FastDNA® kit for soil (Bio101, Inc.), according to the manufacturer's instructions. DNA was quantified as previously described (Chapter 2), and yields were (in units of μg DNA $g^{-1}$ dry soil) 6 for agricultural soil, 13 for riparian soil, and 7 for creek sediment. Three T-RFLP profiles were generated per sample-unit in separate reactions with the endonucleases *Cfo*I (isoschisimer of *Hha*I), *Msp*I, or *Rsa*I (Roche, Co.). Terminal-restriction fragments (T-RFs) with signals above a detection limit of 10 relative fluorescent units were summed for each profile; only fragments with a signal above 3% of the sum of all peaks were included in the analyses.

***nosZ* cloning, sequencing, and phylogenetic analysis.** Fragments of *nosZ* were amplified from DNA that was obtained from riparian soil in July 1999. The *nosZ* fragments were excised from an agarose gel and cloned as previously described (Chapter 2). Clones were screened by sizing 6-FAM labeled PCR products before and after digestion with *Cfo*I and *Rsa*I. Amplified from clones, gel purified *nosZ* fragments were sequenced using the forward *nosZ* primer. Sequencing reactions were done with ABI Prism® BigDye™ Terminator Cycle Sequencing and the samples were analyzed

on an ABI Prism® Genetic Analyzer. Phylogenetic analyses were performed as previously described (Chapter 2).

**Statistical Analyses.** SAS v7.0 (SAS Institute, Inc.) was used for statistical analysis of activity parameters or diversity indices. Differences between habitats were tested by one-factor ANOVA, followed by Tukey's test for mean multiple comparisons. Differences among habitats, accounting for differences among east, middle, and west, positions were examined by two-factor ANOVA. Differences between dates or other specific paired comparisons were evaluated with t-tests. Correlations between denitrification activity and diversity indices were assessed using linear regression.

PC-ORD v4.01 (McCune and Mefford 1999) was used for multivariate analyses of the *nosZ* T-RF profiles. Differences in community composition were assessed graphically using the ordination method of Non-metric Multidimensional Scaling (NMS), based on Sørensen's distance (Kruskal 1964, McCune and Grace 2002). Synonymous with Bray-Curtis distance, Sørensen's distance is a relative distance measure (McCune and Grace 2002). To quantify relationships between *nosZ* distribution and denitrifying activities, NMS ordinations were correlated with activities (Chapter 2). Cluster analyses, with Sørensen's and relative Euclidean distances and linkage methods of Ward's and flexible beta ($\beta = -0.5$) (McCune and Grace 2002), were used to assess distinct groups. To graphically display relative distances among habitats, mean *nosZ* T-RF distribution or activities were analyzed with Sørensen's distance and the weighted pair group method (UPGMA) (Swoffard et al. 1996). A Multi-response Permutation Procedure (MRPP) (McCune and Grace 2002, Mielke

1984), with Sørensen's distance and rank transformation, was used to test for significant differences in community composition among groups defined by habitat, position, date, or near and far from trees. The MRPP *A*-statistic describes the within and between group relatedness relative to that expected by chance (McCune and Grace 2002). It is somewhat analogous to an $r^2$-value (McCune and Grace 2002). To identify T-RFs that differentiated denitrifier communities by habitat, Indicator Species Analysis (Dufrêne and Legendre 1997) was run with 1000 randomizations in a Monte Carlo test.

## RESULTS

**Denitrifying enzyme and $N_2O$-reductase activities.** Nitrous oxide-reductase activity was greater in agricultural soil compared to riparian soil (Fig. 3.1). In slurries that received $NO_3^-$ (5 mM) and $C_2H_2$, $N_2O$ formation was similar (Fig. 3.1A), but in the absence of $C_2H_2$, $N_2O$ formation was lower in the agricultural soil (Fig. 3.1A). In slurries that received $N_2O$ (18 μM) and $C_2H_2$ but not $NO_3^-$, $N_2O$ formation was greater in the agricultural soil (Fig. 3.1B). In duplicate slurries without $C_2H_2$, $N_2O$ disappearance was detected, and more $N_2O$ disappearance was found in agricultural soil (Fig. 3.1B). Except for $N_2O$ disappearance, trends shown in Fig. 3.1 were consistent for all samples.

Based on all samples, the ratio of $N_2O$ produced to DEA, in the presence of 5 mM $NO_3^-$, was substantially higher for riparian soil (0.64 ± 0.02; mean ± standard error, n = 12) compared to agricultural soil (0.19 ± 0.02) or creek sediment (0.32 ± 0.03). Habitat differences based on $rN_2O$ were highly significant

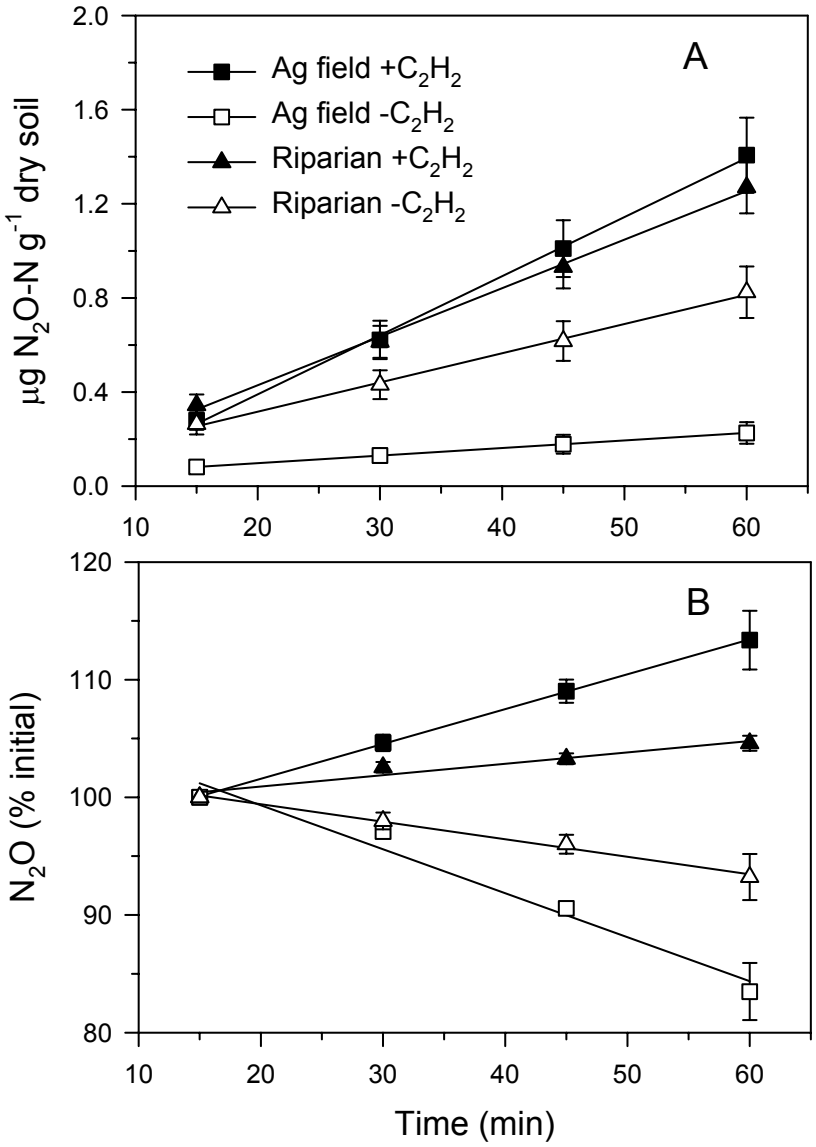



**Fig. 3.1.** Nitrous oxide formation or disappearance in anaerobic soil slurries that received 10 mM glucose, and either 5 mM $NO_3^-$ (A) or 18 μM $N_2O$ (B), with and without $C_2H_2$. Data are from cores obtained 24 April 2001 from the west position in the agricultural field and riparian area. Symbols are means (± SE, n = 3), lines fit by linear regression. In (B), y-axis units are expressed as percent initial $N_2O$, because initial concentrations varied by a SD of ± 1 μM $N_2O$.

($p < 0.0001$, one-factor ANOVA for habitat) and each habitat had a distinct $rN_2O$ signature based on Tukey's multiple comparison among means ($p < 0.01$).

In contrast to $rN_2O$, DEA was similar among habitats ($p = 0.12$, Fig. 3.2A). In slurries that received $NO_3^-$ (5 mM) but not $C_2H_2$, the riparian soil produced over three times more $N_2O$ compared to the agricultural soil or creek sediment ($p < 0.0001$, Fig. 3.2A). Nos activity was about double in agricultural soil ($p = 0.005$, Fig. 3.2A) than in riparian soil or creek sediment. In slurries that received $N_2O$ and $C_2H_2$ but not $NO_3^-$, mean $N_2O$ production was about two-fold higher in agricultural soil ($p = 0.0003$, Fig. 3.2B) than in riparian soil or creek sediment. Except for the noted exception in Fig. 3.1B, in duplicate slurries without $C_2H_2$, $N_2O$ disappearance was not different among habitats ($p = 0.55$, Fig. 3.2B). Mean maximum potential Nos activity was about 70% greater in agricultural soil ($p = 0.02$, Fig. 3.2B) than riparian soil or creek sediment.

The distributions of $rN_2O$, DEA, and $Nos_{maxp}$ as a function of habitat position or sampling date are shown in Table 3.1. Statistical differences in activities between habitats or positions were determined for each sampling date (Table 3.2) or for the same plots sampled on different dates (Table 3.3).

Within habitats, $rN_2O$ showed little variation between plot position (Table 3.1; $p > 0.1$ for position, Table 3.2), and no difference between samples taken from the same plot on different dates (Table 3.1; $p > 0.5$, Table 3.3). In contrast, DEA and $Nos_{maxp}$ were more variable within habitats or on different dates (Table 3.1, 3.2, and 3.3). Highest $Nos_{maxp}$ was detected on 24 April 2001 in soil from the agricultural field in the west position (Table 3.1). On 24 April 2001, $Nos_{maxp}$ differed significantly between the agricultural and riparian soil and between middle and west positions

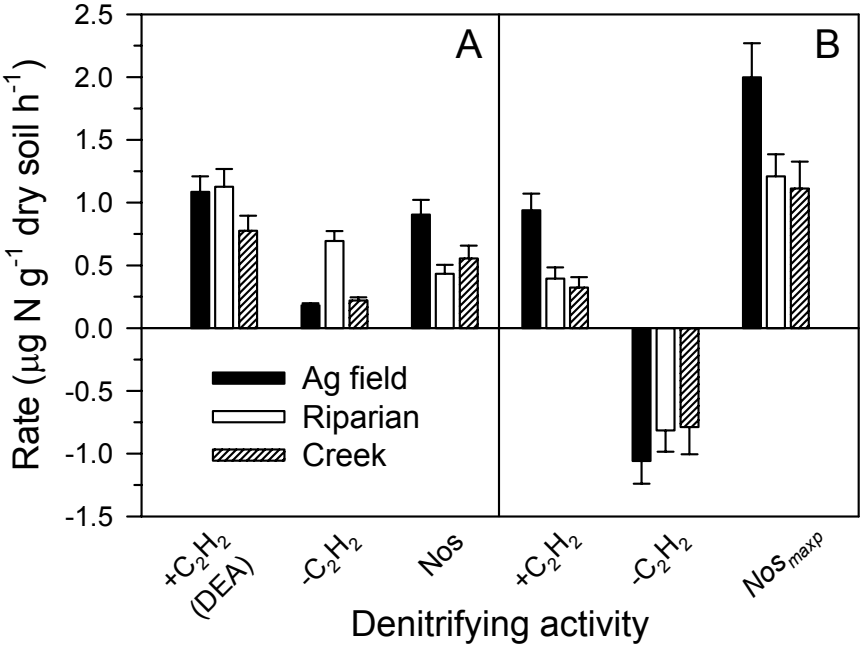



**Fig. 3.2.** Rates of $N_2O$ formation, disappearance (negative values), or Nos activity in anaerobic slurries that received 10 mM glucose, and either 5 mM $NO_3^-$ (A) or 18 μM $N_2O$ (B), with and without $C_2H_2$. Bars are the mean (± SE, n = 12) for each habitat. Nos is an abbreviation for $N_2O$-reductase activity and $Nos_{maxp}$ for maximum potential $N_2O$-reductase activity. Nos was calculated as the difference between $N_2O$ flux rates, with and without $C_2H_2$.

**Table 3.1.** Distribution of denitrifying enzyme and $N_2O$-reductase activities in agricultural and riparian soil and creek sediment.

| Sample | Location | | Activities†, mean (SD, n = 3) | | |
|---|---|---|---|---|---|
| | | | μg N $g^{-1}$ dry soil $h^{-1}$ | | |
| Date* | Habitat | Position | $rN_2O$ | DEA | $Nos_{maxp}$ |
| 4 April | Riparian | East | 0.71 (0.06) | 0.7 (0.2) | 0.8 (0.8) |
| | | West | 0.57 (0.08) | 1.8 (0.2) | 1.7 (0.4) |
| | Creek | East | 0.27 (0.05) | 0.5 (0.1) | 1.0 (0.4) |
| | | West | 0.29 (0.13) | 1.0 (0.5) | 1.7 (0.8) |
| 17 April | Creek | Tree | 0.39 (0.12) | 0.7 (0.6) | 0.5 (1.0) |
| | | No Tree | 0.33 (0.03) | 1.0 (0.2) | 1.3 (0.4) |
| 24 April | Ag field | Middle | 0.16 (0.06) | 1.2 (0.4) | 1.6 (0.7) |
| | | West | 0.13 (0.03) | 1.5 (0.3) | 3.2 (0.1) |
| | Riparian | Middle | 0.66 (0.14) | 0.8 (0.2) | 0.8 (0.3) |
| | | West | 0.60 (0.10) | 1.3 (0.2) | 1.5 (0.4) |
| 16 May | Ag field | East | 0.29 (0.03) | 0.5 (0.1) | 1.1 (0.2) |
| | | West | 0.17 (0.10) | 1.1 (0.1) | 2.1 (0.7) |

†Abbreviations are $rN_2O = N_2O/(N_2O + N_2)$; DEA = denitrifying enzyme activity; $Nos_{maxp}$ = maximum potential $N_2O$-reductase activity.
*All samples were obtained in 2001.

**Table 3.2**. Statistical differences in denitrifying enzyme and $N_2O$-reductase activities between habitats or positions.

| | | *p*-value | | |
|---|---|---|---|---|
| Date | Factor | $rN_2O$ | DEA | $Nos_{maxp}$ |
| 4 April (n‡ = 12)† | Habitat (riparian vs. creek) | <0.0001 | 0.05 | 0.8 |
| | Position (east vs. west) | 0.3 | 0.004 | 0.06 |
| 17 April (n = 6)* | Position (creek tree vs. no tree) | 0.4 | 0.5 | 0.3 |
| 24 April (n = 12)† | Habitat (ag field vs. riparian) | <0.0001 | 0.05 | 0.002 |
| | Position (east vs. west) | 0.4 | 0.03 | 0.004 |
| 16 May (n = 6)* | Position (ag field east vs. west) | 0.1 | 0.004 | 0.06 |

‡n = number of observations.
†Two-factor ANOVA.
*t-test.

**Table 3.3.** Statistical differences in denitrifying enzyme and $N_2O$-reductase activities between dates.

| | *p*-value | | |
|---|---|---|---|
| Habitat (dates) | $rN_2O$ | DEA | $Nos_{maxp}$ |
| Riparian (4 April vs. 24 April, n‡ = 6)* | 0.7 | 0.02 | 0.6 |
| Ag field (24 April vs. 16 May, n = 6)* | 0.5 | 0.1 | 0.05 |

‡n = number of observations.
*t-test.

(Table 3.2). DEA tended to differ more strongly between positions than between habitats (Table 3.2). Creek sediment that was sampled near or far from trees showed no difference in denitrification activity (Table 3.1 and 3.2).

Based on cores obtained on 24 April 2001, mean ± SE (n = 3) bulk $NO_3^-$ concentration (in units of μg N $g^{-1}$ dry soil) for agricultural soil of 2.2 ± 0.7 was twice that for riparian soil of 0.9 ± 0.1. Mean $NH_4^+$ values (in units of μg N $g^{-1}$ dry soil) were 69.7 ± 19.8 for agricultural soil and 2.2 ± 0.2 for riparian soil. In soils obtained from the agricultural field on 16 May 2001, mean ± SE (n = 3) inorganic N concentrations (in units of μg N $g^{-1}$ dry soil) were 2.0 ± 0.3 for $NO_3^-$ and 1.5 ± 0.1 for $NH_4^+$ in the west position and 0.9 ± 0.2 for $NO_3^-$ and 1.3 ± 0.1 for $NH_4^+$ in the east position. Based on these $NO_3^-$ values, concentrations of $NO_3^-$-N in slurries that did not receive exogenous $NO_3^-$ ranged from about 15 to 45 μM. Less than one tenth of this N was reduced to $N_2O$ during incubations.

***nosZ* T-RF profiles and diversity.** Seventy-four *nosZ* T-RFs were detected in this study based on combined *Cfo*I, *Msp*I, and *Rsa*I T-RFLP profiles. A mean of 21 T-RFs were detected in each sample-unit. In *Msp*I and *Rsa*I profiles, one dominant fragment (i.e., *Msp*I 111-112 bp and *Rsa*I 666 bp) was present in every sample, comprising 60% of the mean total abundance in each profile. In contrast, dominant T-RFs in *Cfo*I profiles comprised 30% of the mean total abundance in each profile. Based on diversity indices, greater evenness was found for *Cfo*I profiles compared to *Msp*I and *Rsa*I (Table 3.4). With about six T-RFs per profile, *Msp*I profiles were less diverse compared to *Cfo*I and *Rsa*I (Table 3.4). Based on *Rsa*I only, the agricultural field and creek sediment had greater T-RF richness, evenness, and Shannon's diversity compared

**Table 3.4.** Diversity indices for *nosZ* T-RFLP profiles in agricultural soil, riparian soil, and creek sediment.

| | | Total No. | Mean (SE)√ | | |
|---|---|---|---|---|---|
| Enzyme | Habitat | of T-RFs | Richness† | Evenness‡ | Diversity* |
| *Cfo*I | Ag field | 19 | 7.9 (0.6) | 0.85 (0.01) | 1.73 (0.07) |
| | Riparian | 14 | 7.5 (0.3) | 0.87 (0.01) | 1.74 (0.05) |
| | Sediment | 18 | 7.1 (0.3) | 0.85 (0.03) | 1.66 (0.08) |
| | All samples | 29 | 7.5 (0.2) | 0.86 (0.01) | 1.71 (0.04) |
| *Msp*I | Ag field | 10 | 6.3 (0.4) | 0.77 (0.02)$^{a}$§ | 1.40 (0.06)$^{a}$ |
| | Riparian | 13 | 5.8 (0.4) | 0.63 (0.03)$^{b}$ | 1.10 (0.09)$^{b}$ |
| | Sediment | 12 | 5.6 (0.3) | 0.54 (0.02)$^{c}$ | 0.93 (0.06)$^{b}$ |
| | All samples | 17 | 5.9 (0.2) | 0.65 (0.02) | 1.14 (0.05) |
| *Rsa*I | Ag field | 20 | 9.7 (0.5)$^{a}$ | 0.75 (0.01)$^{a}$ | 1.70 (0.06)$^{a}$ |
| | Riparian | 11 | 6.0 (0.4)$^{b}$ | 0.66 (0.02)$^{b}$ | 1.18 (0.06)$^{b}$ |
| | Sediment | 15 | 8.3 (0.5)$^{a}$ | 0.72 (0.03)$^{ab}$ | 1.52 (0.08)$^{a}$ |
| | All samples | 28 | 8.0 (0.4) | 0.71 (0.01) | 1.46 (0.05) |
| Combined | Ag field | 49 | 23.9 (1.0)$^{a}$ | 0.86 (0.01)$^{a}$ | 2.71 (0.05)$^{a}$ |
| | Riparian | 38 | 19.3 (1.0)$^{b}$ | 0.83 (0.01)$^{ab}$ | 2.44 (0.07)$^{b}$ |
| | Sediment | 45 | 21.0 (0.7)$^{ab}$ | 0.81 (0.01)$^{b}$ | 2.47 (0.05)$^{b}$ |
| | All samples | 74 | 21.4 (0.6) | 0.83 (0.01) | 2.54 (0.04) |

√SE = standard error; n = 12 for habitat, n = 36 for all samples.
†Richness = $S$ = number of T-RFs detected in a profile.
‡ $\text{Evenness} = \frac{H'}{\ln S}$.

*Diversity = Shannon's diversity index $= H' = -\sum_{i}^{S} p_i \log p_i$ ; where $p_i$ = T-RF abundance divided by total abundance in a profile.

§Significant differences ($p < 0.05$) between habitats are shown with superscripts *a*, *b*, or *c*, based on Tukey's procedure for multiple comparisons among means.

to the riparian area (Table 3.4). *Rsa*I T-RF richness or Shannon's diversity was negatively correlated with $rN_2O$ ($r^2 = 0.5$, $p < 0.0001$). Diversity indices based on *Cfo*I profiles showed no difference between habitats, and *Msp*I showed different patterns in evenness compared to *Rsa*I (Table 3.4). Diversity indices showed no difference on different dates or little to no difference between positions within habitats.

**Differences in *nosZ* distribution.** Denitrifier communities formed three distinct groups according to habitat, based on presence/absence of T-RFs in combined *Cfo*I, *Msp*I, and *Rsa*I *nosZ* profiles (Fig. 3.3). Creek sediment communities were unique (Fig. 3.3). Agricultural and riparian soil communities were more closely related but still distinct, with the exception of one agricultural sample-unit that grouped among riparian sample-units (Fig. 3.3). Based on cluster analyses, three distinct groups of denitrifying communities were also found according to habitat, with the exception of the same agricultural sample-unit (data not shown). MRPP *p*-values indicated that community differences between habitats were highly significant. MRPP *A*-statistics (*p*-value) for the following pair-wise comparisons were: 0.45 ($p < 10^{-6}$) for agricultural soil vs. creek sediment, 0.23 ($p < 10^{-5}$) for agricultural vs. riparian soil, and 0.44 ($p < 10^{-6}$) for riparian soil vs. creek sediment. MRPP *A*-statistics above about 0.3 reflect distantly related groups (McCune and Grace 2002). Similar results were obtained with individual or combined profiles based on presence/absence or proportional abundance of T-RFs (data not shown).

MRPP was used further to examine differences in *nosZ* distribution on different dates or by positions within habitats. Communities showed no difference on different dates in either the agricultural or riparian soil ($A \leq 0.03$, $p > 0.3$; 6 observations for

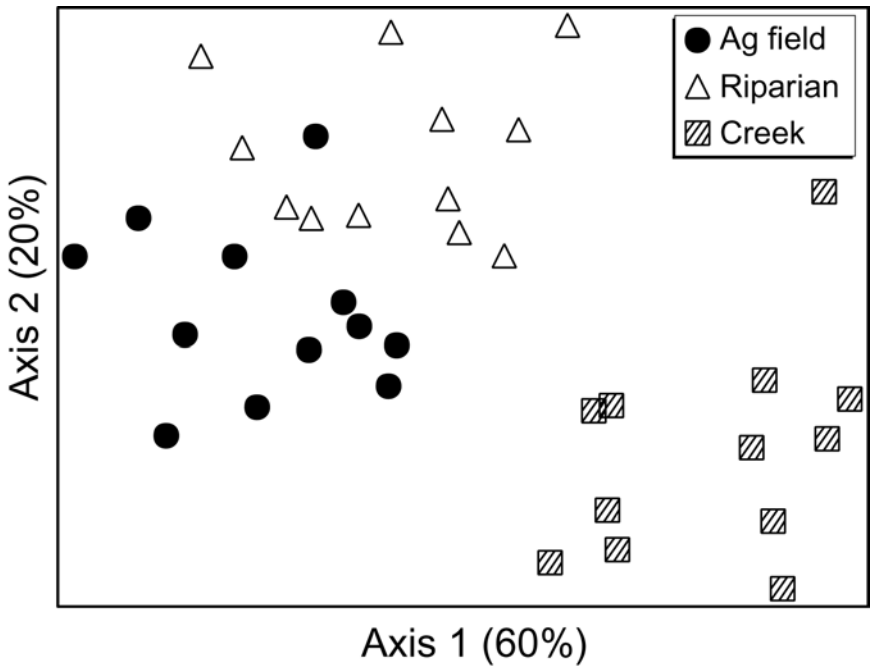



**Fig. 3.3.** Non-metric Multidimensional Scaling (NMS) ordination of denitrifier community composition based on presence/absence of T-RFs in combined *Cfo*I, *Msp*I, and *Rsa*I *nosZ* T-RFLP profiles. Points represent samples taken in this study. The percent variation explained by each axis is shown in parentheses.

each test). Within the agricultural field or riparian area, communities differed between positions ($A$ = 0.26, $p$ = 0.005 for agricultural soil; $A$ = 0.27, $p$ = 0.004 for riparian soil; 12 observations for each test). In the agricultural soil, communities from the west position were in between eastern and middle positions in the NMS plot (data not shown). Riparian communities from the west position tended to group more closely to communities from the agricultural soil in the NMS plot (data not shown). Communities from different positions in the creek showed no difference ($A$ = 0.09, $p$ = 0.14).

**Relationships between *nosZ* distribution and activities.** If relationships between *nosZ* distribution and denitrifying activities were consistent across habitats, then relative differences among habitats should be similar based either on *nosZ* distribution or activities. However, this was not the case, as demonstrated by UPGMA analysis of mean *nosZ* distribution and $rN_2O$ (Fig. 3.4). Agricultural and riparian soil were more closely related based on *nosZ* (Fig. 3.4), but agricultural soil and creek sediment were more closely related based on $rN_2O$ (Fig. 3.4). Relationships among habitats also did not agree based on *nosZ* distribution and other denitrifying activities.

To quantify differing relationships between denitrifier activities and community composition across habitats, activities were correlated with each of four NMS ordinations, one for all samples (i.e., ordination shown in Fig. 3.3) and three ordinations for each combination of paired habitats (Table 3.5; ordinations for paired habitats not shown). Based on all samples, $rN_2O$ was more strongly correlated with *nosZ* distribution than other activities (Table 3.5). However, correlations between $rN_2O$ and NMS ordinations were stronger for paired habitats of agricultural and riparian soil

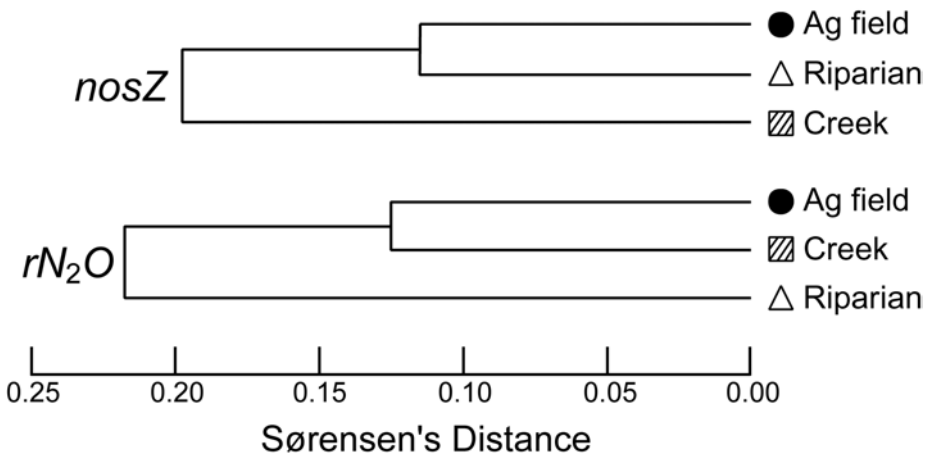



**Fig. 3.4.** UPGMA dendrogram of Sørensen's distance among habitats based on mean *nosZ* distribution and *rN$_2$O*. *nosZ* distribution was based on presence/absence of T-RFs in combined *Cfo*I, *Msp*I, and *Rsa*I T-RFLP profiles. *rN$_2$O* is an abbreviation for $N_2O/(N_2O + N_2)$.

**Table 3.5.** Correlation coefficients ($r^2$) between denitrifier activities and non-metric multidimensional scaling (NMS) ordinations of *nosZ* T-RFLP profiles.*

| Activity‡ | Ag field, Riparian, and Creek NMS | Ag field and Riparian NMS | Ag field and Creek NMS | Riparian and Creek NMS |
|---|---|---|---|---|
| $rN_2O$ | **0.402**† | **0.661** | 0.239 | **0.743** |
| $NO_3^-$ -$C_2H_2$ | 0.307 | 0.288 | 0.153 | **0.665** |
| $NO_3^-$ +$C_2H_2$ (DEA) | 0.211 | 0.181 | 0.325 | 0.223 |
| $NO_3^-$ Nos | 0.276 | **0.469** | 0.330 | 0.153 |
| $N_2O$ -$C_2H_2$ | 0.129 | 0.191 | 0.147 | 0.115 |
| $N_2O$ +$C_2H_2$ | 0.170 | 0.202 | 0.357 | 0.027 |
| $Nos_{maxp}$ | 0.196 | 0.287 | 0.295 | 0.111 |

*$r^2$-values are from correlations between each activity parameter and NMS ordination of presence/absence T-RFs in combined *Cfo*I, *Msp*I, and *Rsa*I *nosZ* profiles.

‡Abbreviations are: $rN_2O$ = $N_2O/(N_2O + N_2)$; $NO_3^-$ -$C_2H_2$ = $N_2O$ formation in the presence of 5 mM $NO_3^-$; DEA = denitrifying enzyme activity; $NO_3^-$ Nos = $N_2O$-reductase activity in the presence of 5 mM $NO_3^-$; $N_2O$ -$C_2H_2$ = $N_2O$ disappearance in slurries that received 18 μM $N_2O$; $N_2O$ +$C_2H_2$ = $N_2O$ formation in the presence of 18 μM $N_2O$ and $C_2H_2$; and $Nos_{maxp}$ = maximum potential $N_2O$-reductase activity.

†$r^2$-values > 0.4 are highlighted in bold.

or riparian soil and creek sediment than for all samples or agricultural soil and creek sediment (Table 3.5).

**Individual *nosZ* T-RFs.** Indicator species analysis was used to identify *nosZ* T-RFs that differentiated habitats (Fig. 3.5). This procedure examines the relative abundance and presence/absence of individual T-RFs in a predefined group compared to that expected by chance (McCune and Grace 2002). T-RFs that were found preferentially in different habitats, as shown in Fig. 3.5, are defined here as indicator T-RFs.

The distribution of proportionally abundant T-RFs differed between habitats (i.e., *Cfo*I 357 bp 476 bp, 700 bp, *Msp*I 111-112 bp, and *Rsa*I 666 bp) (Fig. 3.5). The T-RF *Cfo*I 700 bp was more prevalent in creek sediment (Fig. 3.5). A number of other T-RFs were found preferentially in creek sediment, including: *Cfo*I 233 bp; *Msp*I 111-112 bp and 250 bp; and *Rsa*I 105 bp, 265 bp, 271 bp, 316 bp, and 457 bp (Fig. 3.5). Three T-RFs (i.e., *Cfo*I 233 bp and *Rsa*I 271 bp and 457 bp) were detected in 11 of 12 creek samples but not in any agricultural or riparian samples. The T-RFs *Msp*I 222 bp and *Rsa*I 95 bp were found preferentially in agricultural soil (Fig. 3.5). *Rsa*I 95 bp was detected in 10 of 12 agricultural soil samples. The distribution of *Msp*I 78 bp was similar in agricultural soil and creek sediment, but it was not detected in any riparian samples (Fig. 3.5). *Cfo*I 476 bp was more prevalent in riparian soil than in agricultural soil or creek sediment (Fig. 3.5).

***nosZ* sequences.** Twenty-nine distinct *nosZ* genotypes were identified among 63 clones that were screened based on size of 6-FAM labeled PCR products and *Cfo*I and *Rsa*I T-RFs. The most abundant genotype appeared 12 times and 30% of the

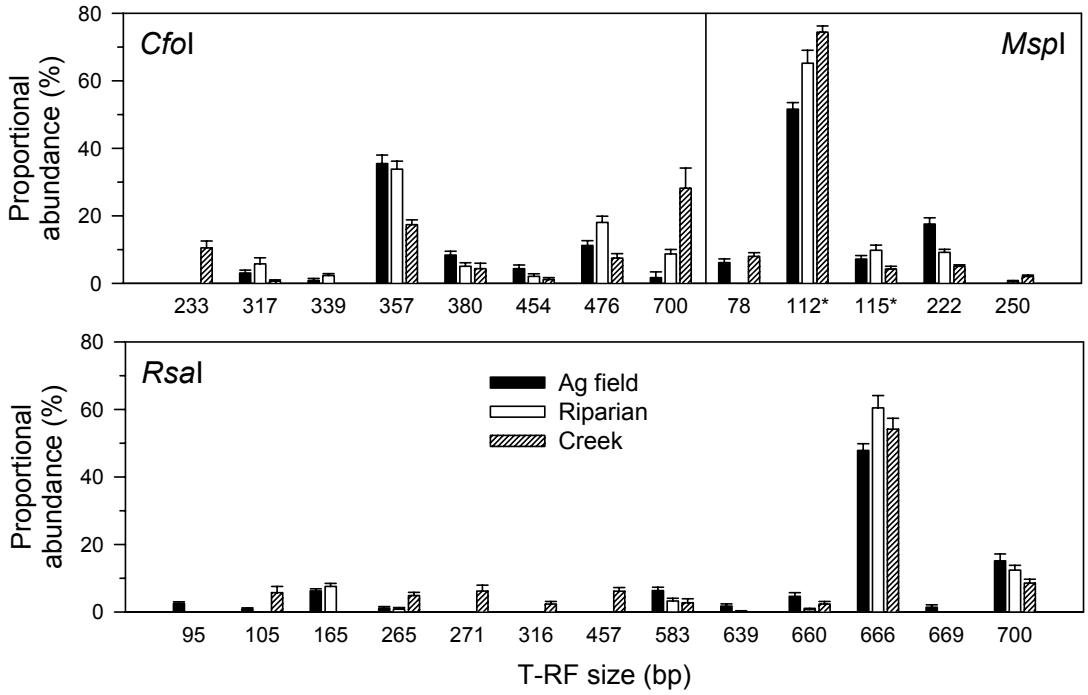



**Fig. 3.5.** *nosZ* T-RFs that differed significantly between habitats, based on Indicator Species Analysis ($p < 0.05$). Each panel shows the restriction endonuclease used to generate the T-RFs. Bars are the mean (± SE, n = 12) for each habitat. *T-RFs *Msp*I 111-112 and *Msp*I 114-115.

genotypes appeared once. Representatives from each of the nine most abundant genotypes were chosen randomly for sequencing. In addition, two genotypes that only appeared once were sequenced.

Sequences of *nosZ* from the riparian soil were distributed throughout *nosZ* phylogenetic trees (e.g., Fig. 3.6). Based on Chapter 2, five major clusters were identified (i.e., A, G, Y, X, and B; Fig. 3.6). Riparian soil *nosZ* sequences were found in all major clusters except Y, which consists of only two clones from a German forest soil (Fig. 3.6). Cluster A was further differentiated into phylogenetic clusters (Fig. 3.6). Each riparian clone contained at least one indicator *nosZ* T-RF (Fig. 3.6) that was detected in direct soil profiles (Fig. 3.5).

To characterize a broader set of *nosZ* fragments, 91 overlapping sequences were subjected to *in silico* sizing of PCR products and *Cfo*I, *Rsa*I, and *Msp*I T-RFs (see Appendix B for complete dataset). In addition to the 11 clones from riparian soil, the dataset consisted of full-length *nosZ* fragments from pure cultures, a German forest soil (Rösch et al. 2002), upland meadow and forest soils in Oregon (Chapter 2), and marine sediments (Scala and Kerkhof 1998). The distribution of these *nosZ* sequences among *nosZ* phylogenetic clusters is shown in Table 3.6. About half of the sequences from cultured denitrifiers were found to belong in phylogenetic cluster A. Of the sequences from Rösch et al. (2002), 70% were distributed evenly between A1a and A2. Eighty percent of the sequences from the H. J. Andrews study were in cluster A1a. With 39 sequences, cluster A1a had the most sequences (Table 3.6), of which 95% were environmental clones from soils. Among the *nosZ* PCR fragments, discrete sizes of 670, 673, 703, 700, and 706 bp were found, reflecting the natural length-heterogeneity

**Fig. 3.6.** Maximum likelihood phylogenetic tree of *nosZ* amino acid sequences (177 positions) from soils, marine sediment, and cultured bacteria. The tree was generated using TREE-PUZZLE (Schmidt et al. 2002) v5.0 for Unix with default settings and the JTT (Jones et al. 1992) model of amino acid substitution. Maximum likelihood quartet puzzling support values (analogous to bootstrap values) are shown above branch nodes, and bootstrap values (1000 replicates) for evolutionary distance (Dayhoff et al. 1979, Felsenstein 1993) below branch nodes. Clones from this study are shown in bold, followed by parentheses with size (bp) of PCR and T-RFs in the order of (PCR-*Cfo*I-*Msp*I-*Rsa*I), with indicator T-RFs marked with asterisks. Clones from Rösch et al. (2002) are identified with CZ and clones from Chapter 2 with HJA. Except for riparian soil clones, accession numbers are shown in parentheses. Scale bar represents an evolutionary distance of 0.1.

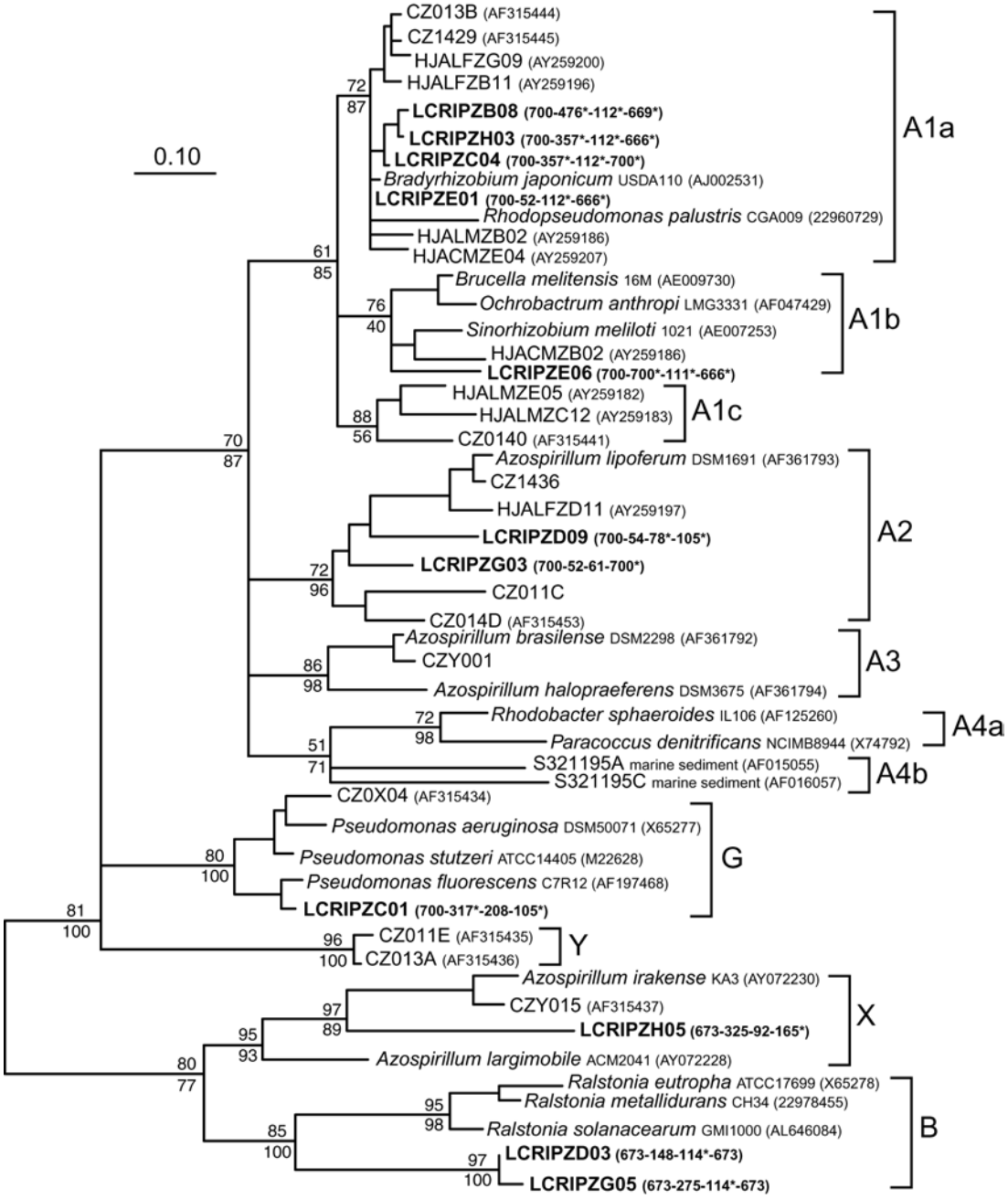

0.10
CZ013B (AF315444)
CZ1429 (AF315445)
HJALFZG09 (AY259200)
HJALFZB11 (AY259196)
LCRIPZB08 (700-476*-112*-669*)
LCRIPZH03 (700-357*-112*-666*)
LCRIPZC04 (700-357*-112*-700*)
Bradyrhizobium japonicum USDA110 (AJ002531)
LCRIPZE01 (700-52-112*-666*)
Rhodopseudomonas palustris CGA009 (22960729)
HJALMZB02 (AY259186)
HJACMZE04 (AY259207)
A1a
Brucella melitensis 16M (AE009730)
Ochrobactrum anthropi LMG3331 (AF047429)
Sinorhizobium meliloti 1021 (AE007253)
HJACMZB02 (AY259186)
LCRIPZE06 (700-700*-111*-666*)
A1b
HJALMZE05 (AY259182)
HJALMZC12 (AY259183)
CZ0140 (AF315441)
A1c
Azospirillum lipoferum DSM1691 (AF361793)
CZ1436
HJALFZD11 (AY259197)
LCRIPZD09 (700-54-78*-105*)
LCRIPZG03 (700-52-61-700*)
CZ011C
CZ014D (AF315453)
A2
Azospirillum brasilense DSM2298 (AF361792)
CZY001
Azospirillum halopraeferens DSM3675 (AF361794)
A3
Rhodobacter sphaeroides IL106 (AF125260)
Paracoccus denitrificans NCIMB8944 (X74792)
A4a
S321195A marine sediment (AF015055)
S321195C marine sediment (AF016057)
A4b
CZ0X04 (AF315434)
Pseudomonas aeruginosa DSM50071 (X65277)
Pseudomonas stutzeri ATCC14405 (M22628)
Pseudomonas fluorescens C7R12 (AF197468)
LCRIPZC01 (700-317*-208-105*)
G
CZ011E (AF315435)
CZ013A (AF315436)
Y
Azospirillum irakense KA3 (AY072230)
CZY015 (AF315437)
LCRIPZH05 (673-325-92-165*)
X
Azospirillum largimobile ACM2041 (AY072228)
Ralstonia eutropha ATCC17699 (X65278)
Ralstonia metallidurans CH34 (22978455)
Ralstonia solanacearum GMI1000 (AL646084)
LCRIPZD03 (673-148-114*-673)
LCRIPZG05 (673-275-114*-673)
B

**Table 3.6.** Distribution of overlapping *nosZ* sequences analyzed in this study.

| | | No. of *nosZ* sequences | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | phylogenetic cluster* | | | | | | | | | | |
| Reference | Source | Total | A1a | A1b | A1c | A2 | A3 | A4a | A4b | G | Y | X | B |
| † | Cultures | 25 | 2 | 4 | 0 | 1 | 4 | 3 | 0 | 6 | 0 | 2 | 3 |
| Rösch et al. (2002) | Soil | 20 | 7 | 0 | 1 | 7 | 0 | 0 | 0 | 1 | 2 | 2 | 0 |
| Rich et al. (Ch. 2) | Soil | 33 | 26 | 1 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| This study | Soil | 11 | 4 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 1 | 2 |
| Scala and Kerkhof | Marine sediment | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | Total | 91 | 39 | 6 | 5 | 12 | 4 | 3 | 2 | 8 | 2 | 5 | 5 |

**nosZ* clusters are based on phylogenetic analyses of *nosZ* sequences as shown in Fig. 3.5 and Chapter 2.
†Too many to name.

of *nosZ* genes among denitrifiers. The 700 bp size was most common. The more divergent *nosZ* sequences in clusters X and B contained 673 bp or 670 bp *nosZ* PCR fragments.

The dataset of 91 overlapping *nosZ* sequences was analyzed for the presence of indicator T-RFs (Table 3.7) that were found in agricultural and riparian soil or creek sediment (Fig. 3.5). The proportionally more abundant indicator T-RFs *Cfo*I 357 bp and 476 bp were found only in group A1a and *Cfo*I 700 bp only in A1a and A1b (Table 3.7). The T-RF *Rsa*I 666 bp was mostly found in A1a. In contrast, *Msp*I 111-112 bp was found in all major clusters except X and B. Less abundant T-RFs, but nevertheless strong indicators among habitats, were not well represented among the *nosZ* sequences. Of the 26-indicator *nosZ* T-RFs, ten were minimally represented (≤ 2 sequences) and five not represented in the database (Table 3.7). Strong indicator *nosZ* T-RFs for creek sediment (*Cfo*I 233 bp) or agricultural soil (*Rsa*I 95 bp) were not represented (Table 3.7).

## DISCUSSION

Although *nosZ* distribution and $rN_2O$ differed among agricultural soil, riparian soil, and creek sediment, denitrifying community composition and activities appeared uncoupled across the agroecosystem. This was because *nosZ* distribution and $rN_2O$ gave different results in terms of similarities among habitats (Fig. 3.4) or relationships between *nosZ* and $rN_2O$ across habitats (Table 3.5). In addition, mean DEA was similar among habitats. In contrast, Rich et al. (Chapter 2) found tighter coupling between denitrifying community composition and functioning in soils from two vegetation types of meadow and adjacent coniferous forest in the Western Cascade Mountains

**Table 3.7.** Distribution of *nosZ* indicator T-RFs in *nosZ* phylogenetic clusters, based on 91 overlapping *nosZ* sequences.

| T-RF | | No. of sequences in each *nosZ* phylogenetic cluster* with T-RF | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Enzyme | Size (bp) | A1a | A1b | A1c | A2 | A3 | A4a | A4b | G | Y | X | B | Total |
| *Cfo*I | 233† | - | - | - | - | - | - | - | - | - | - | - | 0 |
| *Cfo*I | 317 | - | - | - | - | - | - | - | 1 | - | - | - | 1 |
| *Cfo*I | 339† | - | - | - | - | - | - | - | - | - | - | - | 0 |
| *Cfo*I | 357 | 15 | - | - | - | - | - | - | - | - | - | - | 15 |
| *Cfo*I | 380 | - | 1 | - | - | - | - | - | - | - | - | - | 1 |
| *Cfo*I | 454 | 6 | 2 | - | - | - | - | - | 1 | - | - | - | 9 |
| *Cfo*I | 476 | 5 | - | - | - | - | - | - | - | - | - | - | 5 |
| *Cfo*I | 700 | 1 | 2 | - | - | - | - | - | - | - | - | - | 3 |
| *Msp*I | 78 | 2 | - | - | 1 | - | - | - | - | - | - | - | 3 |
| *Msp*I | 111-112 | 30 | 5 | 5 | 6 | 3 | - | 2 | 7 | 1 | - | - | 59 |
| *Msp*I | 114-115 | - | - | - | - | - | - | - | - | - | 1 | 4 | 5 |
| *Msp*I | 222 | 3 | - | - | - | - | - | - | - | 1 | - | - | 4 |
| *Msp*I | 250 | 2 | - | - | - | - | - | - | - | - | - | - | 2 |

(continued)

**Table 3.7 (continued).**

| T-RF | | No. of sequences with T-RF in each *nosZ* phylogenetic cluster* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Enzyme | Size (bp) | A1a | A1b | A1c | A2 | A3 | A4a | A4b | G | Y | X | B | Total |
| *Rsa*I | 95† | - | - | - | - | - | - | - | - | - | - | - | 0 |
| *Rsa*I | 105 | - | - | - | 2 | 1 | 1 | - | 3 | - | - | - | 7 |
| *Rsa*I | 165 | - | - | - | - | - | - | - | - | - | 1 | - | 1 |
| *Rsa*I | 265 | 1 | 1 | - | - | - | - | - | - | - | - | - | 2 |
| *Rsa*I | 271 | - | - | - | - | - | - | - | 1 | - | - | - | 1 |
| *Rsa*I | 316 | - | - | - | 1 | - | - | - | - | - | - | - | 1 |
| *Rsa*I | 457 | - | - | 1 | - | - | - | - | - | - | - | - | 1 |
| *Rsa*I | 583† | - | - | - | - | - | - | - | - | - | - | - | 0 |
| *Rsa*I | 639† | - | - | - | - | - | - | - | - | - | - | - | 0 |
| *Rsa*I | 660 | 2 | - | - | - | - | - | - | - | - | - | - | 2 |
| *Rsa*I | 666 | 23 | 2 | - | 2 | 2 | - | - | - | - | - | - | 29 |
| *Rsa*I | 669 | 1 | - | - | 1 | - | - | - | - | - | - | - | 2 |
| *Rsa*I | 700 | 5 | 3 | - | 5 | - | - | 1 | - | - | - | - | 14 |

**nosZ* clusters are based on phylogenetic analyses of *nosZ* sequences as shown in Fig. 3.5 and Chapter 2.
†No match.

of Oregon. Mean DEA was generally an order of magnitude greater in meadow than in forest soils (Chapter 2). Differences in *nosZ* distribution between meadow and forest soils were in a similar range as for differences in *nosZ* distribution between agricultural or riparian soil and creek sediment (Appendix C). Other factors in the agroecosystem, such as availability of fertilizer-N or contrasting hydrologic regimes may have contributed more to denitrifying activities than community composition. These studies suggest that relationships between denitrifying community structure and functioning may be ecosystem specific.

Urea- and $NH_4^+$-based fertilizers were applied to the agricultural field that we sampled. In a previous year with a similar crop and fertilizer-N application, Horwath et al. (1998) concluded that nitrification in the agricultural field exceeded *in situ* denitrification by more than ten-fold at the same site. In addition, Horwath et al. (1998) showed that concentrations of $NO_3^-$ were four-to-ten-fold greater in agricultural soil than in riparian soil and that *in situ* denitrification was more than an order of magnitude greater in agricultural soil than in riparian soil. Nevertheless, riparian soil had similar DEA compared to agricultural soil [this study and (Davis 2003)]. In addition, riparian soil had lower redox potentials compared to agricultural soil during spring (Wigington et al. 2003). We also found three-times greater $CO_2$ production by riparian soil than agricultural soil in anaerobic slurries amended with only $NO_3^-$ (5 mM), and incubated for 15 d (data not shown), suggesting greater carbon availability in the riparian soil.

Work by Firestone et al. (1980) and Weier et al. (1993) suggested that greater carbon availability, lower redox potentials, and lower $NO_3^-$ concentrations, as found in riparian soil, would favor lower $rN_2O$. Therefore, our results of two-to-three times

higher $rN_2O$ in riparian soil compared to agricultural soil or creek sediment were unexpected. Based on the current study and similar observations of three-times lower $rN_2O$ in agricultural soil compared to adjacent grassland soil (Hénault et al. 2001), differences in carbon and $NO_3^-$ concentrations and redox do not sufficiently explain differences in $rN_2O$. Another factor may be greater repression of Nos than the other reductases leading to $N_2O$ formation (Firestone and Tiedje 1979). However, because DEA and $Nos_{maxp}$ were similar in riparian soil (Fig. 3.2), concentrations of Nos appeared similar to the other N-oxide reductases. Higher $rN_2O$ in riparian soil may have resulted from lower affinity (i.e., higher $K_m$) for $N_2O$ in riparian soil than in agricultural soil or creek sediment. Betlach and Tiedje (1981) showed that variation in $N_2O$ accumulation during denitrification was due to variation in enzyme kinetics among individual N-oxide reductases and not due to inhibition of Nos activity by $NO_3^-$ (10 mM) or $NO_2^-$ (0.5 mM).

Based on *nosZ* distribution, several proportionally abundant denitrifying genotypes (i.e., T-RFs *Cfo*I 357 bp, 476 bp, and 700 bp) were only associated with *nosZ* phylogenetic clusters A1a or A1b (Table 3.6). Cluster A1a and A1b were previously shown to be specific to *Rhizobiaceae* in *α-Proteobacteria* (Chapter 2). Therefore, denitrifiers related to *Rhizobiaceae* may be abundant in the agricultural and riparian soil and creek sediment. Similarly, *Rhizobiaceae*-like denitrifiers may dominate in meadow and forest soils (Chapter 2). However, absolute abundances of individual denitrifying genotypes remain unknown. In addition, the extent of *nosZ* phylogenetic diversity in soil or freshwater habitats is only beginning to be determined, especially for the less proportionally abundant *nosZ* T-RFs that we detected. Unique

*nosZ* T-RFs detected in low abundance in upland soils were specific for *nosZ* based on sequencing (Chapter 2). Although beyond the scope of this study, more comprehensive sequence data is necessary to better identify denitrifying genotypes that differed in distribution among habitats.

In conclusion, $N_2O$-reductase activity and *nosZ* distribution differed among habitats. Community composition of denitrifiers was more unique in creek sediment compared to agricultural and riparian soil. A number of unique *nosZ* T-RFs were found preferentially in creek sediment. The diversity of *nosZ* T-RFs detected in this system was poorly represented in the sequence database, including pure-culture denitrifiers. Relatively few terrestrial and aquatic environments have been examined for both *nosZ* T-RF distribution and phylogenetic diversity. Denitrifiers that differed in distribution among habitats have yet to be identified and characterized.

## ACKNOWLEDGEMENTS

Financial support for this work was provided by the Oregon State University Agricultural Research Foundation and the National Science Foundation Microbial Observatory Program (MCB-9977933).

We are grateful to Steve Griffith, Jenny Davis, and colleagues at the United States Department of Agriculture, Corvallis for establishing the study site. Also, thank you to Don Wirth for his cooperation at the study site.

**Community Composition and Activities of Denitrifying Bacteria in Soils**

## Chapter 4: Conclusions to the Dissertation

Few studies have directly compared denitrifying community composition and activities in natural environments by coupling molecular-genetic techniques and traditional measures of denitrification. Because denitrifying community composition was related to denitrification processes, this work provided impetus for better understanding denitrifying community structure and functioning. In addition, insight was provided into how denitrifying community composition may vary among different soil habitats. By comparing *nosZ* phylogenetics and T-RF profiles, this work gave a starting point for evaluating which denitrifiers might be important in soils. However, much work remains to identify and characterize denitrifiers that differed in distribution among habitats, and to determine specific activities and absolute abundances of individual denitrifiers.

As a first-cut approach, the T-RFLP method appears useful for examining denitrifying communities in previously unstudied ecosystems. Up-and-coming approaches, such as microarray techniques, show promise for quantifying absolute gene abundances (Taroncher-Oldenburg et al. 2003, Wu et al. 2001) or specific activities of individual denitrifiers. However, a prerequisite for using microarrays is comprehensive knowledge of phylogenetic diversity in a system. Coupled with cloning and sequencing, T-RFLP profiles may serve as a guide for microarray design in terms of achieving adequate sequence coverage for target bacterial communities. Other techniques that show promise for examining denitrifying gene abundances or expression include quantitative PCR (Grüntzig et al. 2001) or reverse transcriptase-

PCR (Nogales et al. 2002), but as with microarrays, progress is necessary for these techniques to be generally applicable.

The current sequence database for *nosZ* is limited to *Proteobacteria*. As more functional gene sequences from pure-culture denitrifiers are added to the database, PCR primers and conditions will likely need to be modified accordingly. To detect denitrifiers other than *Proteobacteria* (e.g., *Archaea* and Gram-positive bacteria), T-RFLP approaches may require a number of primer-pairs. Evaluating PCR and T-RFLP biases and detection sensitivity may allow for more quantitative applications of the T-RFLP method.

A major finding of this thesis was that denitrifier community composition differed among habitats. Differences were due to changes in the relative abundance of T-RFs and the presence/absence of less abundant T-RFs. Overall, denitrifying community composition appeared fairly well conserved among habitats because the same dominant T-RFs (*Cfo*I 357, *Msp*I 111-112, and *Rsa*I 666 bp) were detected in all soils. The T-RF *Cfo*I 700 bp was more abundant in creek sediment compared to the other habitats.

Individual T-RFs represented a number of distinct denitrifier genotypes. For example, in the H. J. Andrews study, *Rsa*I 666 bp represented at least 14 distinct denitrifier genotypes based on screening 225 *nosZ* clones. Of the clones with *Rsa*I 666 bp, sequenced representatives from H. J. Andrews soil were closely phylogenetically related in clusters A1a and A1b. In contrast, *Msp*I 111-112 bp was found among most known *nosZ* phylogenetic groups. Whether *Cfo*I 700 bp represents similar denitrifiers in the riparian soil and creek sediment remains unknown.

A few T-RFs, present in low abundance, were consistently found only in certain habitats. These T-RFs were equally strong indicators of different habitats as the more abundant T-RFs. For example, the T-RF *Cfo*I 454 bp was detected in relatively low abundance in all H. J. Andrews meadow soils but generally not detected in forest soils. *Cfo*I 454 bp was present in about half the agroecosystem samples, irregardless of habitat. T-RFs *Cfo*I 233 bp and *Rsa*I 271 bp were detected in all but one creek sediment sample, but in none of the soil samples in either study. Because of uncertainties associated with using T-RFLP profiles to determine relative abundances of denitrifying genotypes, organisms represented by less abundant T-RFs should not be neglected in future attempts to quantify absolute abundances of individual denitrifiers. Whether organisms represented by these low abundance T-RFs are disproportionately more active than organisms represented by dominant T-RFs remains unknown.

Both studies yielded different results regarding relationships between denitrifying community composition and activities. In the H. J. Andrews study, meadow and forest soils showed strong differences based either on DEA or *nosZ* distribution. In contrast, in the agroecosystem study, habitats showed similar DEA but differences in *nosZ* distribution. Dissimilarities in *nosZ* distribution between meadow and forest soils were of a similar range as for dissimilarities in *nosZ* distribution between agricultural or riparian soil and creek sediment. In the agroecosystem study, correlations between $N_2O/(N_2O + N_2)$ and NMS ordinations were in a similar range or higher compared to correlations between DEA and NMS ordinations in the H. J. Andrews study. However, relationships between community composition and activities varied across the agroecosystem habitats. A mechanism explaining these results

remains unknown. Determining population size and specific activity of individual denitrifiers may enable a better explanation for relationships between denitrifier distribution and denitrification processes.

In both studies, denitrifiers related to *Rhizobiaceae*, particular *Bradyrhizobium japonicum*, appeared dominant in the soils. Taxonomic relationships among cultured denitrifiers based on SSU rRNA were in reasonable agreement with phylogenetic relationships based on *nosZ*. However, because of high phenotypic diversity in *Rhizobiaceae*, it is difficult to predict the life-styles of these newly appreciated soil denitrifiers without further study. New culturing approaches, coupled with extensive metagenome analyses of soil DNA, may yield insights into linkages between taxonomic and functional markers of soil denitrifiers.

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# Appendices

## Appendix A: Potential $N_2O$ Consumption in H. J. Andrews Soils

To determine if similar differences between meadow and forest soils were observed based on $N_2O$-reductase activities, potential $N_2O$ consumption was estimated for H. J. Andrews soils.

**Methods.** The capacity for $N_2O$ consumption was determined in long-term incubations of a sub-set of 12 samples, representing each site and vegetation type. These incubations were similar to the DEA conditions, with the following modifications. Sieved soils were kept at 4°C for 17 d prior to the incubation. No nitrate was added to any of the flasks. Each sample was incubated in duplicate. Ultra-high purity $N_2O$ was added to half the duplicates (2200 ppm v/v, final concentration), to measure net $N_2O$ consumption in the absence of acetylene. To the other half of duplicates, acetylene was added to determine background $N_2O$ production from endogenous nitrate. Total $N_2O$ consumption was estimated by adding the absolute mean values of net $N_2O$ consumption in the absence of acetylene, and net $N_2O$ production in the presence of acetylene, over 0.25 h and 216 h.

**Results.** The capacity for $N_2O$ consumption under denitrifying conditions, presumably via nitrous oxide reductase (Nos), showed similar differences between vegetation types (Table A1). The meadow soils consumed about 9-fold greater $N_2O$ in 9 d compared to the forest soils (Table A1). Nos activity in the meadow soils was only apparent after the first 17 to 35 h of the incubation, and $N_2O$ levels in the presence of acetylene reached equilibrium at about 24 h (data not shown).

**Table A1.** $N_2O$ consumption and production by H. J Andrews soils during a 9 d incubation under denitrifying conditions.√

| | | μg $N_2O$-N $g^{-1}$ dry soil‡ | | |
|---|---|---|---|---|
| Site | Vegetation type | Net consumption* | Net production† | Total consumption |
| Carpenter | Meadow | 21.51 (6.01) | 7.76 (0.19) | 29.27 |
| | Forest | 3.42 (0.68) | 0.03 (0.00) | 3.45 |
| Lookout | Meadow | 34.93 (4.80) | 4.46 (0.41) | 39.39 |
| | Forest | 4.35 (5.42) | 0.07 (0.02) | 4.41 |

√Anaerobic conditions, glucose was added (10 mM) but not nitrate.
‡Mean (±1 standard error) are shown, n=3.
*Approximately 50 μg $N_2O$-N $g^{-1}$ dry soil was added at the beginning of the experiment.
†A parallel set was incubated with acetylene (10%); no $N_2O$ was added.

**Appendix B: PCR Product and T-RF Size of 91 Overlapping *nosZ* Fragments.**

**Table B1.** Characteristics of 91 overlapping *nosZ* sequences from this study and GenBank.

| | | | | Fragment size (bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | | T-RFs | | |
| No. | Accession No. | Organisms or clone | *nosZ** cluster | PCR | *Cfo*I | *Msp*I | *Rsa*I |
| 1 | AY259203 | HJACMZB05 | A1a | 700 | 52 | 78 | 700 |
| 2 | NZ_AAAF 01000001 | *Rhodopseudomonas palustris* CGA009 | A1a | 700 | 52 | 112 | 660 |
| 3 | AY259184 | HJALMZD07 | A1a | 700 | 52 | 112 | 666 |
| 4 | | LCRIPE01 | A1a | 700 | 52 | 112 | 666 |
| 5 | AY259187 | HJALMZE02 | A1a | 700 | 54 | 111 | 531 |
| 6 | AY259196 | HJALFZB11 | A1a | 700 | 54 | 112 | 451 |
| 7 | AY259198 | HJALFZF09 | A1a | 700 | 54 | 112 | 451 |
| 8 | AF315443 | CZ1439 | A1a | 700 | 54 | 112 | 666 |
| 9 | AF315445 | CZ1429 | A1a | 700 | 54 | 112 | 666 |
| 10 | AF315446 | CZ1459 | A1a | 700 | 54 | 112 | 666 |
| 11 | AY072231 | CZ1496 | A1a | 700 | 54 | 112 | 666 |
| 12 | AY259210 | HJACMZF05 | A1a | 700 | 54 | 112 | 666 |
| 13 | AY259199 | HJALFZG03 | A1a | 700 | 357 | 112 | 265 |
| 14 | AY259194 | HJALFZB02 | A1a | 700 | 357 | 112 | 451 |
| 15 | AF315444 | CZ013B | A1a | 700 | 357 | 112 | 666 |
| 16 | AY072241 | CZ0135 | A1a | 700 | 357 | 112 | 666 |
| 17 | AY259193 | HJALFZA08 | A1a | 700 | 357 | 112 | 666 |
| 18 | AY259206 | HJACMZD07 | A1a | 700 | 357 | 112 | 666 |
| 19 | | LCRIPZH03 | A1a | 700 | 357 | 112 | 666 |
| 20 | AY259189 | HJALMZE07 | A1a | 700 | 357 | 112 | 700 |
| 21 | AY259200 | HJALFZG09 | A1a | 700 | 357 | 112 | 700 |

(continued)

**Table B1 (continued)**

| No. | Accession No. | Organisms or clone | *nosZ** cluster | Fragment size (bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | | T-RFs | | |
| | | | | PCR | *Cfo*I | *Msp*I | *Rsa*I |
| 22 | | LCRIPZC04 | A1a | 700 | 357 | 112 | 700 |
| 23 | AY259205 | HJACMZD03 | A1a | 700 | 357 | 222 | 451 |
| 24 | AJ002531 | *Bradyrhizobium japonicum* USAD110 | A1a | 700 | 357 | 222 | 666 |
| 25 | AY259209 | HJACMZE12 | A1a | 700 | 357 | 222 | 666 |
| 26 | AY259188 | HJALMZE03 | A1a | 700 | 357 | 250 | 666 |
| 27 | AY259207 | HJACMZE04 | A1a | 700 | 357 | 250 | 666 |
| 28 | AY259191 | HJALMZH12 | A1a | 700 | 454 | 46 | 666 |
| 29 | AY259190 | HJALMZE12 | A1a | 700 | 454 | 111 | 700 |
| 30 | AY259185 | HJALMZC01 | A1a | 700 | 454 | 112 | 295 |
| 31 | AY259211 | HJACMZG11 | A1a | 700 | 454 | 112 | 660 |
| 32 | AY259186 | HJALMZB02 | A1a | 700 | 454 | 112 | 666 |
| 33 | AY259204 | HJACMZC03 | A1a | 700 | 454 | 112 | 666 |
| 34 | AY259201 | HJALFZH03 | A1a | 700 | 476 | 78 | 666 |
| 35 | AF315442 | CZ011H | A1a | 700 | 476 | 111 | 666 |
| 36 | AY259195 | HJALFZB03 | A1a | 700 | 476 | 112 | 666 |
| 37 | AY259212 | HJACMZH06 | A1a | 700 | 476 | 112 | 666 |
| 38 | | LCRIPZB08 | A1a | 700 | 476 | 112 | 669 |
| 39 | AY259208 | HJACMZE05 | A1a | 700 | 700 | 112 | 666 |
| 40 | AE009730 | *Brucella melitensis* 16M | A1b | 700 | 52 | 111 | 700 |
| 41 | AE014528 | *Brucella suis* 1330 | A1b | 700 | 52 | 111 | 700 |

(continued)

**Table B1 (continued)**

| No. | Accession No. | Organisms or clone | *nosZ** cluster | Fragment size (bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | | T-RFs | | |
| | | | | PCR | *Cfo*I | *Msp*I | *Rsa*I |
| 42 | AY259202 | HJACMZB02 | A1b | 700 | 454 | 50 | 666 |
| 43 | AY072229 | *Ochrobactrum anthropi* LMG3331 | A1b | 700 | 454 | 112 | 700 |
| 44 | | LCRIPZE06 | A1b | 700 | 700 | 111 | 666 |
| 45 | AE007253 | *Sinorhizobium meliloti* 1021 | A1b | 700 | 700 | 112 | 265 |
| 46 | AY259180 | HJALMZB06 | A1c | 706 | 47 | 112 | 672 |
| 47 | AY259181 | HJALMZC03 | A1c | 706 | 52 | 112 | 57 |
| 48 | AY259182 | HJALMZE05 | A1c | 706 | 52 | 112 | 457 |
| 49 | AF315441 | CZ0140 | A1c | 706 | 52 | 112 | 672 |
| 50 | AY259183 | HJALMZC12 | A1c | 706 | 361 | 111 | 672 |
| 51 | AF315454 | CZ011C | A2 | 700 | 47 | 112 | 700 |
| 52 | AF315455 | CZ0143 | A2 | 700 | 47 | 112 | 700 |
| 53 | AF315450 | CZ011J | A2 | 700 | 52 | 61 | 700 |
| 54 | AF361793 | *Azospirillum lipoferum* DSM1691 | A2 | 700 | 52 | 61 | 700 |
| 55 | | LCRIPG03 | A2 | 700 | 52 | 61 | 700 |
| 56 | AF315457 | VZ0004 | A2 | 700 | 52 | 112 | 666 |
| 57 | AF315452 | CZ1441 | A2 | 700 | 54 | 61 | 700 |
| 58 | | LCRIPZD09 | A2 | 700 | 54 | 78 | 105 |
| 59 | AF315451 | CZ014H | A2 | 700 | 282 | 61 | 316 |
| 60 | AY259197 | HJALFZD11 | A2 | 700 | 282 | 111 | 105 |

(continued)

**Table B1 (continued)**

| No. | Accession No. | Organisms or clone | *nosZ** cluster | Fragment size (bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | | T-RFs | | |
| | | | | PCR | *Cfo*I | *Msp*I | *Rsa*I |
| 61 | AY259192 | HJALFZA01 | A2 | 700 | 282 | 112 | 669 |
| 62 | AF315453 | CZ014D | A2 | 700 | 355 | 112 | 666 |
| 63 | AF361794 | *Azospirillum halopraeferens* DSM3675 | A3 | 700 | 47 | 111 | 531 |
| 64 | AY074762 | *Azospirillum* sp. A1-3 | A3 | 700 | 47 | 112 | 105 |
| 65 | AF361792 | *Azospirillum brasilense* DSM2298 | A3 | 700 | 52 | 93 | 666 |
| 66 | AF361791 | *Azospirillum brasilense* DSM1690 | A3 | 700 | 52 | 111 | 666 |
| 67 | AF047429 | *Achromobacter cycloclastes* NCIMB11015 | A4a | 706 | 54 | 61 | 672 |
| 68 | X74792 | *Paracoccus denitrificans* NCIMB8944 | A4a | 706 | 54 | 61 | 706 |
| 69 | AF125260 | *Rhodobacter sphaeroides* IL106 | A4a | 703 | 173 | 50 | 105 |
| 70 | AF016055 | S321195A marine sediment | A4b | 703 | 380 | 111 | 468 |
| 71 | AF016057 | S321195C marine sediment | A4b | 700 | 386 | 112 | 700 |
| 72 | AE004760 | *Pseudomonas aeruginosa* PA01 | G | 700 | 52 | 111 | 105 |
| 73 | AB054991 | *Pseudomonas* sp. MT-1 | G | 700 | 54 | 111 | 49 |
| 74 | AF315434 | CZ0X04 | G | 700 | 54 | 111 | 49 |
| 75 | AF361795 | *Alcaligenes faecalis* A15 | G | 700 | 54 | 111 | 49 |
| 76 | AF197468 | *Pseudomonas fluorescens* C7R12 | G | 700 | 54 | 111 | 105 |
| 77 | X65277 | *Pseudomonas aeruginosa* DSM50071 | G | 700 | 54 | 111 | 459 |

(continued)

**Table B1 (continued)**

| | | | | Fragment size (bp) | | | |
|---|---|---|---|---|---|---|---|
| | | | | | T-RFs | | |
| No. | Accession No. | Organisms or clone | *nosZ** cluster | PCR | *Cfo*I | *Msp*I | *Rsa*I |
| 78 | | LCRIPZC01 | G | 700 | 317 | 208 | 105 |
| 79 | M22628 | *Pseudomonas stutzeri* ATCC14405 | G | 700 | 454 | 111 | 271 |
| 80 | AF315435 | CZ011E | Y | 706 | 85 | 222 | 672 |
| 81 | AF315436 | CZ013A | Y | 706 | 145 | 112 | 672 |
| 82 | AF315438 | CZ0137 | X | 673 | 75 | 70 | 673 |
| 83 | AY072230 | *Azospirillum irakense* KA3 | X | 673 | 75 | 70 | 673 |
| 84 | AF315437 | CZY015 | X | 673 | 75 | 115 | 108 |
| 85 | AY072228 | *Azospirillum largimobile* ACM2041 | X | 670 | 325 | 81 | 670 |
| 86 | | LCRIPZH05 | X | 673 | 325 | 92 | 165 |
| 87 | | LCRIPZD03 | B | 673 | 148 | 114 | 673 |
| 88 | | LCRIPZG05 | B | 673 | 275 | 114 | 673 |
| 89 | AL646084 | *Ralstonia solanacearum* GMI1000 | B | 673 | 325 | 50 | 81 |
| 90 | NZ_AAAI 01000322 | *Ralstonia metallidurans* CH34 | B | 673 | 325 | 114 | 81 |
| 91 | X65278 | *Ralstonia eutropha* ATCC17699 | B | 673 | 356 | 115 | 81 |

**nosZ* clusters are based on phylogenetic analyses of *nosZ* sequences as shown in Fig. 3.5 and Chapter 2

## Appendix C: Dissimilarities in Mean *nosZ* Distribution Among Habitats

**Table C1.** Sørensen's distance among habitats based on mean *nosZ* distribution.*

| Habitat comparison | Sørensen's distance |
|---|---|
| Carpenter meadow vs. Carpenter forest | 0.30 |
| Lookout meadow vs. Lookout forest | 0.38 |
| Agricultural soil vs. Riparian soil | 0.23 |
| Agricultural soil vs. Creek sediment | 0.41 |
| Riparian soil vs. Creek sediment | 0.38 |

**nosZ* distribution was based on presence/absence of *nosZ* T-RFs in combined *Cfo*I, *Msp*I, *Rsa*I profiles.