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Soil Biology & Biochemistry 38 (2006) 870–878

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon

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Received 27 January 2005; received in revised form 26 April 2005; accepted 1 August 2005
Available online 6 September 2005

Abstract

In order to examine the effects of disturbance, vegetation type, and microclimate on denitrification and denitrifier community composition, experimental plots were established at the H. J. Andrews Experimental Forest in the Cascade Mountains of Oregon. Soil cores were reciprocally transplanted between meadow and forest and samples were collected after 1 and 2 years. Denitrifying enzyme activity (DEA) was measured using the acetylene block assay and terminal restriction length polymorphism profiles were generated with *nosZ* primers that target the gene coding for nitrous oxide reductase. Nitrate concentrations, C mineralization, and water content were measured to gain additional insights into soil properties controlling DEA. Meadow soils were significantly higher in DEA than forest soils, and the highest DEA levels were observed in cores transferred from the meadow into the forest. Nitrate concentrations were also different between forest and meadow soils, but did not correlate to DEA. DEA was higher in open versus closed cores, suggesting an association between denitrification and the rhizosphere. Denitrifier communities of undisturbed forest and meadow soils shifted through a 4-year period but remained distinct from each other. Similarly, denitrifier communities clustered by vegetation type of origin regardless of manipulation, suggesting that the overall denitrifier communities are well buffered against environmental changes.

Keywords: Denitrification; Forest soils; Microbial community composition; N-cycling

1. Introduction

Denitrification, the process by which mineral N in the forms of nitrate (NO_3^-) and nitrite (NO_2^-) is converted to N_2 gas, represents an important N-cycling transformation. Not only does denitrification lead to a loss of N from soil systems, but incomplete conversion of mineral N to N_2 results in the formation of nitric oxide (NO), which can contribute to ozone formation, and nitrous oxide (N_2O), a greenhouse gas (Öquist et al., 2004; Zumft, 1999). The amounts of N_2O released from forest soils were traditionally thought to be low, approximately 0.2 kg N ha^{-1} yr^{-1} (Bouwman et al., 1995), but a more recent study has shown that denitrification rates can be spatially variable and quite high in the litter layer of coniferous forests (Laverman et al., 2000). Despite typically low NO_3^- concentrations in coniferous forest soils, isotope dilution studies have shown that gross rates of NO_3^- production and consumption can occur at relatively high rates (Davidson et al., 1992; Stark and Hart, 1997). Therefore, under some circumstances denitrification may in fact be an important fate of NO_3^- in these systems. For example, Brooks et al. (1997) showed that denitrification increased during the winter and spring months under snow cover when plant uptake of NO_3^- was low.

Although the rates of denitrification, as well as other N-cycling processes, have been measured extensively (Laverman et al., 2000; Öquist et al., 2004; Parsons et al., 1991), fewer studies have sought to link these rates to the microbial communities. Studies that have utilized molecular

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0038-0717/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2005.08.003

methods to target functional genes have found that denitrifying community composition varies between: cultivated and uncultivated agricultural soils (Stres et al., 2004), riparian and agricultural soils (Rich and Myrold, 2004), wetland and forested uplands (Prieme et al., 2002), and forest and meadow soils (Rich et al., 2003). Temporal studies have shown seasonal shifts in these communities, as well (Mergel et al., 2001; Wolsing and Prieme, 2004). The long-term dynamics and overall environmental effects on these communities are still unknown, however. The goals of this study were to expand our knowledge of denitrifying community dynamics by evaluating the roles of plant community and microclimate on community composition in the Oregon Cascade Mountains where open meadows are interspersed among coniferous forests.

As part of a study designed to more fully understand how the forest and meadow environments might influence N-cycling, denitrification and denitrifying community composition were determined in soil cores that had been reciprocally transferred between adjacent meadow and forest sites. In the light of previous work (Rich et al., 2003) we hypothesized that treatments controlling root in-growth would allow us to examine the importance of vegetation type on denitrifying enzyme activity (DEA) and community composition. Likewise we hypothesized that transferring cores between the shaded, wetter forest and the drier meadow would permit us to evaluate the relative impact of forest and meadow microclimate on denitrifying parameters.

2. Material and methods

2.1. Site description

This study was conducted at the H. J. Andrews Experimental Forest, located in the western Cascade Mountains of Oregon. Two separate sites were selected, Lookout and Carpenter Mountains. The sites were at an elevation of roughly 1500 m and both contain coniferous forest with interspersed meadows. For complete site description and details of vegetation type, refer to Rich et al. (2003); Bottomley et al. (2004).

At each of the two locations 35×35 m grids were established in the forest and adjoining meadow. The organic horizon was removed and a PVC pipe (inner diameter 5 cm) was driven into the mineral soil to a depth of 10 cm. A total of five treatments were included in the study design. In the 'remaining' treatment, soils were cored and then left in place. An equal number of soil cores were transferred to the adjacent vegetation type. Transferred and remaining cores were subdivided into open and closed cores to test for root effects on soil processes. Open cores were placed in mesh bags and closed cores remained in the PVC pipe with mesh enclosing the top end. Soil cores were transferred in September 2000 and collected in September of 2001 and 2002. To control for disturbance effects, background cores were taken at the same time of core collection, as well as at the time of plot establishment and again in 2004, so as to also examine temporal variation in denitrifying communities.

2.2. Soil properties

The soil samples were passed through a 4-mm sieve 1 day after they were collected from the field. A 10-g sample was dried at 105 °C overnight and reweighed to determine gravimetric water content. Another 10-g sample was immediately extracted using 2 M KCl and NO_3^- was measured colorimetrically using an autoanalyzer (Astoria-Pacific, Portland, OR). An additional 30-g sample was placed in a 1-L mason jar and incubated at 25 °C for 28 days. CO_2 was measured at 7, 14, and 28 days using a gas chromatograph equipped with a thermal conductivity detector. The jars remained sealed throughout the incubation, except for 5-min aerations just following the weekly measurements. Aeration ensured that the soils did not become anaerobic.

2.3. Denitrification enzyme activity

Denitrification enzyme activity (DEA) was determined using methods described by Rich et al. (2003). Fresh soil (10 g) was added to a solution containing glucose, NO_3^-, and phosphate buffer. Assay systems were made anaerobic and acetylene was added to block N_2O reductase activity. Production of N_2O was quantified in samples taken at 15 and 75 min using gas chromatography.

2.4. DNA extraction and amplification

A Bio101 soil extraction kit (Q-Biogene, Irvine, CA) was used to extract DNA from each soil sample. Briefly, approximately 0.5 g of soil was added to a mini-prep tube containing garnet beads and cells were lysed using physical disruption. The extract was then purified by binding the DNA to a silicon matrix and rinsing with ethanol containing buffer, before elution with water. Extracted DNA was quantified using a UV spectrophotometer before amplification by PCR.

A primer set targeting a 700-bp region of the *nos*Z gene was used with previously described PCR parameters (Rich et al., 2003). The only modification was an increase from 25 to 30 PCR cycles making it unnecessary to pool products. These PCR products were then purified using a Qiagen DNA clean-up kit (Qiagen, Valencia, CA) in order to eliminate genomic DNA, excess primers, and unused nucleotides.

2.5. Restriction and TRFLP profiles

Products were digested using three restriction enzymes: *Cfo*I, *Rsa*I, and *Msp*I (Promega Corp., Madison, WI).

Digests were run according to manufacturers' specifications by incubating the restriction digest for 3 h at 37 °C and heat inactivating at 65 °C for 15 min.

Restricted samples were submitted to the Oregon State University Center for Gene Research and Biotechnology for analysis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Approximately 1 ng of amplified DNA was submitted for analysis. Samples were re-run if the largest peak in an individual profile was less than 2000 fluorescent units.

2.6. Statistical analysis

DEA and soil properties were analyzed using repeated measures analysis of variance (ANOVA) (Ramsey and Schafer, 2002), included in the SAS software package. Significant interactions between year of sampling, site, and treatments required that the data be analyzed separately for each year and site. Soil disturbance effects were analyzed by comparing background with open remaining cores. Effects of transferring soil and core type were analyzed using the data from remaining and transferred cores and open and closed cores, respectively (i.e. background cores were excluded). Linear regression analysis was also performed to determine if DEA correlated to NO_3^- concentration, C mineralization, and soil water content. Treatment effects were considered significant if the p-value was <0.05.

In the case of terminal restriction fragment length polymorphisms (TRFLP) profiles, length and fluorescence of the terminal restriction fragments (TRF) were determined using GeneScan version 3.5 and Genotyper version 2.5 software (Applied Biosystems). The community data set was then analyzed using multivariate statistical analysis performed with PC-ORD version 4.27 (B. McCune and J. J. Mefford, PC-ORD for Windows: multivariate analysis of ecological data, 4.01 ed. MjM Software, Gleneden Beach, OR, 1999). The total fluorescence was summed for each sample and then used to relativize peaks as a fraction of the total fluorescence. After examining electropherograms of negative controls, peak contributing less than 1.5% of the total fluorescence in a single profile were excluded and the relative fluorescence recalculated, making the sum of each profile equal to 100% (Rich et al., 2003). Peaks were then aligned by base pairs and any peaks less than two base pairs apart were combined for final fragment identifications. The three enzyme profiles were combined to produce composite TRFLP profiles. Differences in sample community profiles were determined using nonmetric multidimensional scaling (NMS) (McCune and Grace, 2002) ordination techniques and Sørenson distance measures. Starting from random configurations, 500 iterations were run to produce NMS ordinations with a final instability criterion of 0.0001. Monte Carlo test results were compared against real data to determine the significance of a given solution. Ordinations were constrained to two axes; the ordination with the lowest final stress was chosen for further analysis. Relationships between DEA and denitrifier community composition were explored with the joint plot feature of PC-ORD (McCune and Grace, 2002), which basically correlates the DEA values with the NMS axis scores of the samples.

Multi-response permutation procedures (MRPP) were used to test the strength and statistical significance of group membership in total community profiles. MRPP is a nonparametric method for testing group differences, similar to multivariate analysis of variance (MANOVA). A p-value <0.05 and an A-statistic >0.1 was considered significant (McCune and Grace, 2002). Indicator species analysis was used to identify possible biomarkers and to identify TRFs responsible for the separation of individual samples into groups.

3. Results

3.1. Denitrification enzyme activity

DEA was an order of magnitude higher in soils originating from the meadow compared to the forest, with values slightly higher in year two than year one and Lookout higher than Carpenter (Fig. 1 (A) and (B)). Forest samples ranged from undetectable levels for some Carpenter samples to 0.92 μg N_2O–N g^{-1} soil d^{-1} for the Lookout forest open transfer samples in year two. In contrast, meadow samples ranged from 0.60 to 4.38 μg N_2O–N g^{-1} soil d^{-1}. DEA was lower in background compared to open remaining cores at Lookout forest in year one, but this was the only disturbance effect observed. Transfer of open cores from Lookout meadow to forest caused an increase in DEA relative to the cores that remained in place. DEA in open cores was usually higher than closed cores. This core effect was statistically significant for meadow soils at both sites in year one and at Lookout in year two.

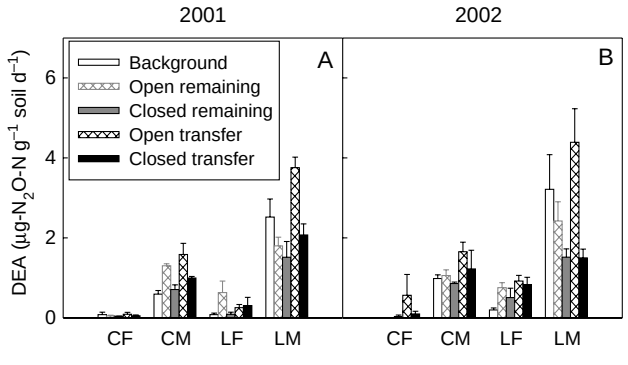

Fig. 1. Denitrification enzyme activity (DEA) values are shown in panels A and B, where bars represent the mean values of three field replicates and error bars show the standard error. Original soil location is listed along the bottom: Carpenter forest (CF), Carpenter meadow (CM), Lookout forest (LF), and Lookout meadow (LM). Treatments include: background (B), open remaining (OR), closed remaining (CR), open transfer (OT), and closed transfer (CT). Panel A is the data for year one and B is year two data.

3.2. Soil properties

Nitrate concentrations were an order of magnitude higher between years one and two (Fig. 2(A) and (B)). Despite the low levels of NO_3^- in year one, statistically significant differences were observed between open and closed, as well as transferred and remaining treatments. Closed cores had more NO_3^- than open cores. Nitrate concentrations were significantly higher at the Carpenter site in cores incubated in the meadow compared to forest, during both years, and at the Lookout site in year two.

Carbon mineralization tended to be higher in year two compared to year one (Fig. 2(C) and (D)). A single disturbance effect was observed in Lookout forest soils during the second year where the open remaining treatment was higher than the background. At Carpenter in year two, C mineralization rates were higher for cores located in the forest compared to the meadow. In year two at Lookout, C mineralization rates were higher in open versus closed cores.

Water contents were significantly higher in cores incubated in the forest compared to meadow-incubated cores (Fig. 2(E) and (F)). A disturbance effect was observed in Lookout forest during year one, but this was the only case where open remaining cores contained significantly more water than backgrounds. Closed cores incubated in the forest tended to contain more water than open cores. This was significant both years at Carpenter and the second year at Lookout.

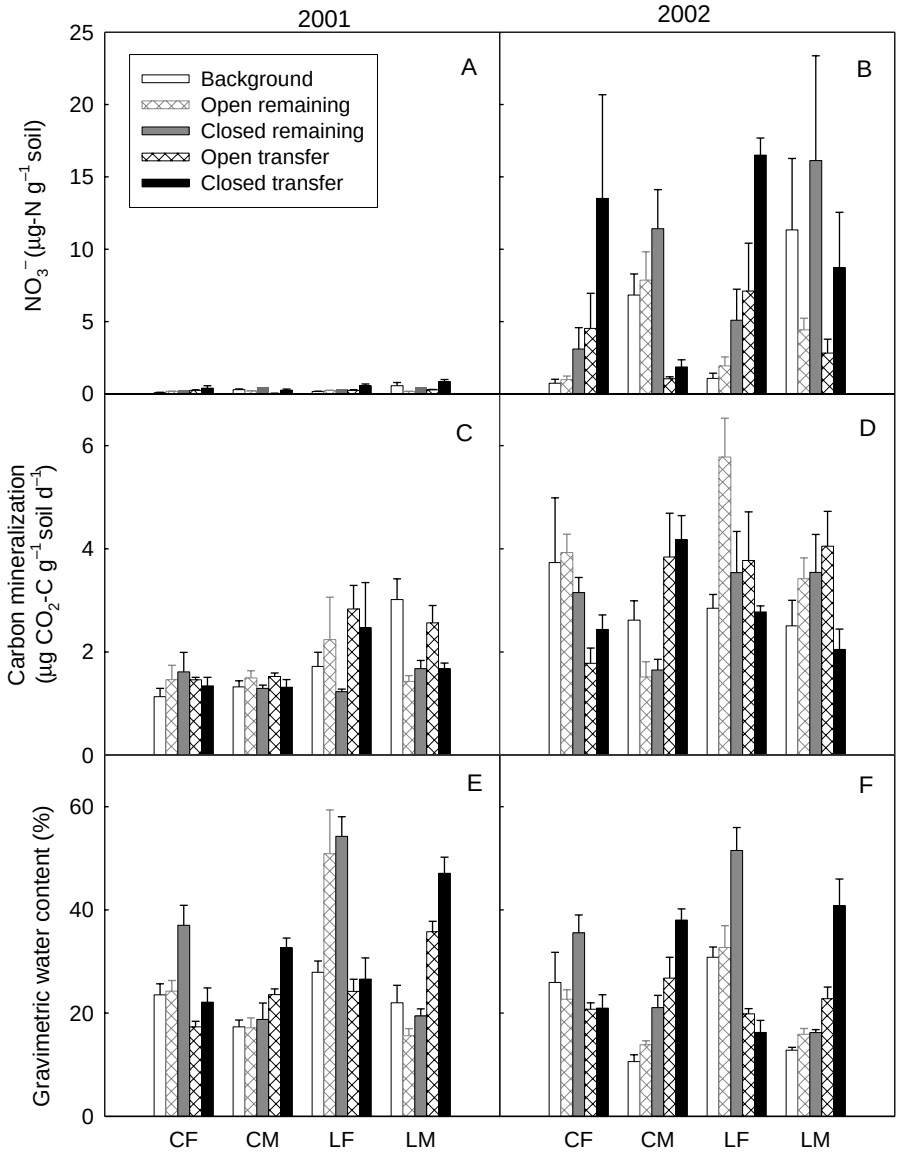

Fig. 2. Panels A–F show soil properties for each location where soil originated. Bars are an average of three field replicates and error bars are standard errors. Original soil location is listed along the bottom: Carpenter forest (CF), Carpenter meadow (CM), Lookout forest (LF), and Lookout meadow (LM). Treatments include: background (B), open remaining (OR), closed remaining (CR), open transfer (OT), and closed transfer (CT). Panels A and B list NO_3^- concentrations for year one (2001) and two (2002). Panels C and D show C mineralization values, and E and F give water content at the time of collection.

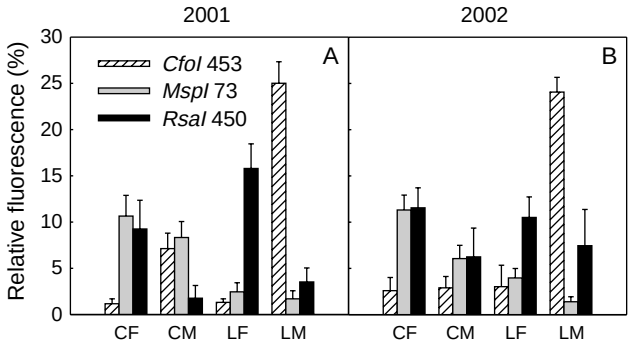

Fig. 3. Panels A and B show the relative fluorescence of three terminal restriction fragments *CfoI* 453, *MspI* 73, and *RsaI* 450. Bars represent the average of 15 cores and include all treatment types. Error bars are the standard error of the mean. Original soil location is listed along the bottom: Carpenter forest (CF), Carpenter meadow (CM), Lookout forest (LF), and Lookout meadow (LM). Panels A and B are year one and two, respectively.

When data for both years was used, no significant correlations was found between DEA and soil NO_3^- concentration, C mineralization, or water content; however, a few significant correlations were found for subsets of the data. DEA was positively correlated with C mineralization for meadow soils in year one ($R^2=0.454$) and DEA was positively correlated with water content of open cores in forest and meadow soils in year one, and in meadow soils in year two (R^2 values ranging from 0.463 to 0.690).

3.3. Community composition

A total of 242 peaks were identified with *Cfo*I, *Rsa*I, and *Msp*I containing 83, 64, and 95 peaks, respectively. When the digest profiles were combined, each sample contained an average of 18 peaks.

Vegetation type (defined by soil origin) was an important factor governing denitrifier community composition ($p<0.001$, $A=0.12$). Indicator species analysis identified 51 TRFs that contributed to the observed differences in vegetation. Many of these fragments were rare, but 16 of these fragments were examined more closely because they were seen in at least 25% of all generated profiles; among these fragments were *CfoI* 453, *MspI* 73, and *RsaI* 450 (Fig. 3). Similar to the results reported by Rich et al. (2003), these fragments appeared to be markers for forest or meadow soil. For example, *CfoI* 453 was a minor peak in Lookout forest soils making up less than 5% of the mean relative fluorescence and present in only 14 of the 30 forest profiles. In contrast, *CfoI* 453 was present in all the Lookout meadow profiles and made up 25% of the mean relative fluorescence (Fig. 3). Fragment *RsaI* 450, on the other hand, was more indicative of forest soils.

When stratified by year and site community profiles differed between selected treatments (Table 1). For example, in year two cores from Lookout forest transferred to the meadow environment differed from those that remained in place. Further investigation of the community profiles showed that indicator peaks, for forest or meadow soils, varied between the remaining and transferred samples (Fig. 4). Although not statistically significant in most cases, the meadow-specific *CfoI* 453 fragment consistently made up a slightly higher proportion of the fluorescence in soils transferred to the meadow compared to those forest soils that remained in place. Likewise, an examination of the forest-specific *RsaI* 450 showed that this TRF was more abundant in meadow soils that were moved to the forest (Fig. 4).

The low *A*-statistic reported for Carpenter forest in year one reflects a relatively high amount of within-group variability associated with denitrifying communities in forest soils (Table 1). The heterogeneous nature of these soil communities may have masked additional treatment effects. For instance, no root effects (closed versus open cores) were observed during either year, and disturbance effects were only observed in Lookout forest (Table 1).

The data were also examined to find possible correlations between the denitrifier community composition and the DEA activity. In year one, forest and meadow communities separated on the NMS ordination and DEA was positively correlated ($r^2=0.54$) with these forest community profiles (data not shown). A similar trend was observed in 2002. No correlation was observed between imposed treatments, however.

3.4. Temporal variability in community composition

Denitrifying community composition was compared in background cores from all four locations through a 4-year

Table 1
Differences between denitrifying community composition under different treatment effects

Year	Site	Soil origin	Disturbance effect	Transfer effect	Root effect
2001	Carpenter	Forest	–	0.041 (0.08)	–
		Meadow	–	0.005 (0.14)	–
	Lookout	Forest	<0.001 (0.28)	–	–
		Meadow	–	–	–
2002	Carpenter	Forest	–	–	–
		Meadow	–	–	–
	Lookout	Forest	0.022 (0.43)	0.039 (0.12)	–
		Meadow	–	–	–

p-Values with *A*-statistics in parentheses were calculated using MRPP. A *p*-value of <0.05 was considered significant.

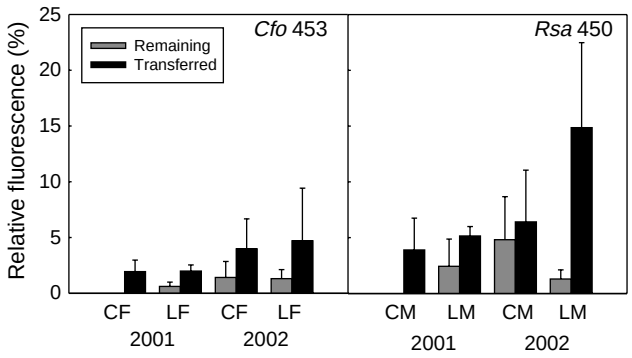

Fig. 4. The graph shows relative fluorescence of two terminal restriction fragments. The abundance of *CfoI* 453, a meadow marker, is compared in soils from Carpenter forest (CF) and Lookout forest (LF), during year one (2001) and year two (2002). The abundance of the forest-specific marker *RsaI* 450 is compared in soils from Carpenter meadow (CM) and Lookout meadow (LM). All bars are the average of six field replicates and error bars represent the standard error of the mean.

period (Fig. 5). Similar to the findings in all reciprocal transfer treatments, background communities differed between the two vegetation types ($p=0.008$, $A=0.13$), but there was no significant difference between the Carpenter and Lookout site ($p=0.708$, $A=-0.01$). Therefore, sites were combined for further statistical analysis. Year zero background samples cluster on the right, year two and four samples are on the left, and year one samples are intermediate. Separation in background communities by year was supported by MRPP ($p<0.001$, $A=0.25$).

Because the year zero and year one samples were extracted and then stored before amplification and analysis of the *nos*Z fragment, we investigated the effects of storage. The initial analysis of the year two samples began within

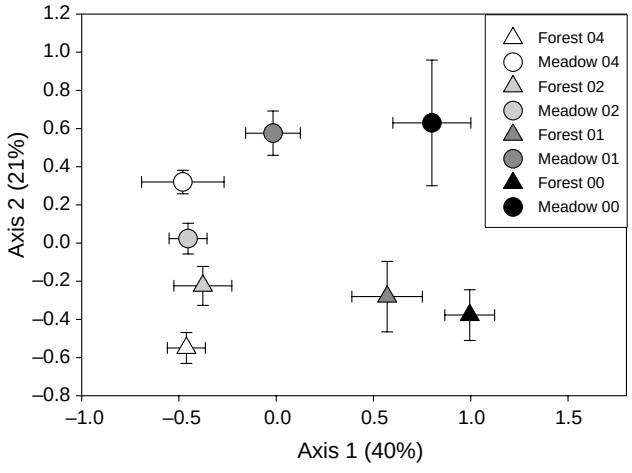

Fig. 5. All terminal restriction fragment length polymorphism (TRFLP) profiles of background cores were ordinated using nonmetrical multidimensional scaling (NMS). The two-dimensional solution had a final stress of 11.14 and a final instability of 0.00001. Percentages associated with axis one and two show the percent variance explained along that axis. Points represent the average of all vegetation-specific soil and are the average of six replicates, three replicate cores from each of the two sites. Error bars are the standard error of the mean.

a month of field collection, but a subset of these extracts was stored under the same conditions as the year zero and year one extracts. After 2 years of storage, the year two extracts were re-amplified and new community profiles were generated. A comparison of community profiles from fresh and stored samples indicated no change in the community fingerprint (data not shown). These results indicate that storage did not significantly alter microbial community structure, but that the communities did change over time.

4. Discussion

The reciprocal transfer treatments allowed us to examine the extent to which a combination of forest and meadow microclimates and plant community composition influenced denitrifying activity and denitrifier community composition in these environments of the Oregon Cascade Mountains. We found that denitrifier community composition was highly dependent on original vegetation type and was quite resistant to changes brought about by disturbance and transfer of soils. Our data confirmed our hypothesis that root in-growth affects denitrification activity in meadow soils. We also found that DEA remained low in forest soils transferred to the meadow despite a large increase in rates of nitrification and NO_3^- concentrations (Bottomley et al., 2004). Because differences were observed in specific TRFs of meadow and forest soil and the composition of background communities indicated shifts over time, some members of the denitrifier community may be dynamic and subject to a complex set of biological and abiological controls.

Most studies from forested ecosystems suggested that NO_3^- availability limits DEA (Griffiths et al., 1998; Groffman and Tiedje, 1989; Henrich and Haselwandter, 1997; Rich et al., 2003; Robertson and Tiedje, 1984; Vermes and Myrold, 1992). We found DEA to be unrelated to soil NO_3^- concentrations (Figs. 1 and 2), which were highly variable year to year; however, there was a significant correlation ($R^2=0.171$) of DEA with the net nitrification rates reported by Bottomley et al. (2004). This correlation was stronger in year one ($R^2=0.505$) than year two ($R^2=0.295$), probably because there was more NO_3^- available the second year (Fig. 2).

We measured soil water content as a proxy for soil aeration but found no overall correlation between soil water content and DEA. Soils that experienced the forest microclimate were always wetter than soils of comparable treatments located in the meadow (Fig. 2), however, and meadow soils incubated in the forest had higher DEA (Fig. 1). Although closed cores were usually wetter than open cores (Fig. 2), which excluded roots, the effect of water content on DEA was only significant for open cores, suggesting a complex interaction among denitrifiers, roots, and soil water content.

Enhanced DEA in most open cores suggests that a rhizosphere effect may have favored a larger or more active denitrifying population. The influence of roots on denitrifier is a balance between positive and negative effects. Respiration by roots and enhanced microbial respiration from root-derived C may decrease O_2 availability, thereby favoring denitrifying bacteria (Klemedtsson et al., 1987; Qian et al., 1997). The C supplied by rhizodeposition is known to stimulate microbial respiration and activity (Butler et al., 2004; Kuzyakov and Domanski, 2000; Qian et al., 1997; Schimel and Bennett, 2004), increasing denitrifier activity and possibly population size (Hall et al., 1998; Mounier et al., 2004; Priha et al., 1999; Smart et al., 1997; Smith and Tiedje, 1979). Although significant differences were not observed in the C mineralization during laboratory incubations, we did not focus upon rhizosphere soil, specifically, and root supplied C may turn over quickly and so would need to be examined in situ (Butler et al., 2004; Kuzyakov and Domanski, 2000). Plant uptake of water and NO_3^- could have a negative impact on denitrifying bacteria; the former by increasing aeration, the latter by direct competition. The open cores in our study were consistently drier than closed cores but this did not have a negative impact on DEA (Figs. 1 and 2), suggesting that conditions conducive to denitrification occur at least some time during the year. Indeed, recent studies indicate that anaerobic microbial processes can occur in wet soil under snow pack during winter and early spring (Brooks et al., 1996). The water content of the open cores likely influenced denitrifiers because, in most cases, DEA was positively correlated to the water content of the open cores. Concentrations of NO_3^- (Fig. 2) and net nitrification rates (Bottomley et al., 2004) were often lower in open versus closed cores, most likely because of plant uptake of NO_3^-. Because DEA was higher in the open cores, it is likely that denitrifiers compete effectively with other microbial and plant sinks for NO_3^- (Chirstensen and Tiedje, 1988; Højberg et al., 1996; Murray et al., 1989). In addition to activity measurements, Mergel et al. (2001), using probes for denitrifying genes, reported an increase in the abundance of *nirS*, *nirK*, and *nosZ* genes in rhizosphere versus bulk soil, further supporting the link between denitrifying bacteria and the rhizosphere.

The clustering of meadow denitrifier community profiles apart from forest profiles lends further support for the coupling of denitrifier communities to plant root activities (Hall et al., 1998; Rösch et al., 2002; Rich et al., 2003; Smart et al., 1997). Denitrification rates and communities differed between meadow and forest, but treatment-imposed changes of DEA generally did not correlate well with community profiles. For example, although DEA rates increased in meadow soils transferred to the forest (Fig. 1), this transfer effect was not usually accompanied by a detectable shift in the community composition (Table 1). The relatively high diversity and numbers of denitrifiers in soil (Cavigelli and Robertson, 2001; Palumbo et al., 2004; Throbäck et al., 2004) may have obscured changes in active but less numerous groups of denitrifiers: we examined the entire *nosZ*-containing community and cannot determine how many of these TRFs are associated with actively denitrifying organisms. A significant increase in a specific subpopulation of denitrifiers might not be detected against a larger *nosZ*-containing population that remained inactive. Alternatively, the lack of correlation between DEA and denitrifier community composition may have resulted from the stimulation in activity, but not growth of certain denitrifiers. For example, we observed a rhizosphere effect (open versus closed cores) on DEA (Fig. 1) but not on denitrifier community composition (Table 1), similar to the responses observed by Mounier et al. (2004) to root mucilage.

The lack of significant changes in denitrifying community composition as a whole suggests that these communities may be well buffered to environmental change. Nevertheless, indicator species analysis identified several TRFs that did change in response to treatments and temporal variations were observed. We observed slight differences in some TRFs when cores were transferred between microenvironments (Fig. 4). It is possible that the dominant members of the denitrifier community (identified previously to be α-proteobacteria related to *Rhizobiaceae* (Rich et al., 2003)) are more sensitive to perturbation than other community members. On the other hand, these specific changes may foreshadow more significant changes that would have occurred if the treatments had been left in place for a longer period of time. In either case, we may find that differences in the *nosZ* gene translate to enzymatic differences that affect N-oxide scavenging capabilities, O_2 tolerance, or pH tolerance (Šimek et al., 2002).

The observed temporal variability in background cores shows that denitrifier community composition is subject to natural change. Previous studies have shown that microbial communities (Lipson et al., 1999) and N-cycling (Lipson et al., 2002) vary seasonally (Griffiths et al., 1998; Priha and Smolander, 1999); however, to our knowledge no other studies have observed denitrifier communities in any natural environment for more than one year.

These findings highlight the need for long-term ecological studies that examine N transformation and community dynamics both seasonally and over several years. The current study and another recent study (Horz et al., 2004) demonstrates that field manipulations provide insight into the nature of microbial communities and their response to environmental change. Our study examined net production of NO_3^- (Bottomley et al., 2004) and one possible sink for NO_3^- (i.e. denitrification), but could be expanded to other possible sinks, such as microbial NO_3^- assimilation. These future studies would help to develop models of N-cycling that couple microbial communities and processes in this environment. Additionally, examining seasonal

patterns of denitrifying activity, denitrifier community dynamics, and plant root activity may help us to better understand plant and microbe interactions.

Acknowledgements

Support was provided by grants from the National Science Foundation Microbial Observatory and IGERT Programs. Thank you to Shawna McMahon for assistance in data collection, Oregon State University's Center for Gene Research and Biotechnology for sample analysis, and to the staff at the H. J. Andrews Experimental Forest LTER.

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