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RESEARCH ARTICLE

# Spatial differences in denitrification and bacterial community structure of streams: relationships with environmental conditions

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**Abstract** In streams, benthic bacterial communities are integral to multiple aspects of ecosystem function, including carbon and nitrogen cycles. Variation both in terms of bacterial community structure (based on taxonomic and/or functional genes) and function can reveal potential drivers of spatiotemporal patterns in stream processes. In this study, the abundance and diversity of 16S rRNA genes and abundance of *nosZ* genes, encoding for nitrous oxide reductase, were related to denitrification and environmental conditions. Denitrification rates varied among the three streams examined, and within a given stream there were significant longitudinal differences. Likewise, bacterial community structure based on analysis of the 16S rRNA gene also differed significantly among streams. However, variation in denitrification rate was not well correlated with environmental or biological variables measured. In addition, relatively large numbers of denitrifiers occurred when denitrification rates were low. In conclusion, although the streams differed in environmental conditions as well as bacterial community structure, these differences did not explain much of the spatial variation in denitrification rates.

**Keywords** Streams · Denitrification · Bacterial communities

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## Introduction

Denitrification, the microbially mediated reduction of nitrate to a nitrogenous gas, is an integral part of the global nitrogen cycle; conditions energetically favorable for denitrification are common in streams (e.g., Mulholland et al. 2008, 2009). Because of the ecological importance of this process, past studies have sought to explain spatiotemporal variations in lotic denitrification rates based on environmental data (Arango and Tank 2008; Inwood et al. 2005; Mulholland et al. 2008, 2009) and broad, general variables related to the microbial community, such as ecosystem respiration (Mulholland et al. 2008). Understanding the relationships between these variables is important for predictions of how biogeochemical processes will respond to changing environmental conditions (Schlesinger et al. 2006).

Basic factors required for denitrification are nitrate, organic matter, and anoxia. Typically, nitrate concentration is among the environmental variables that often correlates with denitrification rates in streams (Martin et al. 2001; Inwood et al. 2005; Arango and Tank 2008; Mulholland et al. 2009). Dissolved organic carbon (DOC) availability, because of its importance as a carbon and energy source for aquatic bacteria (Meyer et al. 1988), may also be limiting to denitrification, but evidence of its importance is not strongly supported in the literature. For example, Herrman et al. (2008), in a study of three Northeast Ohio streams, found that DOC amendments did not affect denitrification rates. Also, Bernhardt and Likens (2002) found that a whole-stream addition of labile DOC did not enhance denitrification rates. In contrast, a relationship between denitrification rates and benthic organic carbon is apparent (Inwood et al. 2005; Arango et al. 2007; Arango and Tank 2008). Finally, other environmental features, such as

hydrological conditions and temperature, can be related to denitrification rates (Herrman et al. 2008). A meta-analysis across diverse aquatic systems, revealed (using multiple regression) that nitrate, oxygen, organic carbon and phosphorus were predictive of denitrification rates (Piña-Ochoa and Álvarez-Cobelas 2006).

Denitrification results from the combined efforts of individual bacteria with a mixture of phylogenetic affinities and functional abilities (Zumft 1997). Community-wide biological measures, including respiration, production, total bacterial number, and total biomass may reveal coarse-scale information about microbial assemblages. However, these variables treat the microbial community as a whole and do not reveal differences in community structure, such as the phylogenetic affinities or abilities of the microbes. Community structure can be examined with well-developed molecular approaches that target either the denitrifier community directly or assess broad taxonomic differences via examination of 16S rRNA genes (Rich and Myrold 2004; Henry et al. 2006). For example, genes that encode for enzymes in the denitrification pathway can be measured to assess relative abundance of the denitrifiers (Henry et al. 2006). Other methods reveal variation in sequences of the target gene in the assemblage, such as terminal restriction-length polymorphisms (TRFLP, Cao et al. 2008; Perryman et al. 2008) or cloning, RFLP and sequencing (Prieme et al. 2002).

Molecular approaches have been applied to examine connections between denitrification rates and bacterial community structure in a variety of environments (Henderson et al. 2010; Song et al. 2011), including streams (Graham et al. 2010; Knapp et al. 2009; Rich and Myrold 2004); however, results of these studies are mixed. For example, Graham et al. (2010), in a study of prairie streams, found that abundance of *nir* genes (encoding for nitrite reductase) was related to denitrification efficiency, whereas denitrification rates appeared to be controlled by nitrate concentration. In an urban stream, the spatial distribution of these genes was highly heterogeneous (Knapp et al. 2009), demonstrating that there is genetic variation that potentially relates to differences in denitrification rates among locations. Likewise, in an intermittent, agriculturally impacted stream, Rich and Myrold (2004) found that sediment communities (based on *nosZ* gene sequences) were distinct from surrounding soil communities but that gene distribution appeared to be uncoupled from function. Thus, spatial variation in gene distribution and diversity are expected, but are not necessarily related to differences in denitrification rates.

In this study, we concurrently examined denitrification rates, environmental conditions presumed to be drivers of denitrification rates, and bacterial community composition and gene abundance. We hypothesized that denitrification rates would be related to specific bacterial and environmental variables, namely nitrate concentration, amounts of organic matter, and denitrifier abundance (based on quantification of the *nosZ* gene) across study sites. In contrast, we predicted that denitrification will be unrelated to measurements that incorporate the composition of the whole microbial community, including total bacterial counts, 16S rRNA gene abundance, and T-RFLP profiles based on 16S rRNA genes. To test these hypotheses, physicochemical data, denitrification rates, and bacterial communities were examined within and among three streams in northeast Ohio that drain watersheds with a range of land-uses.

## Methods

### Study sites

The three streams examined in northeast Ohio (USA) represented a range of environmental conditions. The first stream (which is unnamed) is located on the Mellinger Farm (MF) property near Wooster, OH in Wayne County and is part of the Killbuck Creek watershed. Mellinger Farm has been in operation since 1816, and current land use of the 1.3 $km^2$ property is approximately 50 % row crops (corn and soybeans); the remainder is pasture, mixed hardwood forest, buildings and roads (C. Hoy, personal communication).

The second stream was Silver Creek (SC), near Hiram, OH in Portage County, a tributary of Eagle Creek, located in the Mahoning River watershed. The reach sampled is part of the 1.6 $km^2$ James H. Barrows Hiram College Field Station and has a forested riparian zone.

The final stream, the West Branch of the Mahoning River (WBM), is located near Ravenna, OH in Portage County, also in the Mahoning River watershed. The WBM is a relatively undisturbed site, and all areas sampled had a forested riparian zone. The stream has been the subject of numerous previous studies on stream microbial ecology (e.g., McNamara and Leff 2004; Rubin and Leff 2007; Das et al. 2007).

### Environmental variables

Sediment samples were collected, as described below, in triplicate from each stream in summer and fall 2009 and three sites approximately 50 m apart were sampled per stream (total number of samples per stream per date was 9). This level of sampling was selected to represent the conditions along the reach studied in each stream. In two of the streams (SC and WBM), longitudinal differences in riparian vegetation, sediments, and other channel properties were not visually apparent. However, along the length of

the Mellinger Farm stream, there was variation in conditions. The most upstream site (site 1) had a forested riparian area and immediately upstream of this area was also forested, while site 2, which had deeper water than site 1 and very loose sediments, was between a corn field and a road-fill slope. The most downstream sampling site (site 3) was in a pasture and horses had access to the stream.

In the field, percent canopy cover was determined using a Spherical Crown Densiometer (Model C; Forestry Suppliers, Inc., Jackson, MS). Water temperature, conductivity, pH and dissolved oxygen were measured using a HQd/IntelliCAL Rugged Field Kit (Hach Company, Loveland, CO). Turbidity was determined with a 2100P Turbidometer (Hach Company). Velocity was measured with a Portable Water Flow meter Model 201 (Hach Company) and used, along with average stream width and average stream depth measurements, to calculate discharge.

Water samples (in triplicate from each site in each stream on each date) for nitrate, ammonium (fall samples only), and soluble reactive phosphorus (SRP) were filtered (0.45 μm filters, Gelman Instrument Co., Ann Arbor, MI) and concentrations were measured using a Lachat QuikChem 8000 FIA+ system (Lachat Instruments, Hach Company, Loveland, CO). Detection limits were 0.013 mg N $L^{-1}$, 0.006 mg N $L^{-1}$, and 3.5 μg P $L^{-1}$, respectively. For determination of DOC concentrations, samples were filtered through 0.7 μm glass microfiber filters and acidified with HCl and analyzed via high-temperature combustion on a Scientific Instruments TOC5000 analyzer (Shimadzu, Columbia, MD, detection limit = 0.15 mg C $L^{-1}$). Sediment percent organic matter and particle size distribution were determined as in Santmire and Leff (2006).

## Denitrification rates

Denitrification rates of sediment samples, collected using a plastic corer (6 cm wide × 4 cm deep) in triplicate from each site in each stream, were determined using the chloramphenicol-amended acetylene block technique (Royer et al. 2004). Sites for sample collection were chosen at random with one collection site along each transect at the right bank, one in the center, and one at the left bank. Very large rocks were avoided. For each site, 25 mL of sediment collected in the field was placed in a 75 mL bottle with a septum cap. Each bottle received 50 mL of stream water with chloramphenicol to achieve a final concentration of 1 mM. One replicate bottle without chloramphenicol was included as a control. Bottles were capped tightly and flushed with helium (He) for 5 min to create an anoxic environment. Acetylene gas (15 mL) was added to each bottle and they were incubated at approximately stream temperature. A headspace sample was collected 15 min after acetylene addition and once every hour for 4 h. After each sampling, a mixture of 10 % acetylene and 90 % He gas was added to the bottle to replace the headspace removed. Headspace samples were stored in evacuated vials and analyzed for $N_2O$ on a Shimadzu GC-2014 Gas Chromatograph (Shimadzu Corporation, Columbia, MD). The total mass of $N_2O$ in each bottle was determined from the headspace concentration and the appropriate Bunsen coefficient used to account for partitioning of $N_2O$ between gaseous and aqueous phases. Denitrification rates were calculated as the linear change in total $N_2O$ mass within the bottles during the sampling period.

## Microbiological variables

For total bacterial enumeration, sediment samples were weighed and preserved with formalin and phosphate buffered saline. Samples were stored at 4 °C following sonication in a Branson 2210 ultrasonic bath for 5 min in 0.1 % tetrasodium pyrophosphate to dislodge bacteria from particles. Bacterial abundance was determined using epifluorescence microscopy on samples stained with 4′6-diamidino-2-phenylindole (DAPI) (Kepner and Pratt 1994). Ten fields were counted for each sample and averaged.

Total algal biomass was estimated by measuring chlorophyll *a* as described in Sartory and Grobbelaar (1984). A known area of rock surface (3 rocks per transect) was scraped, filtered onto Type A/E glass fiber filters (Pall Corporation, Port Washington, NY), and frozen at −20 °C. Samples were then submersed in 95 % ethanol and heated at 79 °C for 5 min. Extraction occurred in the dark over 24 h at room temperature. Absorbance was recorded at 665 and 750 nm, and then each sample was acidified with 0.1 N HCl. After 90 s, samples were read again at 665 and 750 nm.

For DNA extraction, triplicate sediment samples were collected from each site (at the same time and same locations as the samples used for denitrification measurements) by coring with sterile 50-mL centrifuge tubes (3 cm wide × 4 cm deep) and samples were frozen at −80 °C. DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's instructions.

The quantity of 16S rRNA genes was determined with quantitative real-time PCR (QPCR). Reaction mixtures contained template DNA, SYBR Green PCR Master Mix (Applied Biosystems) and forward and reverse primers (0.2 μM each, from Fierer et al. 2005). The temperature profile on a Stratagene MX3005P Real-time PCR System (Agilent Technologies) was 95 °C for 10 min then 40 cycles of 94 °C (30 s), 57 °C (1 min) and 72 °C (30 s) (data acquisition step). A dissociation curve was generated via forty 30-second cycles, increasing 1 °C per cycle,

starting at 55 °C. *Pseudomonas aeruginosa* (ATCC number BAA-47; GenBank accession number AE004091) genomic DNA (extracted using the UltraClean Microbial DNA Isolation Kit, MoBio Laboratories) was used as a standard.

To examine denitrifiers, abundance of *nosZ* genes was determined with QPCR as in Henry et al. (2006). Reaction mixtures contained template DNA, SYBR Green PCR Master Mix and forward and reverse primers (from Henry et al. 2006). The temperature profile was 95 °C for 10 min and 40 cycles of 94 °C (45 s), 57 °C (1 min), 72 °C (2 min), and 80 °C (15 s) (data acquisition step). A dissociation curve was generated via forty 30-s cycles increasing 1 °C per cycle, starting at 55 °C. The standard and other conditions were as above for the 16S rRNA gene.

The structure of the bacterial community in benthic samples was determined using terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA gene. Primers were from Blackwood et al. (2005) and reaction mixtures contained template DNA, GoTaq Flexi DNA polymerase (2.5 U), ammonium PCR buffer, $MgCl_2$ (0.5 mM), deoxynucleoside triphosphates (0.2 mM each), forward and reverse primers (0.2 μM each), and bovine serum albumin (0.64 mg $mL^{-1}$). Forward primers were fluorescently labeled with 6-Fam (6-carboxyfluorescein). For PCR amplification, the temperature profile on a PTC-200 DNA Engine Cycler (BioRad) was 94 °C for 3 min and 30–35 cycles of 94 °C (30 s), 57 °C (30 s), and 72 °C (1 min 30 s) followed by a final extension of 72 °C for 7 min. For each sample, PCR products of five reactions were pooled and purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. PCR products were checked using 1 % agarose gels stained with ethidium bromide and digested with endonuclease *Hae*III (as in Wu et al. 2009) at 37 °C with 2 U of restriction endonuclease for 16 h. After digestion, T-RFLP analysis was performed at The Ohio State Plant Microbe Genomics Facility using a 3730 DNA Analyzer (Applied Biosystems). T-RFLP results were analyzed via GeneMapper 4.0 (Applied Biosystems). Attempts to garner enough PCR products for T-RFLP of the *nosZ* gene (following the method of Rich et al. 2003) were not successful, perhaps because of the modest copy numbers present.

### Statistical analysis

Data were tested for normality and equal variance (Shapiro–Wilk and Anderson–Darling tests, $P > 0.05$), and all data required log-transformation. Nested ANOVAs with Tukey's HSD post hoc multiple comparisons were used to test for significant differences in variables between sites within individual streams and within seasons. However, we were unable to normalize nitrate concentrations and instead used the Kruskal–Wallis test to analyze these data. Analyses were performed in SYSTAT 12 (Systat Software Inc, Chicago, IL). Multiple linear regression (with stepwise forward selection) was used to determine relationships between denitrification rate and denitrifier abundance with all other measured variables. Multiple regression was performed in Sigmaplot 11 (Systat Software Inc). Redundancy analysis was used to determine factors that explained significant amounts of variation among T-RFLP profiles and was performed in CANOCO for Windows version 4.5 (ter Braak and Smilauer 2002). Redundancy analysis was selected based on the findings of Blackwood et al. (2003) and is suitable for "environmental interpretation" of microbial communities (Ramette 2007).

## Results

### Environmental variables

Physical and chemical conditions of the streams on the two sampling dates are summarized in Table 1. The agriculturally impacted Mellinger Farm stream had the largest percentage of open canopy and warmer temperatures than the streams with intact riparian zones. In summer, this stream had substantially higher turbidity than the other two streams and, on both dates, much lower discharge.

Nitrate concentrations were similar among streams in the fall based on the Kruskal–Wallis (KW) test ($P > 0.05$), but were higher in MF compared to WBM and SC during the summer (Fig. 1; KW, $P < 0.001$). Further, the two forested streams (WBM and SC) had higher nitrate concentrations in the fall than in the summer. In contrast, concentrations were higher in summer than in fall (KW, $P < 0.001$) in the agriculturally impacted stream (MF), but this was driven by high nitrate concentrations at the most downstream site in summer.

Ammonium concentration (only measured in fall) was 4.5 times higher in the agriculturally impacted stream (MF) than the other two streams (nested ANOVA, $P < 0.001$). Concentrations in that stream were highest at the most downstream site, although differences among sites were not significant (Table 2). SRP concentrations were below detectable levels in the summer in the forested streams and were higher in MF ranging from below detection to 23.5 μg $L^{-1}$. SRP concentrations varied along the length of the MF stream with lower concentrations at the most upstream sites [mean = 9.4 (site A) and 3.1 (site B) μg $L^{-1}$], and 2× higher concentrations at the most downstream site [mean = 19.9 μg $L^{-1}$ (site C)]. In fall, SRP was detectable in all streams but not in all samples. Like nitrogen concentrations, the most downstream site had the highest SRP concentrations in MF.

**Table 1** Mean (±1 SE) summer and fall percent open canopy, temperature, pH, specific conductivity, turbidity, discharge, dissolved oxygen, benthic percent organic matter, and chlorophyll *a* concentrations by stream for each sampling date

| Stream | SC | MF | WBM |
|---|---|---|---|
| % Open canopy | | | |
| Summer | 26.5 ± 4.5 | 77.5 ± 11.3 | 19.6 ± 2.7 |
| Fall | 46.6 ± 1.7 | 81.3 ± 6.6 | 49.5 ± 3.0 |
| Temperature (°C) | | | |
| Summer | 18.9 ± 0.10 | 21.4 ± 0.41 | 18.9 ± 0.13 |
| Fall | 8.5 ± 0.06 | 7.2 ± 0.09 | 12.3 ± 0.36 |
| pH | | | |
| Summer | 8.1 ± 0.01 | 7.6 ± 0.10 | 8.4 ± 0.06 |
| Fall | 7.6 ± 0.03 | 7.9 ± 0.02 | 6.7 ± 0.05 |
| Specific conductivity (μS $cm^{-1}$) | | | |
| Summer | 470 ± 1 | 545 ± 25 | 606 ± 2 |
| Fall | 456 ± 3 | 553 ± 7 | 424 ± 6 |
| Turbidity (NTU) | | | |
| Summer | 10 ± 0 | 46 ± 19 | 8 ± 1 |
| Fall | 4 ± 0 | 4 ± 0 | 3 ± 0 |
| Discharge (L $s^{-1}$) | | | |
| Summer | 60 | 0.43 | 80 |
| Fall | 60 | 1.0 | 90 |
| Dissolved oxygen (mg $L^{-1}$) | | | |
| Summer | 7.9 ± 0.08 | 7.9 ± 0.80 | 8.6 ± 0.11 |
| Fall | 10.8 ± 0.03 | 12.3 ± 0.07 | 7.5 ± 0.12 |
| % Organic matter | | | |
| Summer | 1.7 ± 0.25 | 6.8 ± 1.00 | 2.8 ± 1.10 |
| Fall | 1.3 ± 0.15 | 6.2 ± 0.74 | 1.0 ± 0.11 |
| Chl *a* (mg $m^{-2}$) | | | |
| Summer | 6.6 ± 3.5 | 11.5 ± 7.6 | 4.8 ± 2.0 |
| Fall | 22.5 ± 7.4 | 8.2 ± 3.2 | 8.7 ± 1.2 |

Values are averages of the sites sampled in each stream ($n = 9$)

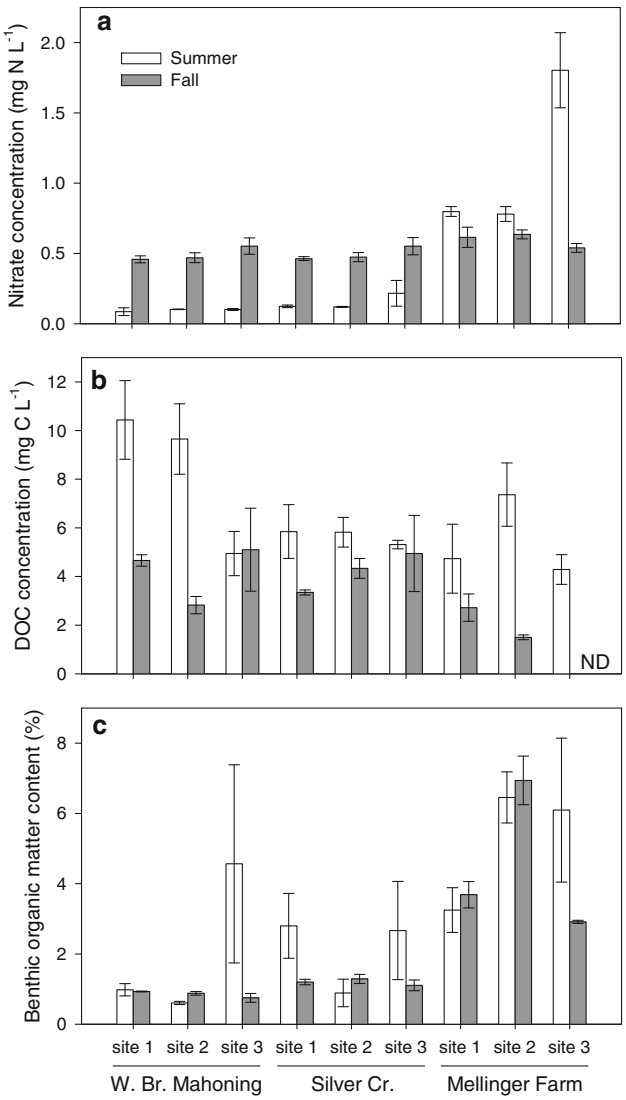


**Fig. 1** **a** Nitrate concentrations, **b** dissolved organic carbon concentrations and **c** benthic percent organic matter at each site in the three streams examined on two dates. On the *x*-axis, sites *1*, *2*, and *3* represent the three sites sampled in each stream from the most upstream to the most downstream site. *ND* no data. Values are means of three replicates and standard errors

Differences in DOC concentrations varied by stream and season (stream by season interaction, $P < 0.05$, Fig. 1). Across streams, DOC concentrations were significantly higher in summer than in fall and there were significant differences among streams. Benthic organic matter content also varied among streams ($P < 0.05$); MF had 3 times higher benthic organic matter content (mean = 4.9 %) than the other two streams (Fig. 1, mean WBM = 1.5 % and SC = 1.7 %).

## Microbiological variables

Chlorophyll *a* (chl *a*) concentrations were variable across streams and dates (Table 1, nested ANOVA stream by date interaction $P < 0.05$). Concentrations were lowest in WBM in summer; this site had a high percentage of canopy coverage. MF in fall had the lowest chl *a* concentrations in spite of having an open canopy. Also in MF, chl *a* concentrations at the most downstream site were more than seven times higher than the two most upstream sites (data not shown).

The abundance of bacterial cells per gram DM (based on DAPI staining) was significantly higher in the two forested streams (mean WBM = 2.7 and SC = 2.5 × $10^7$ cells $g^{-1}$ DM) than in MF (Fig. 2, mean MF = 1.0 × $10^7$ cells $g^{-1}$ DM). In addition, numbers in fall (mean = 2.4 × $10^7$ cells $g^{-1}$ DM) were significantly higher than in summer (nested ANOVA $P < 0.05$, mean = 1.6 × $10^7$ cells $g^{-1}$ DM).

**Table 2** Average (and standard error) concentrations of ammonium and SRP in fall

| Stream | Site | Ammonium (μg N $L^{-1}$) | SRP (μg P $L^{-1}$) |
|---|---|---|---|
| WBM | A | 8.3 (1.9) | 1.1 (0.4) |
| | B | 9. 9 (1.4) | 0.4 (ND) |
| | C | 6.0 (3. 6) | 1.0 (0.1) |
| SC | A | 15.6 (5.7) | 2.7 (2.2) |
| | B | 5.5 (1.69) | 1.1 (ND) |
| | C | 9.5 (2.9) | 0.2 (ND) |
| MF | A | 41.2 (12. 9) | 0.6 (0.2) |
| | B | 24.8 (10.9) | 3.0 (0.6) |
| | C | 58.3 (10.5) | 3.5 (1.7) |

ND represents cases where only one of the three replicate samples from a given site gave detectable values

*WBM* West Branch of Mahoning, *SC* Silver Creek, *MF* Mellinger Farm

The number of copies of the 16S rRNA gene varied significantly among streams. MF and SC had greater numbers of copies of this gene than WBM (Fig. 2; average across both dates was 4.8, 3.5, and $2.0 \times 10^7$ copies $g^{-1}$ DM, respectively). Number of copies of the *nosZ* gene were also highest in MF compared to the other two streams (Fig. 2, average across both dates was MF = 0.37, WBM = 0.04, SC = $0.13 \times 10^7$ copies $g^{-1}$ DM sediment).

Potential relationships of abundances of both genes to environmental variables were examined based on multiple linear regression (MLR). MLR showed that abundance of 16S rRNA gene copy number was unrelated to environmental drivers, but *nosZ* abundance was significantly related to benthic organic matter and temperature (Table 3).

To examine bacterial community structure, redundancy analysis was used to examine T-RFLP data. This analysis revealed that there were differences in bacterial community composition among streams (Table 4). One notable exception was the similarity of the two forested streams in summer; physiochemical conditions were also similar in these two streams on this date. Although differences between pairs of streams were typically statistically significant, the percentage of variation that was explained by these differences was low (maximum of 30.2 %).

## Denitrification rates

Denitrification rates were highly variable across streams in both summer and fall (Fig. 3). WBM had significantly higher denitrification rates than the other two streams ($P < 0.05$, means across dates: WBM = 0.568, SC = 0.243 and MF = 0.521 ng $N_2O$–N $g^{-1}$ DM $h^{-1}$). In addition, the interaction between stream and date was

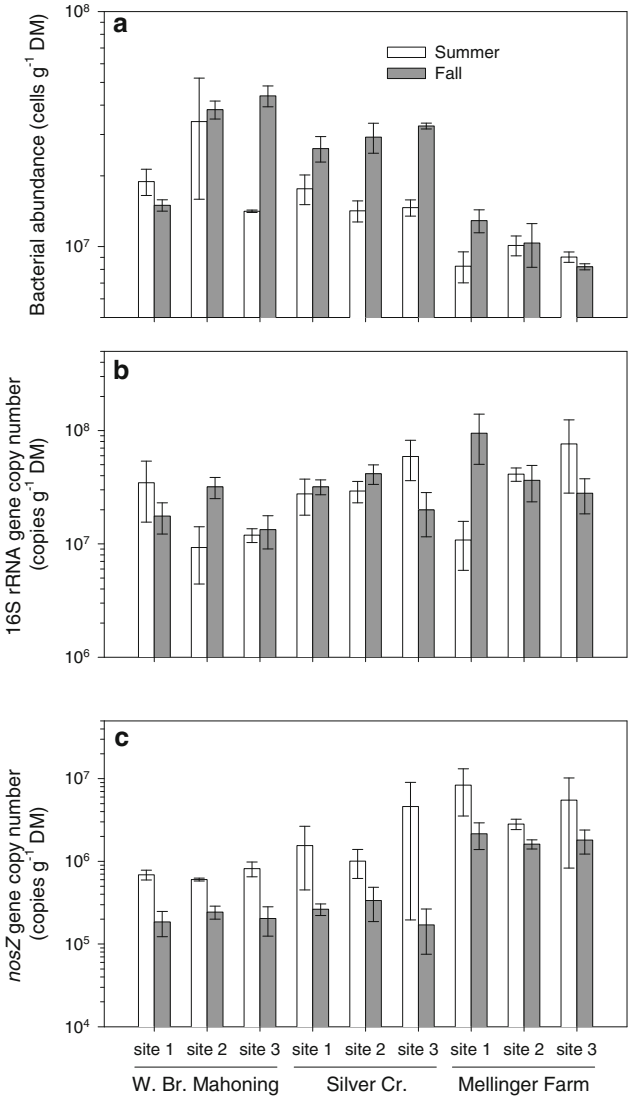


**Fig. 2** **a** Bacterial abundance, **b** number of copies of the 16S rRNA gene and **c** number of copies of the *nosZ* gene at each site in the three streams examined on two dates. On the *x*-axis, sites *1*, *2*, and *3* represent the three sites sampled in each stream from the most upstream to the most downstream site. *ND* no data. Values are means of three replicates and standard errors

significant and there were significant differences among sites within a stream ($P < 0.05$). Surprisingly, MF had a very low denitrification rate in the summer (mean = 0.184 ng $N_2O$–N $g^{-1}$ DM $h^{-1}$) with the lowest measured rate of all of the streams. In fall, MF denitrification rates increased longitudinally downstream with a rate 15 times higher at the most downstream site compared to the most upstream site.

When examining factors influencing the variation in denitrification rate using MLR, denitrification rates were positively related to benthic organic matter content (Table 3). Surprisingly, denitrification rates were negatively related to nitrate concentration. Although MLR results were

**Table 3** Summary of stepwise multiple linear regression results including coefficients, total and partial $R^2$, and $P$ values for each significant variable explaining *nosZ* abundance (overall $P < 0.001$; $n = 54$) and denitrification rates (overall $P = 0.005$; $n = 54$)

| Step # | Independent variable | Coefficient | Total $R^2$ | $\Delta R^2$ | $P$ value |
|---|---|---|---|---|---|
| *nosZ* abundance [log (copies $g^{-1}$ DM)] | | | | | |
| 1 | Log benthic organic matter content (%) | 0.824 | 0.30 | 0.30 | <0.001 |
| 2 | Log temperature (°C) | 0.856 | 0.40 | 0.10 | 0.019 |
| | Intercept | 4.663 | | | <0.001 |
| Denitrification [log (ng $N_2O$–N $g^{-1}$ DM $h^{-1}$)] | | | | | |
| 1 | Log nitrate (mg N $L^{-1}$) | −0.635 | 0.09 | 0.09 | 0.003 |
| 2 | Log benthic organic matter content (%) | 0.504 | 0.19 | 0.10 | 0.016 |
| | Intercept | −4.061 | | | |

**Table 4** Results of redundancy analyses of 16S rRNA T-RFLP profiles

| | WBM vs. SC | | WBM vs. MF | | SC vs. MF | |
|---|---|---|---|---|---|---|
| | Summer | Fall | Summer | Fall | Summer | Fall |
| $P$ value | 0.448 | **0.014** | **0.001** | **0.001** | **0.001** | **0.002** |
| % Variation explained | 6.5 | 12.6 | 21.8 | 30.2 | 21.9 | 22.3 |

Bolded values indicate a statistically significant relationship ($P < 0.05$)

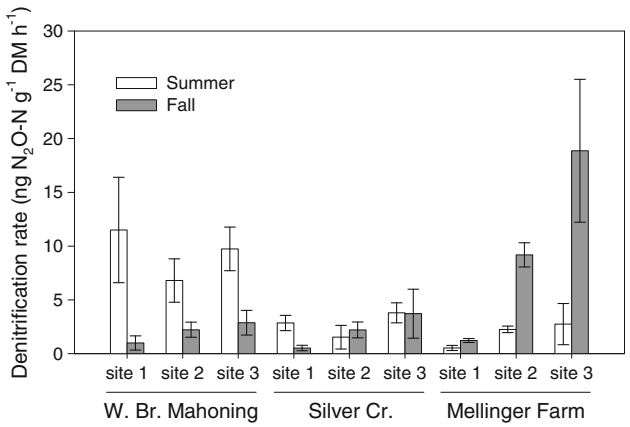


**Fig. 3** Denitrification rates at each site in the three streams examined; values are means of three replicates and standard errors. On the *x*-axis, sites *1*, *2*, and *3* represent the three sites sampled in each stream from the most upstream to the most downstream site

statistically significant, the MLR only explained a total of 19 % of the variation in the denitrification data.

## Discussion

Environmental variables we predicted would drive variation in denitrification rates (i.e., nitrate and organic matter) showed significant, although unexpected patterns. Many studies have found denitrification rates to be strongly correlated with $NO_3^-$ concentrations (e.g., Martin et al. 2001; Mulholland et al. 2009). Previous studies have also found that sediment organic matter content is related to denitrification rates (Inwood et al. 2005; Arango et al. 2007; Arango and Tank 2008; Beaulieu et al. 2009). In contrast, in our study, denitrification rate was weakly negatively correlated with nitrate concentration and positively (although also weakly) correlated with benthic organic matter content. Overall, in the present study, environmental variables typically thought to drive denitrification were not related to rates, and variation in specific environmental conditions among sampling locations may be responsible.

Several factors may have contributed to the lack of expected relationship between nitrate concentrations and denitrification. First, nitrate concentration was negatively correlated with discharge (Pearson's $r = -0.64$), suggesting that that site selection may have played a role and that accounting for differences in discharge may override the negative nitrate:denitrification relationship. Second, at a particular stream site, denitrification rates sometimes deviated from what might be expected based on environmental conditions. For example, there were low denitrification rates in the most agriculturally impacted stream and high rates in one of the forested streams. Specifically, in summer, the Mellinger Farm stream exhibited low denitrification rates relative to the other streams and rates varied among sites within the stream. Denitrification rates in this stream averaged 0.07 mg N $m^{-2}$ $day^{-1}$ in summer (values are expressed here on a per area basis to facilitate comparison to other studies), whereas Royer et al. (2004) in a study of agriculturally impacted Illinois streams found rates ranging from <2.4 to 375 mg N $m^{-2}$ $day^{-1}$ over the course of a year. Likewise, Kemp and Dodds (2002) found that downstream from agriculturally impacted locations, in two streams (Kings and Shane Creek) denitrification rate averaged 0.40 and 0.39 mg N $m^{-2}$ $day^{-1}$. By comparison, in summer, the two forested streams

(WBM and SC) averaged 1.45 and 0.52 mg N $m^{-2}$ $day^{-1}$, respectively, while in fall Mellinger Farm rates averaged 1.36 mg N $m^{-2}$ $day^{-1}$. Moreover, the WBM had unexpectedly high rates of denitrification in light of the relatively low nitrate concentrations. In addition, the rates observed in Mellinger Farm are surprising in light of the environmental conditions associated with land use in this watershed. Specifically, in summer this stream had higher temperatures, higher nitrate concentrations, and greater percentages of benthic organic matter. Streams with high nitrate concentrations frequently have high denitrification rates (Martin et al. 2001; Inwood et al. 2005; Arango and Tank 2008; Mulholland et al. 2009). However, the nitrate concentrations in the streams examined in this study are lower than those of the highly agriculturally impacted streams examined by Royer et al. (2004) and Arango and Tank (2008). Nevertheless, compared to streams in Arango and Tank (2008), given the concentrations of nitrate in MF, we would have expected denitrification rates that were 1–2 orders of magnitude higher than were found in summer.

Potential causes of the low rates observed in MF include inhibitory compounds (such as sulfur ions, Gould and McGready 1982). Another possibility is that the denitrifiers were restricted in function in the environment by lack of labile organic carbon or limitations in extent of anaerobic zones; perhaps the assays were not of sufficient length to facilitate a detectable response to altered conditions in the laboratory. In addition, the acetylene block method has shortcomings and may have inhibited nitrification causing it to be decoupled from denitrification (Knowles 1990; Seitzinger et al. 1993). Lastly, perhaps the conditions were highly reducing and dissimilatory reduction of nitrate to ammonia (DNRA) was favored over denitrification (Burgin and Hamilton 2007); high concentrations of ammonia in this stream are consistent with this possibility.

Responses of denitrification rate to availability of nitrate are transduced by the denitrifier community (Wallenstein et al. 2006). In our study, in spite of the relatively low denitrification rates at Mellinger Farm, the number of *nosZ* gene copies detected was not reduced. Denitrifiers are typically facultative anaerobes (Wallenstein et al. 2006) and so factors that affect their abundance may include those unrelated to denitrification. This possibility is supported to a degree by the observation of higher total bacterial numbers based on 16S rRNA gene abundance at Mellinger Farm. However, when *nosZ* copies are expressed as a percent of 16S rRNA gene abundance, in summer, this stream had relative abundances that were twice that of the other streams.

Abundance of the *nosZ* gene was related to temperature and benthic organic matter but not denitrification. In contrast to the present study, in a study of agriculturally impacted Indiana streams, Baxter et al. (2012) found that *nosZ* abundance was related to denitrification rates; rates were also related to sediment organic content. However, much of the variation in *nosZ* abundance was not explained by variables measured, suggesting that other factors, such as pore water chemistry, are important. In addition, Johnson et al. (2012) found that addition of labile DOC to one of these Indiana streams did not impact *nosZ* gene abundance or denitrification rate. The *nosZ* gene is only one of many functional genes in the genomes of denitrifiers; thus, the abundance of this gene is likely influenced by many properties of the organisms and their environment.

In addition to differences in denitrification rates and bacterial abundances, overall bacterial community structure (based on examination of the 16S rRNA genes) differed among streams. Variation in environmental conditions among the streams was likely a major contributor to these differences; especially given the largest differences were between the agriculturally impacted stream and the two forested streams. Even in those cases, in each pair-wise comparison of the streams, these differences explained at most 30 % of the variation, indicating that other factors were responsible for the majority of differences observed. A variety of environmental factors are thought to be potential drivers of bacterial community structure in streams and there is no comprehensive generality that can be drawn based on publications to date (Findlay 2010). Some of these factors, such as temperature, pH, and substrate properties (Fierer et al. 2007; Santmire and Leff 2006, respectively), are fairly straightforward, while others the quality of the DOC pool, in particular, are more difficult to ascertain (Wu et al. 2009). In addition, prior studies of stream bacterial communities suggest that factors that drive structure vary spatial and temporally (McNamara and Leff 2004; Olapade and Leff 2005), which is likely responsible for the lack of clear generalities (Findlay 2010).

In conclusion, differences in environmental conditions among streams were apparent as were variations along the length of some streams. Such differences were reflected in the measured attributes of the bacterial community. However, neither the environmental conditions nor the bacterial community metrics were strongly related to denitrification rates and rates in the agriculturally impacted stream were relatively low on one date in spite of high nitrate concentrations. In addition, the occurrence of large numbers of denitrifiers (based on *nosZ* copy numbers) when rates were relatively low suggests that communities are poised to respond when favorable conditions return.

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