

Factors Controlling Sediment Denitrification in Midwestern Streams of Varying Land Use

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Abstract

We investigated controls on stream sediment denitrification in nine headwater streams in the Kalamazoo River Watershed, Michigan, USA. Factors influencing denitrification were determined by using experimental assays based on the chloramphenicol-amended acetylene inhibition technique. Using a coring technique, we found that sediment denitrification was highest in the top 5 cm of the benthos and was positively related to sediment organic content. To determine the effect of overlying water quality on sediment denitrification, first-order stream sediments were assayed with water from second- and third-order downstream reaches, and often showed higher denitrification rates relative to assays using site-specific water from the first-order stream reach. Denitrification was positively related to nitrate (NO_3^-) concentration, suggesting that sediments may have been nutrient-limited. Using stream-incubated inorganic substrata of varying size classes, we found that finer-grained sand showed higher rates of denitrification compared to large pebbles, likely due to increased surface area per volume of substratum. Denitrification was measurable on both inorganic substrata and fine particulate organic matter loosely associated with inorganic particles, and denitrification rates were related to organic content. Using nutrient-amended denitrification assays, we found that sediment denitrification was limited by NO_3^- or dissolved organic carbon (DOC, as dextrose) variably throughout the year. The frequency and type of limitation differed with land use in the watershed: forested streams were NO_3^- -limited or co-limited by both NO_3^- and DOC 92% of the time, urban

streams were more often NO_3^- -limited than DOC-limited, whereas agricultural stream sediments were DOC-limited or co-limited but not frequently limited by NO_3^- alone.

Introduction

The global nitrogen (N) cycle has been modified by human activities that increase the pool of biologically available N, including fossil fuel combustion, fertilizer production, and watershed disturbance [38]. This increase may alter aquatic ecosystem structure and function (e.g., species diversity and productivity [9, 22, 38]). Streams often receive large amounts of nitrate N (NO_3^-) via agricultural runoff (e.g., [6, 21]), which may then be transported downstream. Microbial transformations, such as denitrification, may mitigate the amount of N transported to downstream ecosystems [1, 13, 25, 28, 29]. Denitrification converts NO_3^- to N gases (nitrous oxide, N_2O , and dinitrogen, N_2), generally using carbon as an electron donor, resulting in a reduction of bioavailable N. A broad spectrum of bacteria facultatively denitrify when enzyme synthesis (e.g., nitrate reductase) is induced by low oxygen availability [42]. Understanding the mechanisms that control sediment denitrification in streams is essential for determining the importance of denitrification in local, regional, and global N cycles.

Currently, it is unclear which environmental factors have the greatest influence on denitrification activity, as well as what methods are most appropriate for measuring denitrification rates [3, 16, 17, 28, 30, 32, 35]. There remain uncertainties regarding the relative importance of sediment denitrification in stream N cycling due to an incomplete understanding of the factors controlling denitrification, as well as inconsistencies in methods. This restricts our ability to interpret N cycling data and

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formulate effective management plans. Potential factors influencing denitrification are repeatedly incorporated into ecosystem modeling of N removal (e.g., [6, 31, 37]), and are commonly used for supporting management and policy decisions. Thus, understanding the environmental factors that affect denitrification rates is critical to understanding N removal in aquatic ecosystems.

The following factors have been previously identified as potential direct controls on aquatic sediment denitrification: (1) NO_3^- availability [15, 20, 24, 28], (2) organic carbon (C) availability [20, 28], and (3) dissolved oxygen concentration [15, 28]. Potential indirect or secondary controls that may influence sediment denitrification include temperature [19, 24], sediment depth [7, 19], sediment size and composition [8, 24], substrata type ([29], Tank and Arango, unpublished data), the presence of coupled nitrification–denitrification as a nitrate supply [14, 27, 40], land use [13, 14], hydrology [10–12], and whether the system is lotic or lentic (see review in [30]). The significance of each factor to overall denitrification rates varies among streams and over time within an individual stream.

The method used to estimate denitrification also varies among studies making interpretations difficult. One common method used to quantify denitrification is the acetylene (C_2H_2) inhibition technique (e.g., [32]), which allows measurement of N_2O accumulation by blocking the conversion of N_2O to N_2 in the denitrification pathway. This procedure is used with or without nutrient amendments, and/or the addition of chloramphenicol to sediment cores or slurries [3, 28, 32]. Denitrification has also been estimated by using ecosystem mass balance calculations. This approach relies on estimates of N inputs and outputs to a given ecosystem (e.g., a watershed) and the difference is attributed to denitrification (e.g., [6, 18, 31, 37]). More recently, isotopic tracer studies using $^{15}\text{NO}_3^-$ and its subsequent depletion or conversion to $^{15}\text{N}_2$ or $^{15}\text{N}_2\text{O}$ have been used as an alternative method for estimating denitrification either in cores [3, 40] or the whole-reach [4, 17, 23]. Results must be interpreted within the context of the method employed.

To clarify which factors control stream sediment denitrification, and to place these factors in the context of previous studies, we designed four experiments to identify primary controls on denitrification. The following questions directed our research: (1) Does denitrification vary with depth into sediment? (2) How does streamwater source and associated water-column nutrients affect stream sediment denitrification? (3) Does variation in sediment quality influence associated denitrification rates? (4) Are patterns of nutrient limitation in stream sediment denitrification influenced by land use or season? We quantified sediment denitrification rates by using a standardized microcosm assay (chloramphenicol-amended C_2H_2 inhibition [32]) on sediments from

headwater streams within the Kalamazoo River Watershed, Michigan, USA.

Methods

Site Description. Nine streams draining subwatersheds of the Kalamazoo River Basin in southwestern Michigan were selected and classified as forested (FX, FY, FZ), agricultural (AX, AY, AZ), or urban (UX, UY, UZ) based on dominant land cover determined by in-field observations and geographical information system (GIS) analysis [13]. Within each subwatershed, a first-order headwater site was sampled. Downstream locations were also sampled at second- and third-order reaches to determine if water quality changes associated with stream size influenced denitrification rates from sediments collected at first-order sites (see Experiment 2). After the general denitrification assay description, specific modifications for each experiment are detailed.

General Denitrification Assay: Chloramphenicol-Amended Acetylene Inhibition Technique. The C_2H_2 inhibition method was used to quantify denitrification rates [32] for all experiments. Assay bottles were amended with chloramphenicol to eliminate new production of nitrite reductase enzyme [5, 32]. For all denitrification assays, four replicate graduated media bottles were filled with 25 cm^3 of sediment slurry. One replicate bottle was used as a no- C_2H_2 control. Bottles were then filled to 75 mL with site-specific streamwater amended with chloramphenicol (final concentration, 6 mM chloramphenicol), and sealed with a screw-cap fitted with a butyl septum. For each replicate bottle, a subsample of sediment was placed in an ashed and preweighed aluminum pan for determination of sediment dry mass (DM) and ash-free dry mass (AFDM).

Each assay bottle was then purged with ultrapure helium gas for 5 min, swirling several times to ensure distribution into sediments. The purging needle was removed immediately after the venting needle in order to maintain positive pressure. Pure C_2H_2 (15 mL) was then added to each bottle (total pressure 10 kPa). Headspace gas samples (5 mL) were collected over time after shaking each bottle and 4 mL of sampled gas was injected into labeled Vacuainers, subsequently sealed with a silicone rubber bead. Headspace replacement gas (10% C_2H_2 , 90% He) was then added to the assay bottle to maintain pressure. For each assay bottle, five gas samples were collected approximately every hour for 5 h after C_2H_2 addition. Headspace N_2O concentrations from samples were measured on a gas chromatograph equipped with an electron-capture detector (Hewlett Packard 5890 series II; Porapak Q column, oven temperature 70°C , 320°C detector temperature, N_2 carrier flow $\sim 35\text{ mL}$

min⁻¹) and were corrected for temperature and partial pressure by using the appropriate Bunsen coefficient from the equation in [34], followed by a correction for sampling. Production rates of N₂O (in µg N₂O h⁻¹) were then calculated as the statistically significant linear slope of N₂O concentration plotted against time and expressed per g sediment mass (DM and AFDM).

Experiment 1: Effect of Sediment Depth on Denitrification. In October 2002, we collected benthic sediments from an agricultural headwater stream to determine the influence of sediment depth on denitrification rates. Previous work confirmed that sediment from this agricultural stream had high denitrification rates [13], which increased the likelihood of identifying potential variation in denitrification with depth into the stream benthos. Sediments were collected by pushing a vertical corer (diameter, 6 cm) into the streambed, sealing the top to create suction, and removing the corer and intact benthic sediment. Vertical fractions were collected from the core in the field by pressing the entire core through the top while measuring and collecting the desired depth fractions (0–2, 2–5, 5–10, and 10–15 cm). To obtain sufficient material for replicate assay bottles, the coring and fractioning process was repeated at nine points across the width of the stream, and all nine fractions of each depth were pooled in containers, covered with streamwater, and placed on ice for transport to the laboratory. Unfiltered water was collected from the site for use with the denitrification assay. Additionally, a filtered 60-mL water sample was collected, transported on ice, and frozen for analysis of dissolved nutrient content. Previous experiments have shown that sediment transport and storage in the laboratory for less than 24 h does not influence measured denitrification rates [39]. Laboratory denitrification assays were conducted on each sediment slurry depth fraction as described above.

Experiment 2: Influence of Stream Size and Associated Water Quality on Sediment Denitrification. Sediments from first-order sites were incubated with unfiltered streamwater from second- or third-order downstream reaches and compared to assays incubated with *in situ* water (first-order streamwater). We sampled nine first-order sites from streams draining watersheds with forested, agricultural, or urban dominated land use (three each) to examine possible influences of land use on downstream changes in water quality and sediment denitrification response to overlying water. In May 2002, composite sediment samples were collected from approximately the top 5 cm of the benthos in each first-order stream, sampling in a zigzag pattern across the width of the streambed. Sediment samples were placed on ice, transported back to the laboratory, and refrigerated for overnight storage until denitrification assays were begun.

Unfiltered water was collected from the first-order sites as well as the second- and third-order reaches downstream (except site UX where only two reaches were sampled). A filtered water sample was also collected from each reach for analysis of dissolved NO₃⁻, DOC, and specific ultraviolet absorbance (SUVA) as an index of carbon quality (detailed methods described below).

Experiment 3: Effect of Substratum Size and Inorganic Content on Denitrification Rates. We examined the effect of substratum characteristics on stream denitrification to determine whether biofilms on inorganic substrata of varying sizes contribute to denitrification activity in streams. Natural inorganic substrata (sand and pebbles) were collected from the benthos of a third-order stream in north central Indiana, rinsed thoroughly with distilled water, and fractioned into specific size classes by using mesh sieves resulting in sand (0.25–1 mm), small pebble (2–4 mm), and large pebble (4–8 mm) fractions. Each size fraction was dried and ashed at 550°C for 5 h. Small bags of substratum size classes were created by placing ~25 cm³ of each substratum fraction into nylon socks that were then closed with a knot and plastic cable tie. In September 2002, replicate substratum bags (*N* = 9 for each size class) were anchored to the stream bed with a stake and placed in the same stream channel within a ~1 m² area. After a 1-month incubation, bags were carefully recovered with minimal disruption, placed in plastic resealable bags, saturated with streamwater, and transported on ice back to the laboratory. In addition, native stream sediments were collected slightly upstream of the incubation site (as described in Experiment 2) for determination of ambient sediment denitrification rates. Denitrification assay bottles were prepared for each substratum type as well as the native sediment using four techniques: (A) unrinsed sediment—one substratum bag was cut open and its contents emptied into an assay bottle (four replicate bottles from four replicate substratum bags); (B) rinsed sediment—one substratum bag was emptied into an assay bottle to which was added 5 mL unfiltered streamwater, the bottle was gently swirled to suspend unattached fine particulates, and this “supernatant” was quickly poured into another assay bottle; (C) rinse water or “supernatant” from treatment B that contained unattached fine particulates (four replicate bottles of rinsed sediment from four replicate bags; all supernatant combined in one bottle per size class); and (D) a “no-chloramphenicol” control otherwise identical to the unrinsed sediment treatment (using 1 substratum bag). All bottles were assayed using the same streamwater (chloramphenicol-amended except for treatment D) to eliminate differences in dissolved nutrient availability. In this experiment, sediment DM and AFDM for each bottle was quantified by filtering the entire contents onto an ashed and preweighed filter

(Gelman A/E, 1.0 μm nominal pore size) after completion of the denitrification assay.

Experiment 4: The Influence of Dissolved NO_3^- and Organic C on Sediment Denitrification. We performed nutrient-amended sediment denitrification assays to evaluate whether nutrient limitation of sediment denitrification varies in streams draining subwatersheds dominated by different land use and whether there were seasonal patterns in nutrient limitation. Stream sediments were collected from each of the nine first-order streams during February, April, June, August, October, and December 2002, as described in Experiment 2 above. Assay bottles containing sediment slurry, unfiltered streamwater, and chloramphenicol were amended with either NO_3^- (+4.7 mg N L^{-1} above ambient using 1 mL of a 350 mg L^{-1} stock solution), or dextrose (representative of labile organic C; +30 mg C L^{-1} above ambient, using 1 mL of 2.25 g C L^{-1} stock solution), or both nutrients, in addition to ambient controls (triplicates for each treatment and site).

Analysis of Water Column Dissolved Nutrients and Sediment Quality. Filtered water samples were analyzed for NO_3^- by using ion chromatography ([36], modified according to Applications Note 133; Dionex Corporation, Sunnyvale, CA, USA; IonPac AG14 guard column, AS14 analytical column). Dissolved organic carbon (DOC) content was determined on a Shimadzu 5000A carbon analyzer after acidifying samples to pH of 2–4 [2]. SUVA was calculated as the absorbance at 254 nm of unacidified sample divided by DOC concentration [2], and indicates relative aromaticity [41].

Sediment organic content was calculated by obtaining the difference in mass between dried samples (55°C for 24 h minimum) and samples ashed in a muffle furnace (at 550°C for 1.5 h), rewetted, and redried. These values were used to express denitrification rates per g DM and per g AFDM. The percent organic content was calculated as $\text{AFDM}/\text{DM} \times 100$ for each sediment sample. For Experiment 4, an additional sediment subsample from each site was dried, ground, and analyzed on an elemental analyzer for N and C content (expressed as % N or % C; Costech Analytical Technologies, Inc. ECS 4010).

Statistical Analyses. Statistical analyses were performed by using SAS Analyst (System for Windows V8; SAS Institute, Inc., Cary, NC, USA). Data were transformed where necessary to meet the statistical assumption of normal distribution, generally by log transformation, and significance was determined at the 0.05 α level (95% confidence). Linear regression or correlation analysis (using Bonferroni corrections) was used to relate sediment denitrification rates to potential controlling factors (reported with the r^2 or R value,

respectively). Depending on the experiment, significance of depth, water source, substrata type or treatment was assessed by using analysis of variance (ANOVA) followed by Tukey-adjusted least square (LS) means test for individual treatment comparisons. For Experiment 4, we assumed that nutrient limitation of stream sediment denitrification by NO_3^- and/or DOC (as dextrose) occurred when amendment of the denitrification assay bottle with NO_3^- and/or dextrose caused a significant increase in denitrification rate (relative to rates of the unamended controls) by using a two-way factorial ANOVA for each site and date [33]. The relative response of denitrification to nutrient amendment (calculated as the ratio of the nutrient-amended denitrification rate divided by the unamended ambient rate) was also calculated for each site, treatment, and date [33]. Response ratios among treatments and streams were analyzed with ANOVA and correlated with environmental factors (water column NO_3^- , DOC, NH_4^+ , SRP, and SUVA; sediment N, C, and organic content).

Results

Experiment 1: Effect of Sediment Depth on Denitrification. Both the 0–2 and 2–5 cm sediment fraction exhibited significantly higher denitrification activity than either 5–10 or 10–15 cm fractions (ANOVA, $p < 0.0001$, Fig. 1). The total denitrification rate (calculated as the sum of means from each depth fraction) was 94.5 $\mu\text{g N}_2\text{O g AFDM}^{-1} \text{h}^{-1}$, and more than 88% of this denitrification activity was associated with the top 5 cm of sediment. The mean denitrification rate from all depths was 23.6 $\mu\text{g N}_2\text{O g AFDM}^{-1} \text{h}^{-1}$ (SE = 5.30). Denitrification in the deepest sediment fraction (10–15 cm) was 1 order of magnitude lower than rates associated with the shallowest sediment (0–2 cm). Denitrification was linearly related to percent organic content of sediments when all samples were pooled across depths ($r^2 = 0.26$, $p = 0.042$; Fig. 2). Streamwater NO_3^- was 4.8 mg L^{-1} and DOC was 2.7 mg C L^{-1} , compared to an annual average of 5.0 and 4.8, respectively. Water column NO_3^- and C were not likely limiting denitrification because nutrient amendment experiments did not increase denitrification (see Experiment 4), and NO_3^- was well above the threshold limitation level calculated for streams in this area (0.4 mg N L^{-1}) [13].

Experiment 2: Influence of Stream Size and Associated Water Quality on Sediment Denitrification. Overlying streamwater was found to influence sediment denitrification, as evidenced by differences in sediment denitrification activity (scaled per g AFDM) when headwater sediments were incubated with streamwater collected from second- and third-order downstream reaches. Water

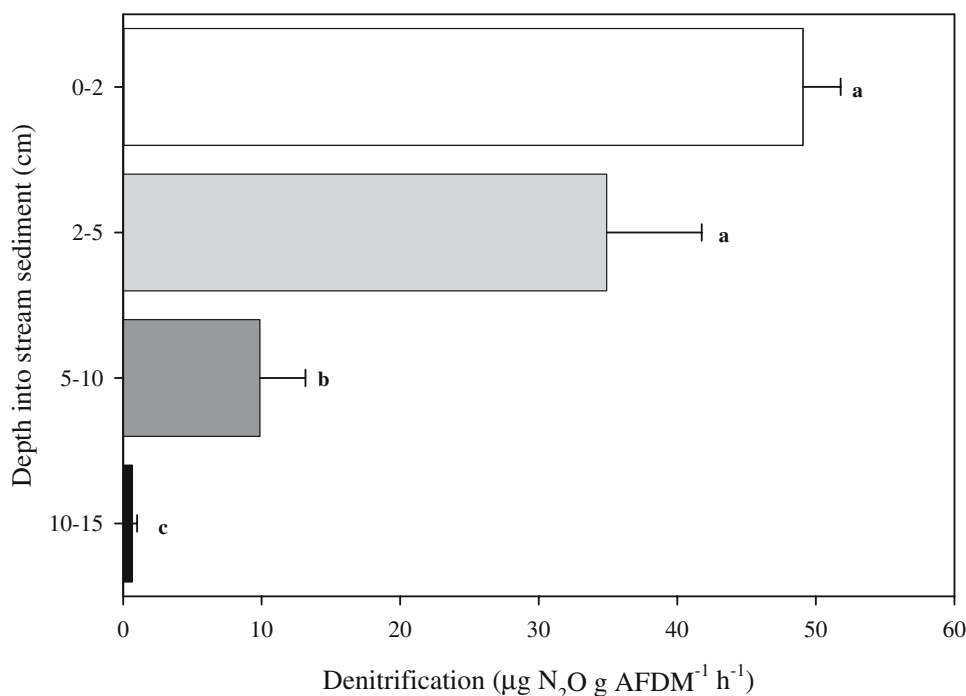


Figure 1. Sediment denitrification rate (+1 SE) within each fraction into the streambed at headwater site AY during October 2002. Different letters indicate statistically different groups, $p < 0.0001$ (ANOVA followed by LS means test).

source (first- to third-order stream) significantly influenced sediment denitrification rates in seven of nine subwatersheds (repeated-measures ANOVA, $p < 0.008$ for all cases). In six of those seven cases, sediment denitrification rate increased with streamwater from downstream reaches (second- and third- order), although denitrification rates associated with second- and third-order streamwater were often similar (LS means, $p >$

0.05). Overall, denitrification rates were linearly related to NO_3^- concentration of the water source ($r^2 = 0.31$, $p = 0.003$; Fig. 3). Additionally, discharge at the stream-water source was positively correlated with denitrification rates ($R = 0.44$, $p = 0.02$). Other measured habitat parameters were not significantly correlated with sediment denitrification when exposed to different streamwater sources, including DOC, SUVA,

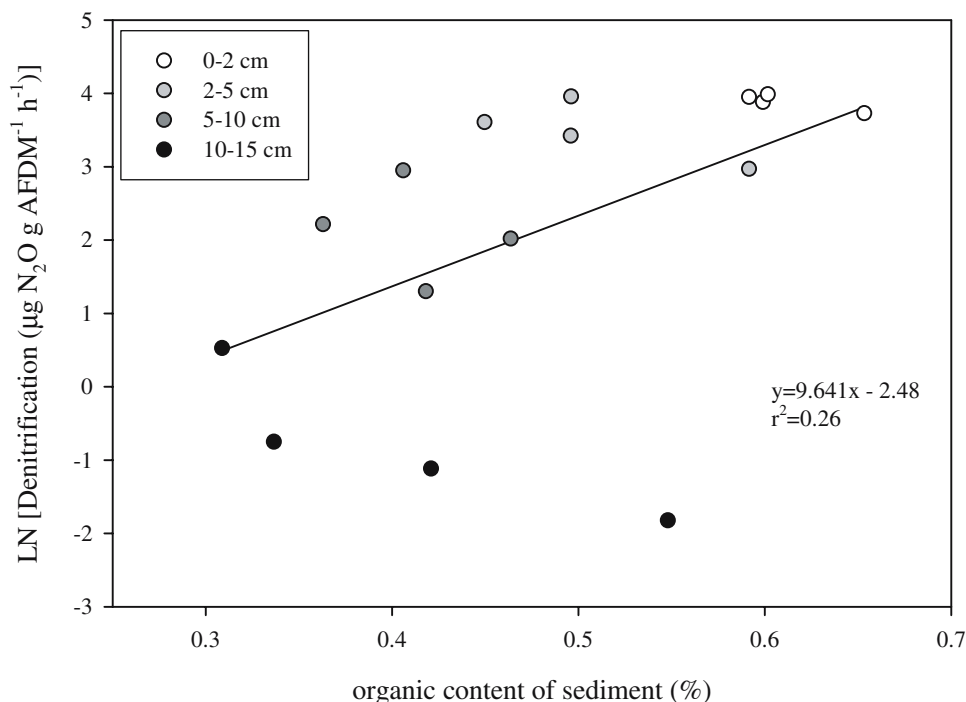


Figure 2. Denitrification rates versus corresponding percent organic content for all depth fractions from Experiment 1. The regression line and equation are also shown.

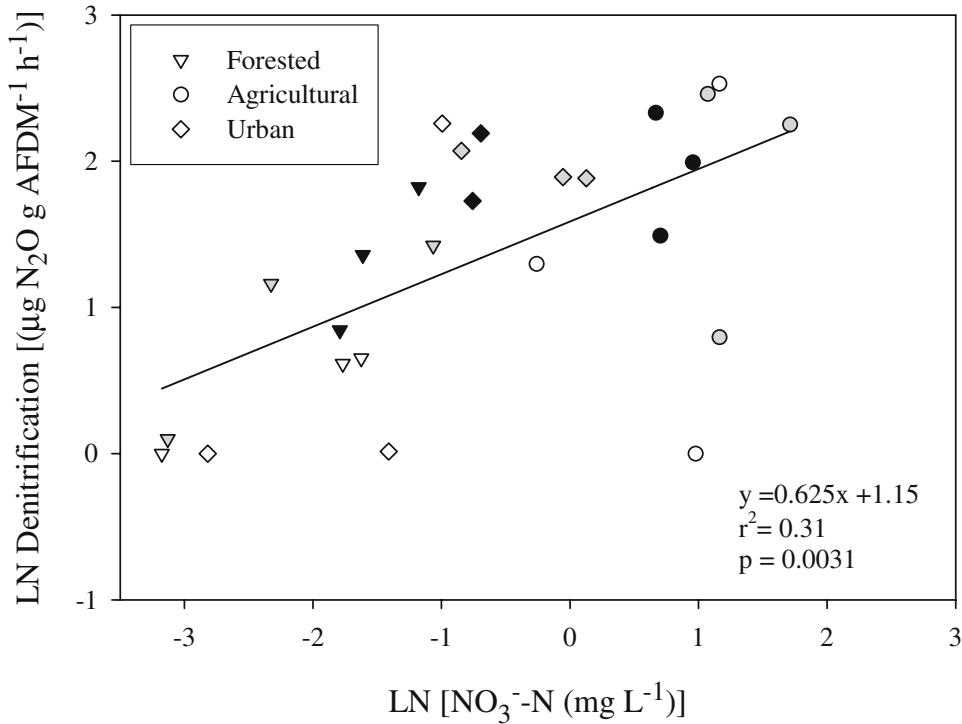


Figure 3. Stream sediment denitrification rates resulting from incubating headwater sediments with either first-, second-, or third-order downstream reach water (white, gray, or black symbols, respectively) are shown with the linear regression.

watershed area, or percent of each land use type ($p > 0.05$).

Experiment 3: Effect of Substratum Size and Inorganic Content on Denitrification Rates. Both substratum size (sand, small pebble, or large pebble) and rinsing treatment affected denitrification rates, but statistical significance sometimes changed when denitrification was expressed per g AFDM or g DM (Fig. 4). In all cases, rinsed substrata (treatment B) had similar denitrification rates relative to unrinsed substrata (treatment A) within each size class (ANOVA, $p > 0.05$). Ambient sediments had higher denitrification rates than incubated experimental substrata (ANOVA, $p < 0.0001$), and denitrification rates tended to decrease as inorganic substrata size increased, although the differences were generally not significant (ANOVA followed by LS means comparison, $p > 0.05$). “Supernatant” assays generally had higher denitrification rates compared to other treatments (except large pebble size), and the difference was greatest when rates were scaled per g DM. Substrata organic content predicted 66–91% of the variability in denitrification rates for the unrinsed and rinsed sediment when data from all substrata were pooled ($r^2 = 0.91$ and 0.66 , respectively, $p < 0.0001$; Fig. 5).

Experiment 4: Nutrient Limitation of Sediment Denitrification by Dissolved NO_3^- and Organic C. All sites were limited by either NO_3^- , DOC, or both at least once during the study year. The frequency of each type of limitation (NO_3^- , DOC, or colimitation) varied among

land-use types with forested streams being NO_3^- -limited in all but two instances (Fig. 6). Urban streams were also more often NO_3^- -limited than DOC-limited. In contrast, agricultural streams were most frequently DOC-limited.

When data were grouped by land use, significant differences in relative response to nutrient amendments were observed during April and June in forested and agricultural streams, respectively. When data were grouped by nutrient treatment (N, C, or N + C amendment), NO_3^- and SRP concentrations were negatively related to relative response for N-amended sediments ($r^2 = 0.379$ and 0.178 , $p < 0.0001$ and $= 0.0074$, respectively; Fig. 7). NO_3^- concentrations were also negatively related to relative response for N + C amended sediments ($r^2 = 0.326$, $p = 0.0002$; Fig. 7), whereas none of the independent parameters were significantly correlated with the relative response to C amendment.

Discussion

Experiment 1: Effect of Sediment Depth on Denitrification. Denitrification activity was highest in the top 5 cm of benthic sediment, which accounted for more than 88% of total denitrification. Garcia-Ruiz *et al.* [7] also describe decreasing denitrification activity with depth in the upper 7 cm of stream benthos by using a similar technique, but found a sharp decline between 0–1 and 1–2 cm fractions. Our experiment did not delineate depth fractions at the 1-cm scale, which may explain the slight difference in observations. Furthermore, sediments

in this study were unconsolidated, promoting denitrification in deeper fractions due to less limitation on nutrient diffusion relative to consolidated sediments. Our results are similar to those reported for shallow marine bay sediments [19] and riparian soils (down to 100 cm [10]). Although denitrification in deeper sediments could be limited by nutrient availability, sediment denitrification rates in this study likely were not limited by NO_3^- in streamwater because concentrations were above the threshold of 0.4 mg N L^{-1} identified previously for sediment denitrification in these streams [13]. Under *in*

situ conditions, denitrification associated with deeper sediments may be primarily C-limited.

Sediment organic content decreased with depth into the benthos, a pattern also observed by Groffman *et al.* [10], and suggesting organic C limitation. Additionally, water column DOC concentrations measured at the time of the experiment (2.7 mg C L^{-1}) were lower than the annual average for this site (4.8 mg C L^{-1} [13]), and C limitation did occur in August when ambient DOC was 3.7 mg C L^{-1} (see Experiment 4), further suggesting that organic C availability may have limited denitrification.

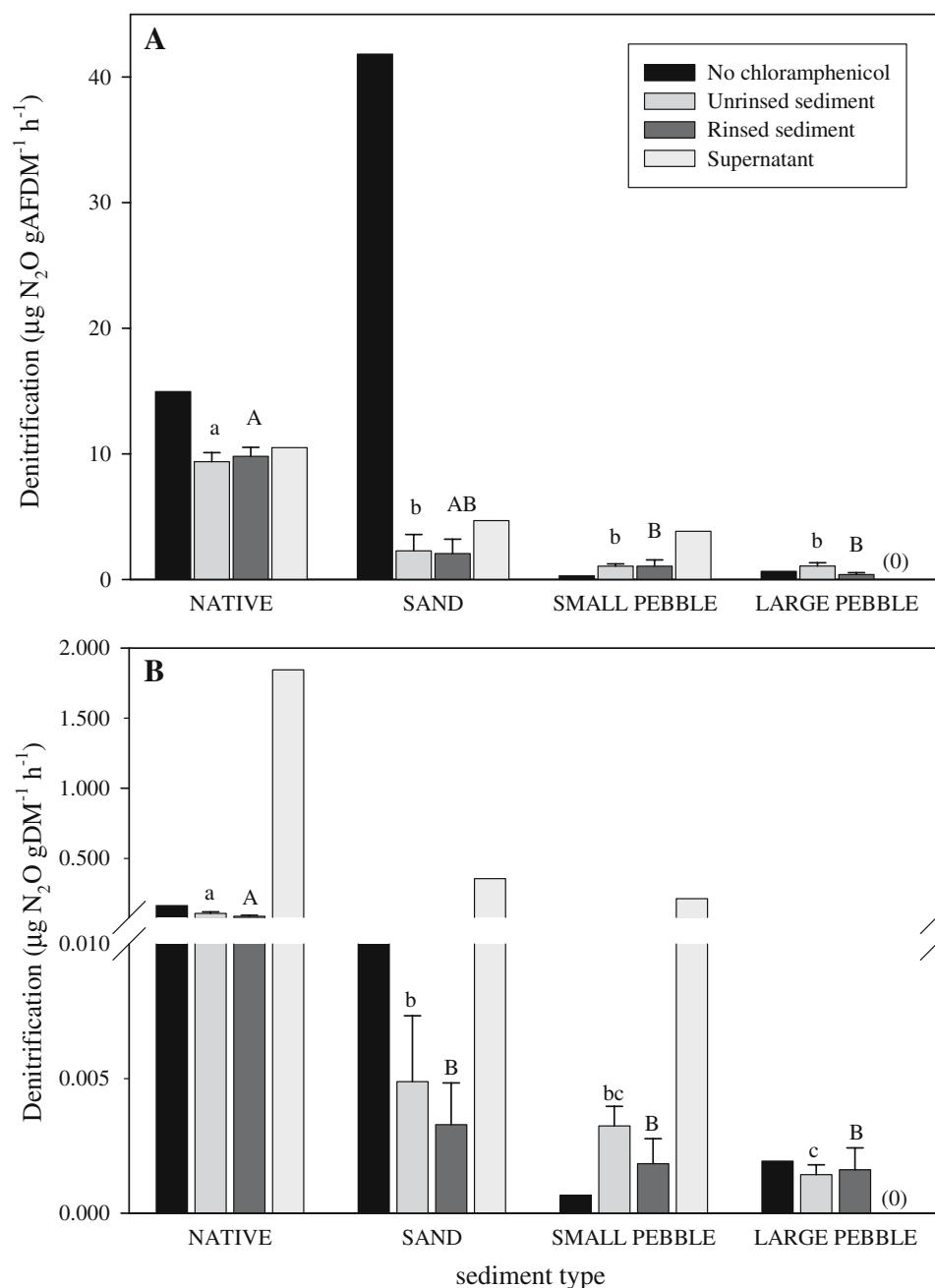


Figure 4. Sediment denitrification expressed per g AFDM (A) and DM (B) resulting from experimentally incubated sediments of varying size classes. Lowercase letters indicate similarities within the “unrinsed sediment” treatment, whereas uppercase letters indicate similarities in the “rinsed sediment” treatment according to ANOVA followed by LS means test. “No chloramphenicol” controls were not replicated and the “supernatant” treatment reflects a pooled sample from replicates of the rinsed treatment.

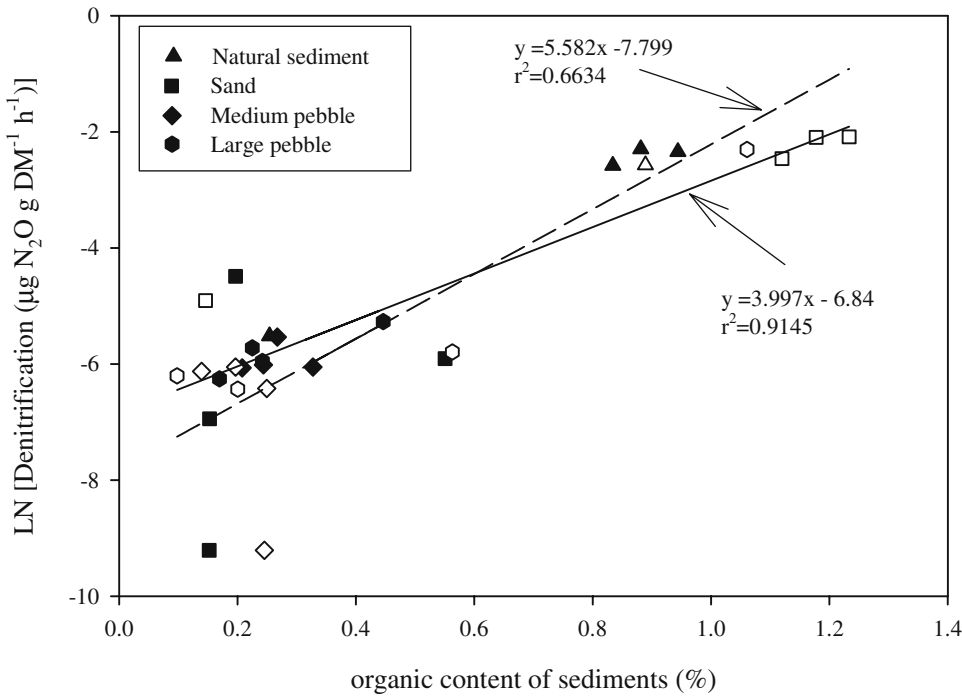


Figure 5. Denitrification activity versus organic content. Linear regressions for unrinsed (black symbols) and rinsed samples (white symbols) are shown.

Experiment 2: Influence of Stream Size and Associated Water Quality on Sediment Denitrification. Incubating sediments with streamwater from downstream sites influenced denitrification activity associated with sediments from first-order streams. In general, denitrification rates tended to be higher when incubated with water from downstream reaches. Garcia-Ruiz *et al.* [8] also found a general increase in denitrification rates from upstream to downstream river sites in the United Kingdom, but did not separate effects due to changing water quality and changing sediment quality

as we did. The increase in denitrification from upstream to downstream river sites in the UK was attributed to a combination of factors including reduced current velocity, increases in sedimentation, %C and % N, fine sediment, phosphate, and NO_3^- [8]. In a similar study, Pattinson *et al.* [24] observed increasing denitrification downstream, as well as concurrently increasing NO_3^- concentrations, but found no statistically significant correlations.

Our study isolated potential changes in denitrification due to properties of the overlying water. We found a

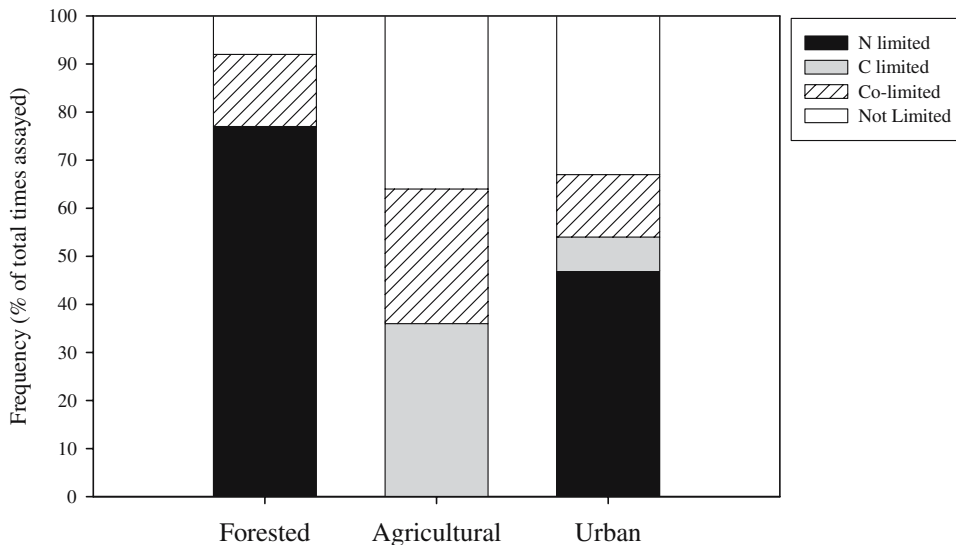


Figure 6. Frequency of each type of nutrient limitation as a percentage of total times forested, agricultural, or urban streams were assayed.

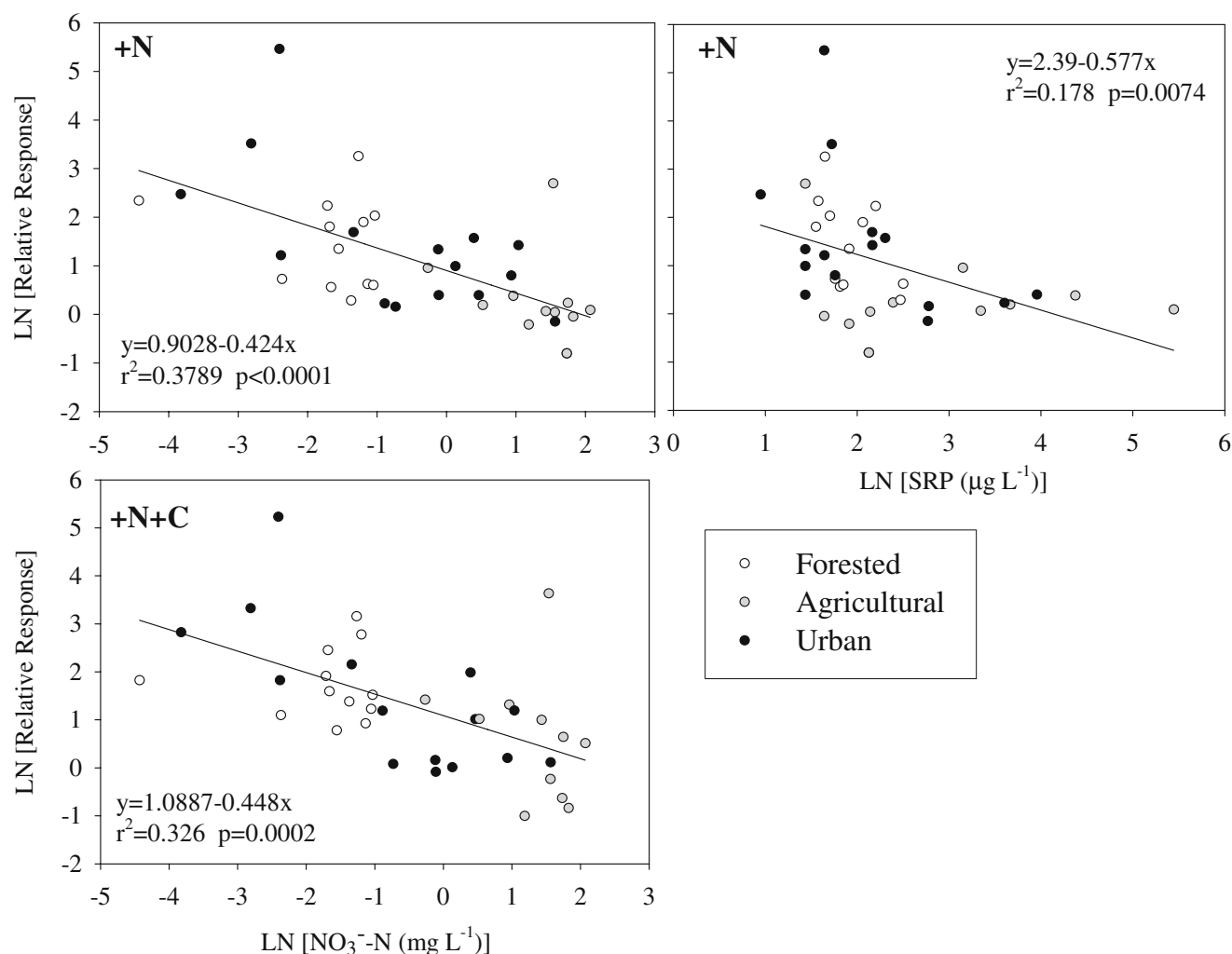


Figure 7. Linear relationship between logarithmically transformed environmental parameters and the response of stream sediment denitrification to amendment with NO_3^- (+N), or NO_3^- and DOC (+N + C) relative to unamended sediments.

strong linear relationship between water NO_3^- concentration and sediment denitrification, supporting the idea that NO_3^- concentration is a primary factor controlling sediment denitrification (e.g., [13, 20]). Furthermore, neither water column DOC concentration nor quality (as SUVA) correlated significantly with sediment denitrification. Interestingly, NO_3^- was not correlated with discharge or watershed area, likely due to the variation in associated land use. Both sites where there were no significant differences in denitrification observed with overlying water had relatively high denitrification rates, suggesting that denitrification activity may have been at its maximum under those sediment and biofilm conditions. Additionally, in AX there was a decrease in NO_3^- in the downstream reaches that may have contributed to the decreasing longitudinal trend in denitrification rates. Other possible factors controlling denitrification that

could be influenced by land-use type include sediment load, light availability, pesticide contamination, and trace metal concentrations; however, we did not assess them in our study.

Experiment 3: Effect of Substratum Size and Inorganic Content on Denitrification Rates. Denitrification was affected by substrata size and generally decreased with larger substrata. This trend may be related to a reduction in surface area available for microbial colonization [24] because smaller substrata (e.g., sand) may have greater surface area per volume of substratum, resulting in increased microbial colonization and denitrification rates. Denitrification rates associated with ambient stream sediments were higher than rates associated with experimentally incubated substrata, suggesting that microbial biofilms on natural sediments were more

developed than incubated sediments or that the sediment structure in natural sediments was more conducive to denitrification.

Both rinsed and unrinsed substrata had measurable denitrification rates, as did the fine particulate supernatant material from rinsing. Denitrification rates from rinsed and unrinsed substrata were similar, indicating that denitrification resulting from microbial biofilms colonizing inorganic substrata—in addition to microbial denitrification on fine particulate organic material loosely associated with sediment—may both be important contributors to overall stream denitrification. This novel comparison implies that when conducting assays such as this, one should fully assess methodological variation in denitrification estimates that may disproportionately affect either the attached biofilm or associated fine particulate abundance. For example, using denitrification measurements obtained from the fine particulate treatments would have resulted in an estimate of up to 2.4 times that of the entire substrata community upon calculating the areal denitrification rate (data not shown).

Results from Experiment 3 support the hypothesis that denitrification occurs on both organic and inorganic substrata. Consequently, it may not be appropriate to normalize the rate of N_2O production using only one estimate of substrata mass (i.e., expressing denitrification either per g AFDM or per g DM) especially when comparing denitrification rates among substrata with different organic content. For example, when denitrification rates from highly organic sediments are scaled per g AFDM, both the substrata organic content and the microbial organic content are included in that mass, whereas the g AFDM of very inorganic sediments would be a better estimate of the microbial organic content alone because substrata organic content would not influence the overall denitrification rate. Using g DM to scale denitrification activity could be similarly ineffective for comparing across substrata types. More accurate comparisons might be made by examining denitrification rates that have been individually scaled to multiple parameters including g AFDM, g DM, bacterial cell count (or other *direct* microbial biomass indicator), substrata volume, and stream bed area. Ultimately, for comparisons among streams that might contain substrata with varying organic matter content, scaling denitrification per unit area of streambed would provide a more useful way to express rates, but only if substrata representative of that heterogeneity are included in the denitrification estimates.

Experiment 4: The Influence of Dissolved NO_3^- and Organic C on Nutrient Limitation of Sediment Denitrification. All streams showed nutrient limitation of sediment denitrification at least once during our

study. NO_3^- most frequently limited sediment denitrification in forested and urban sites, in contrast to agricultural sites where dextrose (i.e., DOC) was the dominant form of nutrient limitation. Kemp and Dodds [15] also reported stimulation of denitrification with NO_3^- amendment to a variety of substrata types from a low NO_3^- (0–30 $\mu\text{g N L}^{-1}$) prairie stream. In reservoir sediments, Wall [39] found that NO_3^- limitation occurred more frequently in fall and winter when ambient NO_3^- was lower. Significant inhibition of denitrification was sometimes observed with NO_3^- and/ or dextrose amendment, but was not examined further in this study. Wall [39] hypothesized that competitive inhibition of denitrification with NO_3^- and/ or DOC amendment, as observed in reservoir sediments, could result from stimulation of dissimilatory nitrate reduction (DNR), which also utilizes NO_3^- and DOC, although DNR was not measured in this study.

In our agricultural sites, where NO_3^- was high, denitrification was most frequently limited by dextrose. Few previous studies have shown the presence of primary DOC limitation of stream denitrification, but this may be because most stream denitrification studies have been conducted in low-nutrient systems (e.g., [15, 20]). For example, dextrose amendment to low-nutrient streamwater only yielded a significant increase in sediment denitrification when combined with NO_3^- amendment [20]. Despite the moderate levels of NO_3^- found in their study sites, Garcia-Ruiz *et al.* [7] did not find significant increases in stream sediment denitrification when DOC was added, although DOC amendment did reduce the proportion of N_2O produced relative to other N gases. We were unable to assess this possible effect of DOC amendment in our sites because we measured only N_2O production.

In a comprehensive laboratory experiment examining soil denitrification, Smith and Tiedje [32] concluded that phase I denitrification (exponential increase) was likely limited by electron donors (i.e., DOC), but phase II

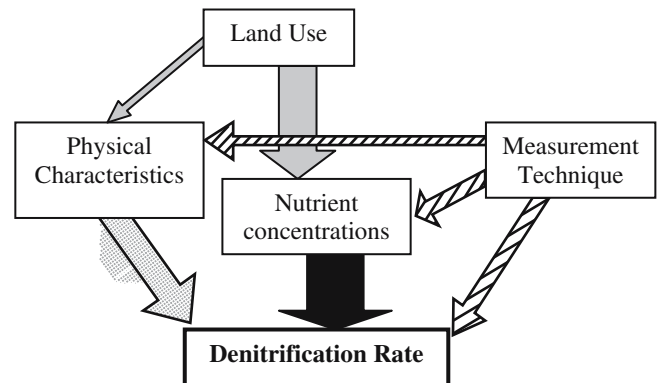


Figure 8. Sequence of factors influencing observed stream sediment denitrification rates. Arrow width approximates relative importance of each factor.

denitrification rates increased in response to DOC amendment as a result of stimulated bacterial growth rather than a per cell increase in denitrification. We minimized the potential for this by using chloramphenicol and having short assay incubation times. Royer *et al.* [28] suggested that DOC quality, rather than actual concentration, may limit sediment denitrification because organic C limitation was seen in only one stream, despite the similarity of ambient DOC concentrations among their three agricultural streams. However, in our study, DOC quality was not correlated to the relative response of denitrification. In general, it appears that denitrification can be limited by DOC when NO_3^- is abundant as in our agricultural sites, although such limitation may be a function of the availability of alternative organic carbon sources within sediments [26]. Use of a single, highly labile type of DOC (e.g., dextrose) in experimental assays may not reveal *in situ* organic carbon limitation of sediment denitrification.

The relative response of sediment denitrification to nutrient amendments was a function of in-stream conditions (e.g., NO_3^- and SRP concentrations) and watershed land use, where agricultural streams tended show a smaller response to nutrient amendment. The negative relationship between the relative response to NO_3^- amendment and NO_3^- concentration indicates that stream sediment denitrification was less limited by NO_3^- when more was present in the water column. In our study, streamwater NO_3^- concentrations were generally high, and therefore not NO_3^- -limited, although denitrifiers might have been limited by other biotic processes (e.g., microbial growth, competition for space, or carbon source). Consequently, *in situ* sediment denitrification could plateau resulting in a limited capacity to mitigate pulses of NO_3^- entering a stream.

Conclusions

Because nutrient cycling in aquatic ecosystems can be highly variable and depend on a number of environmental factors, eliminating variability in laboratory and field techniques would facilitate comparing denitrification patterns among studies. Figure 8 illustrates multiple factors that may concurrently influence observed denitrification rates: land use directly influences nitrate concentrations as well as sediment characteristics [13], which both influence denitrification rates. Additionally, methodology may affect denitrification rates indirectly by altering physical and nutrient conditions, or by directly influencing new enzyme production (e.g., use of chloramphenicol).

Our results indicate that NO_3^- concentration and sediment quality are central in predicting sediment denitrification rates in streams. Because denitrification occurs on both organic and inorganic substrata, denitrification estimates scaled for only one portion of total sed-

iment mass (e.g., AFDM or DM) should be interpreted cautiously. Additionally, microbial denitrifiers may be variably limited by either NO_3^- or DOC, depending on water column characteristics, and these patterns can be associated with land use. If rate estimates used for regional N models were obtained by using laboratory incubations, they should be interpreted in the context of their nutrient status, sediment depth, and longitudinal location within a river network.

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