Benthic organic carbon influences denitrification in streams with high nitrate concentration

CLAY P. ARANGO,* JENNIFER L. TANK,* JAMIE L. SCHALLER,† TODD V. ROYER,‡ MELODY J. BERNOT§ AND MARK B. DAVID†

*Department of Biological Sciences, University of Notre Dame, Notre Dame, IN, U.S.A.
†Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana, IL, U.S.A.
‡School of Public and Environmental Affairs, Indiana University, Bloomington, IN, U.S.A.
§Department of Biological Sciences, Murray State University, Murray, KY, U.S.A.

SUMMARY

1. Anthropogenic activities have increased reactive nitrogen availability, and now many streams carry large nitrate loads to coastal ecosystems. Denitrification is potentially an important nitrogen sink, but few studies have investigated the influence of benthic organic carbon on denitrification in nitrate-rich streams.

2. Using the acetylene-block assay, we measured denitrification rates associated with benthic substrata having different proportions of organic matter in agricultural streams in two states in the mid-west of the U.S.A., Illinois and Michigan.

3. In Illinois, benthic organic matter varied little between seasons (5.9–7.0% of stream sediment), but nitrate concentrations were high in summer (>10 mg N L$^{-1}$) and low (<0.5 mg N L$^{-1}$) in autumn. Across all seasons and streams, the rate of denitrification ranged from 0.01 to 4.77 l g$^{-1}$ N g$^{-1}$ DM h$^{-1}$ and was positively related to stream-water nitrate concentration. Within each stream, denitrification was positively related to benthic organic matter only when nitrate concentration exceeded published half-saturation constants.

4. In Michigan, streams had high nitrate concentrations and diverse benthic substrata which varied from 0.7 to 72.7% organic matter. Denitrification rate ranged from 0.12 to 11.06 l g$^{-1}$ N g$^{-1}$ DM h$^{-1}$ and was positively related to the proportion of organic matter in each substratum.

5. Taken together, these results indicate that benthic organic carbon may play an important role in stream nitrogen cycling by stimulating denitrification when nitrate concentrations are high.

Keywords: agricultural streams, benthic organic carbon, denitrification, dissolved organic carbon, high nitrate, nitrogen cycle

Introduction

Reactive nitrogen (N) in the form of nitrate (NO$_3^-$) and ammonium (NH$_4^+$) has increased as a result of anthropogenic activities including fertiliser use and fossil fuel combustion (Galloway, 1998). Anthropogenic activities contribute 140 Tg N year$^{-1}$ to the biosphere and have effectively doubled N availability compared with historic inputs (Vitousek et al., 1997). Long-term monitoring of the Mississippi River and the Gulf of Mexico illustrates the negative consequences of increased N availability in aquatic ecosystems, where excess anthropogenic N stimulates extensive algae blooms that senesce and decompose, causing large zones of hypoxia (Turner & Rabalais, 1991; Rabalais, Turner & Scavia, 2002).
Non-point sources of N, primarily contributed by agriculture, now exceed point sources as the largest contributor of N to U.S.A. surface waters (USEPA, 1996; Goolsby et al., 2001). Precipitation transports nitrogenous fertiliser applied in excess of crop demand to aquatic ecosystems (Carpenter et al., 1998), leaching up to 80% of applied N into ground and surface waters (Howarth et al., 1996). Drainage tiles installed in agricultural fields expedite N export by rapidly draining NO$_3^-$-rich sub-surface runoff into streams (David et al., 1997; Petry et al., 2002). Feedlot runoff and leaky manure containment ponds associated with a high density of livestock may also increase water-column N concentrations via non-point source pollution (Carpenter et al., 1998), which is difficult to regulate.

Denitrification can reduce in-stream N permanently by removing it from stream ecosystems as nitrous oxide (N$_2$O) and dinitrogen (N$_2$) gases (Alexander, Smith & Schwarz, 2000). A respiratory process used by facultatively anaerobic microbes, denitrification requires supplies of NO$_3^-$, organic carbon (C), and the presence of anoxic habitat (Knowles, 1982; Seitzinger, 1988). In this dissimilatory metabolic pathway, organic C compounds serve as electron donors and NO$_3^-$ serves as an oxidant. Denitrifying microbes harness the energy released by the simultaneous oxidation of reduced C to CO$_2$ and the reduction of NO$_3^-$ to N$_2$O and/or N$_2$. Because denitrification is an anaerobic process, many studies have investigated how the concentration of dissolved oxygen mediates denitrification rates in streams (e.g. Duff, Triska & Oremland, 1984; Christensen et al., 1990; Schaller et al., 2004), but other studies have emphasised the role of NO$_3^-$ supply in controlling denitrification rates (e.g. Holmes et al., 1996; Pattinson, García-Ruiz & Whitton, 1998; Martin et al., 2001). The prevalence of denitrification studies in relatively low NO$_3^-$ streams (<1 mg NO$_3^-$-N L$^{-1}$) has emphasised how NO$_3^-$ supplies control denitrification at the expense of understanding the role of C in stream denitrification (but see Hedin et al., 1998; LeFebvre, Marmonier & Pinay, 2004). Furthermore, studies that have examined how C influences denitrification have focused on dissolved (DOC) rather than particulate organic C (POC), and POC can positively influence denitrification in two ways. First, denitrifiers are limited to using dissolved substances that can be actively or passively transported across their cell membranes, but abiotic leaching and exoenzyme activity can extract DOC from POC, providing denitrifiers with a C source for NO$_3^-$ reduction (Seitzinger, 1988). Secondly, aerobic decomposition of POC can influence denitrification by reducing oxygen concentrations and expanding the anaerobic habitat.

Because NO$_3^-$ is plentiful in agricultural streams in the Midwestern U.S.A., the latter are ideal systems for studying how organic C influences denitrification. Previous research in Illinois and Michigan shows that the addition of NO$_3^-$ does not increase denitrification rate, suggesting C limitation and also that high stream-water NO$_3^-$ concentrations represent a fair proxy for NO$_3^-$ availability at the point of denitrification despite the likelihood that porewater and stream-water concentrations differ. In the Midwestern U.S.A., agricultural streams typically drain former wetlands, which contribute recalcitrant DOC to streams (Royer & David, 2005), and the sandy sediments often entrain POC, which leaches DOC. Therefore, sediment POC (expressed as sediment organic matter; %OM) may represent a better measure of C availability, at the point of denitrification in the anoxic benthos, than stream-water DOC. Inorganic N concentration in agricultural streams in Illinois frequently exceeds 10 mg NO$_3^-$-N L$^{-1}$ in early summer and then decreases below 0.5 mg NO$_3^-$-N L$^{-1}$ in late summer and autumn, a pattern driven by interactions between agricultural practices, hydrology and biotic demand (David et al., 1997; Royer, Tank & David, 2004), whereas sediment organic matter does not generally vary between summer and autumn. We predicted that the rate of denitrification in Illinois streams would be closely related to sediment organic matter only when NO$_3^-$ concentrations were high. Michigan streams have relatively high NO$_3^-$ concentrations (>0.5 mg N L$^{-1}$) that do not vary seasonally, as in Illinois streams. However, Michigan streams have diverse POC sources, hereafter referred to as substrata, the organic fraction of which varies by nearly two orders of magnitude. Given these relatively high NO$_3^-$ concentrations, we predicted that denitrification rates in Michigan would be positively related to the organic fraction of the substrata across all streams.

**Methods**

**Site description**

Our eight study streams were located in east-central Illinois and southwest Michigan, where arable (row-
crop) agriculture dominates land use. The study streams, described in detail by Royer et al. (2004) and Inwood, Tank & Bernot (2005), have little riparian vegetation, are deeply channelised and have flashy hydrographs. However, Michigan streams generally have more riparian vegetation than Illinois streams, and consequently receive more allochthonous organic matter. The study streams ranged from first to third order. As anticipated, Michigan streams had high stream-water NO$_3$ concentrations whereas NO$_3$ concentrations in Illinois streams were high during summer sampling but low during autumn (Table 1). Sediment organic matter in Illinois streams had low spatial variability whereas Michigan streams had patchy organic matter accumulations dominated by four substratum types that ranged in organic fraction (%OM) over two orders of magnitude: sand, fine benthic organic matter (FBOM, organic matter that passes through a 1-mm sieve but is retained by a 63-μm sieve), coarse benthic organic matter (CBOM, organic matter retained by a 1-mm sieve) and bacterial biofilms (Table 2). Bacterial biofilms were distinguished from FBOM by having a green/brown colour suggesting an autotrophic component. Previous research in these streams has demonstrated that NO$_3$ limits denitrification in Illinois only in autumn (Wall et al., 2005; Inwood, Tank & Bernot, 2007). Together, these different conditions allowed us to assess the importance of benthic organic matter to in-stream denitrification in situations where NO$_3$ concentrations are high and unlikely to limit denitrification (Michigan streams and Illinois in summer); further, the seasonally distinct NO$_3$ concentrations in Illinois streams allowed us to compare the importance of benthic organic matter when NO$_3$ was low and high.

Field sampling

We sampled Illinois streams twice in 2002, once in early summer when stream NO$_3$ concentrations are typically high and once in late summer/early autumn when stream NO$_3$ concentrations are typically low (David et al., 1997; Royer et al., 2004). In each Illinois stream, we delineated a sampling grid of five transects spaced 12-m apart along a 50-m stream reach. At each transect we collected sediment cores at 0.25, 0.5 and 0.75 channel width by sampling the top 5 cm of the stream bottom using a 28 cm$^2$ corer. We sampled to a depth of 5 cm because previous studies found

| Location, order, width, discharge, nitrate and dissolved organic carbon (DOC) concentrations at the time of sampling |
|---|---|---|---|---|---|---|
| Stream name and code | Location (latitude, longitude) | Coordinates | Stream order | Width (m) | Stream temperature ($^\circ$C) | Discharge (L s$^{-1}$) | Nitrate (mg N L$^{-1}$) | DOC (mg C L$^{-1}$) |
| Big Ditch Outlet (BDO) | Illinois 40°16’06”N, 88°59’55”W | 41.06 | 2 | 8.6 | 6.6 | 16.8 | 19.8 | 1030 | 35.1 | 17.8 | 0.17 | 1.7 |
| Black Slough (BLS) | Illinois 39°57’07”N, 88°10’01”W | 39.95 | 1 | 3.3 | 1.9 | 17.7 | 21.1 | 324 | 1820 | 13.4 | 5.6 | 1.3 |
| Lake Fork, Kaskaskia River (LFK) | Illinois 39°57’09”N, 88°29’48”W | 39.95 | 3 | 14.1 | 9.6 | 26.3 | 21.3 | X | 12.6 | 0.10 | 2.1 | 0.9 |
| Sand Creek (SNC) | Michigan 42°30’35”N, 85°54’35”W | 42.5 | 1 | X | 0.4 | X | X | X | X | 9.9 | X | 0.79 |
| Swan Creek (SWC) | Michigan 42°46’17”N, 85°43’11”W | 42.77 | 1 | X | 0.7 | X | X | X | 279 | X | 1.1 | 3.9 |
| Black Creek (BLC) | Michigan 42°46’17”N, 85°43’11”W | 42.77 | 2 | X | 2.0 | X | X | X | 279 | X | 1.1 | 3.9 |
| Tributary to Little Rabbit River (RAB) | Michigan 42°46’17”N, 85°43’11”W | 42.77 | 1 | X | 1.5 | X | X | X | 16.4 | X | 5.5 |

that >90% of sediment denitrification occurs in the top 5 cm of the sediments in these streams (Inwood et al., 2007). Because Illinois stream sediments were visually uniform, we did not distinguish between sediment types and considered each core equally representative of the stream bottom. We sampled Michigan streams once in autumn 2003 but because the Michigan streams had patchy distributions of distinct benthic substrata, we used a stratified design to sample each substratum randomly from 10 locations within a 100-m stream reach. We sampled sand, FBOM, CBOM and biofilms by selecting a known area with 100% cover of a given substratum type. We used cores to sample sand as described above, but we sampled CBOM by taking material off the stream bottom, and we sampled FBOM and biofilms using a turkey baster to suck the material off the stream bottom. Of the 10 samples for each substratum, we retained five for calculating substratum standing stock and pooled the remainder into one composite substratum sample from each stream for denitrification assays.

Denitrification assays

We measured denitrification in the laboratory using the chloramphenicol-amended acetylene (C$_2$H$_2$) block method (Smith & Tiedje, 1979; Royer et al., 2004; Inwood et al., 2005). Acetylene (C$_2$H$_2$) blocks the final step of the complete denitrification pathway allowing N$_2$O, which is more easily measured than N$_2$, to accumulate in the assay bottles. Chloramphenicol is an antibiotic that inhibits $de$ novo protein synthesis (Brock, 1961) and reduces bottle effects associated with laboratory slurry incubations, allowing for more accurate estimates of $in$ situ rates (Smith & Tiedje, 1979). Sediments from Illinois streams were incubated at stream temperature, but Michigan substrata were incubated at room temperature to minimise variability not associated with substratum characteristics. We incubated four analytical replicates of each pooled sample in 125-mL media bottles. For Illinois stream sediments, and for sand, FBOM and biofilm substrata from Michigan sites, we made slurries and added 25 cm$^3$ to each bottle; CBOM samples were broken into small pieces to facilitate homogenisation before adding them to each bottle (approximately 10-g wet weight). Bottles were filled to 75 mL with chloramphenicol-amended site water for a final concentration of 5 mM chloramphenicol (Schaller et al., 2004; Inwood et al., 2005). Each bottle was sealed with a septum cap, purged with ultra high purity helium (He) to create anoxia, and vented to relieve excess pressure. We added 15 mL of C$_2$H$_2$ to three of the four assay bottles, and the fourth bottle received no C$_2$H$_2$ to control for background N$_2$O production. We incubated the bottles for 4 h and took four headspace samples throughout the incubation (0:15, 1:30, 2:45, 4:00 hours). Prior to headspace sampling, we shook the bottles for 10 s to equilibrate N$_2$O between the water and headspace. Using a 5-mL syringe, we took a 4-mL headspace sample from each bottle and immediately injected it into a 3.5-mL pre-evacuated vial. We returned the assay bottles to the original positive pressure by replacing the sub-sample with 4 mL of 10% C$_2$H$_2$ in He balance.

We analysed headspace sub-samples by manually injecting 100 µL into a Varian 3600 gas chromatograph (Palo Alto, CA, U.S.A.) equipped with a Porapak Q column, electron capture detector, and a valve to vent C$_2$H$_2$ away from the detector (injector temp, 120 °C; column temp, 40 °C; detector temp, 320 °C, ultra high purity N$_2$ carrier gas, 30 mL min$^{-1}$). Total concentration of N$_2$O at each sampling period was calculated

Table 2 Mean (SE) values for substratum characteristics in the study streams

<table>
<thead>
<tr>
<th>Location</th>
<th>Season sampled</th>
<th>Substratum type</th>
<th>Standing stock (g AFDM m$^{-2}$)</th>
<th>Benthic cover (%)</th>
<th>Organic matter (%)</th>
<th>Chlorophyll-a (µg cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illinois</td>
<td>Summer</td>
<td>Homogenous sediment</td>
<td>X</td>
<td>100</td>
<td>7.0 (0.2)</td>
<td>X</td>
</tr>
<tr>
<td>Illinois</td>
<td>Autumn</td>
<td>Homogenous sediment</td>
<td>X</td>
<td>100</td>
<td>5.9 (0.3)</td>
<td>X</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>Sand</td>
<td>69.5 (10.1)</td>
<td>36.2 (5.7)</td>
<td>0.7 (0.1)</td>
<td>2.3 (0.7)</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>Biofilm</td>
<td>84.8 (4.9)</td>
<td>5.1 (1.8)</td>
<td>9.9 (1.4)</td>
<td>3.3 (1.5)</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>CBOM</td>
<td>272.7 (79.1)</td>
<td>17.5 (3.6)</td>
<td>72.7 (2.3)</td>
<td>X</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>FBOM</td>
<td>343.4 (108.2)</td>
<td>40.6 (5.9)</td>
<td>18.0 (2.1)</td>
<td>7.5 (3.5)</td>
</tr>
</tbody>
</table>

AFDM, ash-free dry mass; CBOM, coarse benthic organic matter; FBOM, fine benthic organic matter; X, data not collected.

© 2007 The Authors, Journal compilation © 2007 Blackwell Publishing Ltd, Freshwater Biology, 52, 1210–1222
using the appropriate Bunsen coefficient to determine the amount of gas dissolved in water at a given headspace concentration (see Inwood et al., 2005, for detailed equations). We plotted N$_2$O concentration against time and calculated denitrification rate as the slope of the line of best fit ($R^2 \geq 0.92$ for all rates). We expressed N$_2$O production rate as denitrification rate by converting N$_2$O to N and normalising by substratum dry mass (DM) ($\mu$g N g$^{-1}$ DM h$^{-1}$). Many published denitrification rates are scaled only by stream bottom surface area. To facilitate inter-study comparisons, we scaled our rates to area (mg N m$^{-2}$ h$^{-1}$) by multiplying DM normalised rates by substratum standing stocks (g m$^{-2}$). Because we measured denitrification in slurries, this may have somewhat overestimated our rates compared with other studies that measured denitrification in situ or by using intact cores.

For rates measured in Michigan, we calculated nutrient spiralling metrics for denitrification using the methods of Royer et al. (2004). Briefly, we calculated the uptake velocity of NO$_3^-$ due to denitrification ($V_{i,dn}$) as:

$$V_{i,dn} = \frac{U}{C}$$  \hspace{1cm} (1)

where $U$ is the substratum denitrification rate scaled to area (mg N m$^{-2}$ s$^{-1}$) and $C$ is the stream-water NO$_3^-$ concentration (mg N m$^{-3}$). We calculated the loss rate ($-k$) of NO$_3^-$-N from the water column via denitrification as:

$$-k = \frac{V_{i,dn}}{h}$$  \hspace{1cm} (2)

where $h$ is stream depth (m). We converted fraction of load s$^{-1}$ to % of load day$^{-1}$.

**Substratum standing stocks**

Sub-samples of all substrata were dried at 60 °C, weighed to obtain DM, ashed at 550 °C and re-weighed to obtain ash-free dry mass (AFDM). We calculated %OM of each sub-sample as the ratio of AFDM to DM, and this value represents mass lost upon ignition. We also combusted oven-dried samples in a Costech Elemental Analyser (Valencia, CA, U.S.A.) to measure substratum %C. To calculate total reach standing stock of each substratum, we multiplied substratum standing stock by its proportional abundance in the reach, determined by identifying benthic cover at 10-cm intervals along 10 regularly placed transects in each reach. For sand, FBOM and biofilm samples collected in Michigan, we retained a third set of sub-samples for chlorophyll-$a$ (chl-$a$) analysis. Chlorophyll-$a$ was extracted using the hot ethanol method (Sartory & Grobbelaar, 1984). We measured chl-$a$ using a Turner Designs TD-700 fluorometer (Sunnyvale, CA, U.S.A.) at 436 nm excitation wavelength and 680 emission wavelength and determined concentration using a calibration curve made from spectrophotometrically analysed concentrated stock solution.

**Water chemistry**

We used filtered water samples collected from each site to measure NO$_3^-$-N and DOC. Water samples were filtered with 0.7-µm Whatman GF/F glass fibre filters (Brentford, Middlesex, U.K.) and collected in acid-washed bottles, prerinsed with site water, stored on ice in the field, and frozen upon return to the laboratory no later than 12-h after collection. Nitrate concentration was measured using a DIONEX 600 ion chromatograph (Sunnyvale, CA, U.S.A.) with ED50 electrochemical detector and AS14A guard and analytical columns (USEPA, 1993). Dissolved organic carbon samples were acidified to pH 2 and measured on a Shimadzu TOC-5000 analyser (Columbia, MD, U.S.A.) using the combustion-infrared method (APHA, 1995) or persulphate oxidation using a Dohrmann-Xertex DC-80 analyser (Sunnyvale, CA, U.S.A.) equipped with a Horiba infrared gas analyser (Model PIR-2000).

**Statistical analyses**

Statistical analyses were performed using SYSTAT 11 (Systat Software, Richmond, CA, U.S.A.). To meet the assumptions of parametric statistics, we transformed non-normal data using either a logarithmic, or logarithmic followed by power, transformation and statistical significance was determined at the 0.05 level. For Illinois streams sampled in two seasons, we determined seasonal influence on stream-water NO$_3^-$ and DOC concentrations and sediment %OM using paired t-tests. We used simple linear regressions to analyse relationships between denitrification rates and %OM within streams, among streams and sampling periods, and between NO$_3^-$ load removed via denitrification and substratum %C. In the Michigan streams, we analysed how substratum type influenced denitrification rates and chl-$a$ using one-way ANOVA. When an ANOVA detected significant differences among means, we used Tukey’s post hoc
multiple comparison test to determine which means differed.

**Results**

*Stream water and sediment characteristics*

We anticipated that all the Illinois sites would have \(\text{NO}_3^-\) concentrations below 0.5 mg N L\(^{-1}\) during autumn sampling. Although site BLS had higher \(\text{NO}_3^-\) concentration than expected, stream-water \(\text{NO}_3^-\) concentrations were significantly higher in summer than in autumn in the Illinois streams \((P = 0.047)\) (Table 1). Stream-water DOC concentration did not vary between seasons, despite the high value at Lake Fork, Kaskaskia River (LFK) in the autumn (Table 1). In Michigan streams, \(\text{NO}_3^-\) concentration ranged from 0.4 to 6.4 mg N L\(^{-1}\) and DOC concentration from 2.4 to 3.9 mg C L\(^{-1}\) (Table 1).

Stream sediments in Illinois were a homogeneous mixture of sand, mud and fine organic particles, and we did not differentiate among these sediments by constituent substratum pools. Although mean sediment %OM was relatively low in the summer and autumn, it was significantly higher in summer than in autumn \((P < 0.001)\) (Table 2). This seasonal difference (1.1%) was numerically small compared with the range of %OM found in Michigan streams (0.7–72.7%) (Table 2). Of the Michigan substrata, CBOM had the highest %OM while sand had the lowest \((P < 0.001)\), and FBOM and CBOM (both as g AFDM m\(^{-2}\)) had the highest standing stocks \((P = 0.023)\), whereas sand and biofilm had the lowest (Table 2). Sand and FBOM combined comprised 71–80% of the benthic cover (Table 2). Sand, FBOM and biofilm each had an autotrophic component as measured by chl-\(a\) biomass, but we detected no differences in chl-\(a\) among substrata.

*Denitrification rates*

We measured denitrification in three replicates of each substratum from each site, and the fourth replicate was a control for background \(\text{N}_2\text{O}\) production. Standard error of our denitrification measures averaged 15% of the mean within a substratum from a site (e.g. sand denitrification in Swan Creek) compared with 30% of the mean among substrata from all sites (e.g. sand denitrification in all Michigan streams). This indicates greater variability in denitrification on the same substratum among sites than within a substratum from a single site. The low variability within a site also suggests that our method for measuring denitrification is reasonably precise even with relatively low replication.

Denitrification rates were generally higher in summer compared with autumn in Illinois streams (1.50–4.77 compared with 0.01–1.00 \(\text{ng N} \text{g}^{-1} \text{DM h}^{-1}\)) (Table 3) despite generally lower stream-water temperatures caused by the larger proportion of streamflow originating from cool, sub-surface tile flow to the stream in summer. However, the difference in denitrification was marginally insignificant \((P = 0.061)\), probably because of small sample sizes \((n = 3\) for each season). Overall, average denitrification rates on sediments were positively related to \(\text{NO}_3^-\) concentration among streams and between seasons in Illinois \((R^2 = 0.83, P = 0.012)\) (Fig. 1), but there was no relationship between denitrification rates and DOC concentrations. For a season within each Illinois stream, we regressed denitrification rate against sediment %OM. At \(\text{NO}_3^-\) concentrations <1 mg N L\(^{-1}\),

### Table 3 Mean (SE) denitrification rates and amount of nitrate load removed for sampled substratum types averaged across the streams \((n = 3\) in Illinois, \(n = 5\) in Michigan)

<table>
<thead>
<tr>
<th>Location</th>
<th>Season sampled</th>
<th>Substratum type</th>
<th>Denitrification ((\mu)g N g(^{-1}) DM h(^{-1}))</th>
<th>Denitrification (mg N m(^{-2}) h(^{-1}))</th>
<th>Nitrate load removed by denitrification; (-k) (%day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illinois</td>
<td>Summer</td>
<td>Homogenous sediment</td>
<td>3.0 (0.4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Illinois</td>
<td>Autumn</td>
<td>Homogenous sediment</td>
<td>0.4 (0.2)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>Sand</td>
<td>0.1 (0.0)</td>
<td>2.0 (0.9)</td>
<td>10.2 (3.8)</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>Biofilm</td>
<td>2.3 (0.6)</td>
<td>1.7 (0.8)</td>
<td>15.1 (4.9)</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>CBOM</td>
<td>11.1 (2.6)</td>
<td>3.8 (1.7)</td>
<td>39.4 (18.1)</td>
</tr>
<tr>
<td>Michigan</td>
<td>Autumn</td>
<td>FBOM</td>
<td>4.2 (1.5)</td>
<td>8.5 (3.8)</td>
<td>118.0 (61.4)</td>
</tr>
</tbody>
</table>

CBOM, coarse benthic organic matter; FBOM, fine benthic organic matter; X, data not collected.

specific denitrification rate (μg N g⁻¹ DM h⁻¹) by the substratum standing stock (g m⁻²) and then weighting the resulting areal rate (mg N m⁻² h⁻¹) by the proportional abundance of the substratum. The summation of the weighted rates yields an estimate of the whole-stream denitrification rate. We found no relationship between whole-stream denitrification and either water-column NO₃ concentration or DOC concentration.

**Discussion**

**Comparison of denitrification rates**

Denitrification can be measured by different methods, which can confuse cross-study comparisons. We used C₂H₂-inhibition, which can underestimate actual denitrification when NO₃ is low and denitrification is strongly coupled with nitrification (Seitzinger et al., 1993). Therefore, for better comparability, we compared our substratum-specific rates with previously published C₂H₂-inhibited rates. Furthermore, we measured denitrification with slurries, which generally give higher estimates than intact cores, so we used chloramphenicol to reduce our over-estimation of actual values (Smith & Tiedje, 1979). When making comparisons, we also note when we compare our slurry-measured rates to those from intact cores.

Among Michigan streams, substratum-specific denitrification rates varied over two orders of magnitude, indicating that some substrata were ‘hotspots’ of denitrification (sensu McClain et al., 2003). Our measures were similar to the few studies that also reported substratum-specific denitrification rates, even though none reported the entire suite of substrata that we measured. For example, denitrification on sand was identical to García-Ruiz, Patterson & Whitten (1998a) at 2.0 mg N m⁻² h⁻¹, though they used intact cores. Denitrification on FBOM (27.4 μg N g⁻¹ AFDM h⁻¹) was lower than reported by Bonin, Griffiths & Caldwell (2003) (57 and 100 μg N g⁻¹ AFDM h⁻¹), but they measured denitrification potential (i.e. C₂H₂-inhibited assays amended with NO₃ and DOC), which likely increased denitrification relative to our unamended assays. Using the same method as ours, Triska & Oremland (1981) measured a denitrification rate of 0.66 μg N g⁻¹ DM h⁻¹ on decomposing periphyton communities; the biofilm we sampled was a similar substratum because of the autotrophic...
presence mixed with detrital material, but we measured somewhat higher rates (2.30 µg N g⁻¹ DM h⁻¹). Each substratum had physical characteristics (e.g. particle size) and a chemical composition (e.g. C content) that likely enhanced or diminished denitrification, and the similarity of our results to other studies suggests that substrata may have intrinsically different denitrification rates. Whereas a particular substratum may support inherently high denitrification rates, the absolute magnitude probably depends primarily on site-specific variability in the controls of denitrification. For example, denitrification in Michigan streams was highest on FBOM and CBOM (Table 3), and Kemp & Dodds (2002) reported the same using intact cores in Kansas streams. However, our denitrification rates are several orders of magnitude higher than theirs (8.5 and 3.8 mg N m⁻² h⁻¹ compared with 0.004 and 0.005 mg N m⁻² h⁻¹ on FBOM and CBOM, respectively). Differences between Kansas and Michigan streams may explain this disparity. Streams in Michigan had higher NO₃⁻ (0.42–6.4 mg N L⁻¹) than in Kansas (0.005–0.774 mg N L⁻¹), which would likely cause higher denitrification rates in Michigan. Also,

FIG. 2 The relationship between denitrification rate and the organic fraction of the sediment in Illinois streams. Plots (a)–(f) correspond to the datum with the same letter (a–f) in Fig. 1: (a) LFK, autumn; (b) BDO, autumn; (c) BLS, autumn; (d) LFK, summer; (e) BLS, summer; and (f) BDO, summer.
Michigan streams are largely heterotrophic (Hamilton et al., 2001) compared with autotrophic Kansas streams (Dodds et al., 2000), so primary producers may outcompete denitrifiers for $\text{NO}_3^-$/$\text{CO}_3^-$ (Kemp & Dodds, 2002) or may repress denitrification by pumping oxygen into the benthos (Rysgaard et al., 1994).

We scaled our substratum-specific denitrification rates to a whole-stream rate using habitat-weighting so we could compare our rates to many other published data. Whole-stream rates we calculated from slurries probably overestimate denitrification, but our data compare with those from many denitrification studies in streams from widely differing biomes (Fig. 5), despite methodological differences. Because some benthic substrata supported higher denitrification rates than others, the habitat-weighting technique probably provided a reasonable whole-stream measure of \textit{in situ} denitrification rates in the patchy Michigan streams. As an indirect estimate of whole-stream denitrification, habitat-weighting incorporates more error than a direct measure. However, given sufficient replication within each substratum and thorough characterization of the habitat-weighting factors, it should provide a reasonable estimate of whole-stream denitrification.

Factors controlling denitrification rates

Denitrification requires $\text{NO}_3^-$, organic carbon and anoxic conditions (Knowles, 1982; Seitzinger, 1988), and comparing among biomes suggests that denitrification rate increases across a broad range of $\text{NO}_3^-$ concentrations (Fig. 5). Denitrification is a metabolic process, however, and is subject to saturation with respect to reactants (i.e. $\text{NO}_3^-$ and C); we can therefore analyse denitrification rate using uptake kinetics. Examined in this context, denitrification will approach saturation when $\text{NO}_3^-$ concentrations exceed

---

Fig. 3 Denitrification rates ($n = 3$ for each datum, ±SE) in Michigan streams in relation to the organic fraction of the substrata.

Fig. 4 The removal rate of stream-water nitrate via denitrification ($n = 3$ for each datum, ±SE) in relation to carbon content of substrata in Michigan streams.

Fig. 5 Whole-stream denitrification rates calculated for the Michigan streams are consistent with denitrification rates measured in streams from diverse biomes. The denitrification rates presented here were measured using many different methods including whole-stream $^{15}\text{NO}_3^-$ releases, membrane inlet mass spectrometry (MIMS), \textit{in situ} $\text{C}_2\text{H}_2$ block chambers, and laboratory $\text{C}_2\text{H}_2$ slurries. In general, denitrification rates are positively related to $\text{NO}_3^-$ concentrations. Data presented are from Triska & Oremland (1981), Duff et al. (1984), Christensen et al. (1990), Nielsen et al. (1990), Duff, Pringle & Triska (1996), Holmes et al. (1996), García-Ruiz et al. (1998a), Pattinson et al. (1998), Bernhardt and Likens (2002), Kemp and Dodds (2002), Bohlke et al. (2004), Laursen and Seitzinger (2004), Mulholland et al. (2004), Richardson et al. (2004), Royer et al. (2004), Schaller et al. (2004), Pribyl et al. (2005).
the half-saturation constant. A study using intact cores to measure denitrification found half-saturation constants between 0.18 and 1.27 mg NO$_3$-N L$^{-1}$ along a river continuum (García-Ruiz, Patтинson & Whitton, 1998b), and a study using slurries in estuarine environments found half-saturation constants between 0.38 and 0.74 mg NO$_3$-N L$^{-1}$ (Seitzinger, 1998). Others have used a two-dimensional Kolmogorov–Smirnov test (Garvey, Marshall & Wright, 1998) to determine a threshold concentration beyond which factors other than NO$_3$ control denitrification. These thresholds, 0.4 mg NO$_3$-N L$^{-1}$ in small streams (Inwood et al., 2005) and 0.9 mg NO$_3$-N L$^{-1}$ in a river and its receiving reservoir (Wall et al., 2005), were within the range of half-saturation constants measured using intact cores. Because the thresholds were measured using C$_2$H$_2$-block slurries, they are directly measured using intact cores. Because the thresholds were measured using C$_2$H$_2$-block slurries, they are directly comparable with our data. In our study, stream-water NO$_3$ concentrations equalled or exceeded these threshold values (but see Big Ditch Outlet and LFK in autumn, Table 1), suggesting NO$_3$ saturation of denitrification, but we found a positive relationship between denitrification and NO$_3$ concentration among streams and sampling periods in Illinois (Fig. 1), suggesting NO$_3$ limitation of denitrification. Although these results appear contradicting, they depict two distinct relationships among the data. First, among streams and seasons, denitrification rates are positively related to NO$_3$ concentration (Fig. 1). Second, within a season at a given stream, denitrification is positively related to the sediment organic fraction only when NO$_3$ is high (Fig. 2).

Denitrification may also be limited by the presence of anoxic microhabitats, which are created by the aerobic decomposition of organic matter. Anoxia often occurs deep in stream sediments (e.g. Holmes et al., 1996; Morrice et al., 2000; LeFebvre et al., 2004) and is related to whether advection or diffusion drive oxygen flux into the benthos. In the sand-bottom streams we studied, Tank (unpublished) found an anoxic layer just below the first few mm of sediment. This suggests that the active zone of denitrification begins immediately below the sediment surface, and the shallow depth to anoxia may be related to fine particles that slow penetration of oxygenated stream-water into the benthos (Vervier et al., 1992; Pretty, Hildrew & Trimmer, 2006). Anoxia may additionally occur in particle-associated microsites in an otherwise oxic environment (Sorensen, Jorgensen & Revsbech, 1979; Sakita & Kusuda, 2000). We measured denitrification on substrata (i.e. FBOM, CBOM and biofilm) taken from the oxic streambed surface. Although using anoxia to measure denitrification in the laboratory exposes these substrata to radically different conditions than in the field, the high denitrification rates we measured demonstrate the potential for denitrification on anoxic microsites. These microsites may supplement the denitrification that occurs at depth in the sandy stream sediments, increasing overall denitrification capacity in these streams.

Denitrifying bacteria are heterotrophic microbes that use DOC as a C substrate for assimilatory and dissimilatory metabolism, so they may also be limited by organic C. We define two distinct forms of organic C in our study streams: DOC and POC. Dissolved organic C was <0.7 μm, the mesh size we used to filter the water in which we measured DOC, whereas POC was >63-μm diameter and included FBOM and CBOM. Studies from soils (e.g. Burford & Bremner, 1975; Jandl & Sollins, 1997), riparian zones (Hill et al., 2000) and streams (Inwood et al., 2005) have shown positive relationships between DOC and denitrification. Denitrification assays performed in similar streams to ours found that denitrification was C limited when NO$_3$ concentrations were high (Inwood et al., 2007). However, we did not observe a relationship between benthic denitrification rates and stream-water DOC in any of our streams, suggesting that sediment POC is a more important C source for denitrifiers than stream-water DOC.

Sediment POC produces DOC directly via abiotic leaching and indirectly via microbial degradation with exoenzymes (Fenchel, King & Blackburn, 2000) or exudates from shredding macroinvertebrates (Meyer & O’Hop, 1983). We found a strong relationship between denitrification rates and substratum %OM across a range of high NO$_3$ concentrations in the Michigan streams (Fig. 3), and when NO$_3$ was high in Illinois streams (Fig. 2). We also found that stream-water NO$_3$ load removed via denitrification was positively related to substratum %C content (Fig. 4), with FBOM and CBOM generally removing more NO$_3$ than sand or biofilms (Table 3). Sediment POC may facilitate denitrification by leaching DOC or by creating more extensive anoxic habitat via heterotrophic decomposition. Particulate organic C amendments have stimulated denitrification in NO$_3$-rich groundwater (Schipper & Vojvodić-Vuković, 2002) and in
marine sediments (Dahllof & Karle, 2005) although the mechanism for increased denitrification (i.e. increased C availability as an electron source or increased anoxic habitat) was not identified. However, POC has been used as the sole C source for denitrification in wastewater treatment applications (e.g. van Oostrom & Russell, 1994; Rocca, Belgiorno & Meric, 2005), demonstrating the feasibility of POC as an electron source for denitrification. The relationships we found between denitrification and substratum %OM, a metric of POC, but not between denitrification and whole-stream DOC indicate that POC plays an important role in regulating stream denitrification when NO\textsubscript{3} concentrations are high. However, more research is required to identify when POC influences denitrification via direct or indirect mechanisms.

These results have important implications when considering streams as ecosystems that may attenuate NO\textsubscript{3} export to downstream waterbodies (e.g. Alexander et al., 2000; Peterson et al., 2001). To promote drainage from agricultural landscapes, management agencies commonly clear riparian vegetation and remove woody debris from streams to hasten water flow (Allan & Flecker, 1993). Practices that promote C removal from agricultural streams may accentuate already high NO\textsubscript{3} concentrations typical of these systems by limiting the denitrification potential of the stream. Engineers manipulate C levels to optimise NO\textsubscript{3} removal in remediation settings (e.g. van Oostrom & Russell, 1994; Schipper & Vojvodić-Vuković, 2002), and the same principles could apply to optimise denitrification in agricultural streams with chronically high NO\textsubscript{3} concentrations. Allowing large organic matter (e.g. LWD) to accumulate in agricultural streams would create more heterogeneous flow conditions and generate settling zones in which FBOM and CBOM could accumulate. These organic matter accumulations could provide a C source for denitrifiers and may enhance the spatial extent of anoxia in stream sediments, further stimulating denitrification.

Acknowledgments

This manuscript was greatly improved by comments from two anonymous reviewers and Alan Hildrew. The authors thank Sarah Duhammel, Erin Hotchkiss, Lareina Wall, Jon Loftus and Jake Beaulieu for assistance in the field, and Stuart Orr and Kris Premier for assistance in the laboratory. Dennis Birdsell and Rian Galloway provided technical assistance for analyses performed at Notre Dame’s Center for Environmental Science and Technology. C. Arango received financial support from the Schmidt Presidential Fellowship during sample collection and analysis, and from the Bayer Predoctoral Fellowship while writing this manuscript. This research was funded in part by NSF-DEB 0111410.

References


(Manuscript accepted 15 February 2007)