

Benthic organic carbon influences denitrification in streams with high nitrate concentration

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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 µg 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 µg 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 (NO₃⁻) and ammonium (NH₄⁺) has increased as a result of anthropogenic activities including fertiliser use and fossil fuel combustion (Galloway, 1998). Anthro-

genic 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).

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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_2O) 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_2O 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 ($<1 \text{ mg NO}_3^- \text{-N L}^{-1}$) 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 denit-

rifiers 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 $10 \text{ mg NO}_3^- \text{-N L}^{-1}$ in early summer and then decreases below $0.5 \text{ mg NO}_3^- \text{-N L}^{-1}$ 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 ($>0.5 \text{ mg N L}^{-1}$) 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

Table 1 Location, order, width, temperature, discharge, nitrate and dissolved organic carbon (DOC) concentrations at the time of sampling

Stream name and code	Location	Coordinates (latitude, longitude)	Stream order	Width (m)		Stream temperature ($^{\circ}\text{C}$)		Discharge (L s^{-1})		Nitrate (mg N L^{-1})		DOC (mg C L^{-1})			
				Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn
Big Ditch Outlet (BDO)	Illinois	40°16'06"N, 88°19'35"W	2	8.6	6.6	16.8	19.8	1030	35.1	17.8	0.17	1.7	3.5		
Black Slough (BLS)	Illinois	39°57'07"N, 88°10'10"W	1	3.3	1.9	17.7	21.1	324	1820	13.4	5.6	1.3	2.1		
Lake Fork, Kaskaskia River (LFK)	Illinois	39°50'09"N, 88°29'18"W	3	14.1	9.6	26.3	21.3	1270	X	12.6	0.10	2.1	12.1		
Sand Creek (SNC)	Michigan	42°35'36"N, 85°56'35"W	1	X	0.4	X	X	X	9.9	X	0.79	X	3.3		
Swan Creek (SWC)	Michigan	42°30'24"N, 85°57'34"W	1	X	0.7	X	X	X	4.5	X	0.42	X	3.9		
Black Creek (BLC)	Michigan	42°46'53"N, 85°52'35"W	2	X	2.0	X	X	X	115	X	6.4	X	2.4		
Dorr and Byron Creek (DBC)	Michigan	42°44'17"N, 85°45'11"W	3	X	2.7	X	X	X	279	X	1.1	X	3.5		
Tributary to Little Rabbit River (RAB)	Michigan	42°44'17"N, 85°48'34"W	1	X	1.5	X	X	X	16.4	X	5.5	X	3.1		

X, not collected.

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

Location	Season sampled	Substratum type	Standing stock (g AFDM m ⁻²)	Benthic cover (%)	Organic matter (%)	Chlorophyll- <i>a</i> (µg cm ⁻²)
Illinois	Summer	Homogenous sediment	X	100	7.0 (0.2)	X
Illinois	Autumn	Homogenous sediment	X	100	5.9 (0.3)	X
Michigan	Autumn	Sand	69.5 (10.1)	36.2 (5.7)	0.7 (0.1)	2.3 (0.7)
Michigan	Autumn	Biofilm	84.8 (49.4)	5.1 (1.8)	9.9 (1.4)	3.3 (1.5)
Michigan	Autumn	CBOM	272.7 (79.1)	17.5 (3.6)	72.7 (2.3)	X
Michigan	Autumn	FBOM	343.4 (108.2)	40.6 (5.9)	18.0 (2.1)	7.5 (3.5)

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

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 (C₂H₂) block method (Smith & Tiedje, 1979; Royer *et al.*, 2004; Inwood *et al.*, 2005). Acetylene (C₂H₂) blocks the final step of the complete denitrification pathway allowing N₂O, which is more easily measured than N₂, 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 cm³ 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 C₂H₂ to three of the four assay bottles, and the fourth bottle received no C₂H₂ to control for background N₂O 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 N₂O 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% C₂H₂ 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 C₂H₂ away from the detector (injector temp, 120 °C; column temp, 40 °C; detector temp, 320 °C, ultra high purity N₂ carrier gas, 30 mL min⁻¹). Total concentration of N₂O at each sampling period was calculated

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 N₂O 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 N₂O production rate as denitrification rate by converting N₂O to N and normalising by substratum dry mass (DM) ($\mu\text{g N g}^{-1} \text{DM h}^{-1}$). Many published denitrification rates are scaled only by stream bottom surface area. To facilitate inter-study comparisons, we scaled our rates to area ($\text{mg N m}^{-2} \text{h}^{-1}$) 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 NO₃⁻ due to denitrification ($V_{f,\text{dn}}$) as:

$$V_{f,\text{dn}} = U/C \quad (1)$$

where U is the substratum denitrification rate scaled to area ($\text{mg N m}^{-2} \text{s}^{-1}$) and C is the stream-water NO₃⁻ concentration (mg N m^{-3}). We calculated the loss rate ($-k$) of NO₃⁻-N from the water column via denitrification as:

$$-k = V_{f,\text{dn}}/h \quad (2)$$

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 reweighed 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 NO₃⁻-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 NO₃⁻ 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 NO₃⁻ 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 NO_3^- concentrations below 0.5 mg N L^{-1} during autumn sampling. Although site BLS had higher NO_3^- concentration than expected, stream-water 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, 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). However, 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 meas-

ured 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 N_2O 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 – $1.00 \text{ } \mu\text{g N g}^{-1} \text{ DM h}^{-1}$) (Table 3) despite generally lower stream-water temperatures caused by the larger proportion of stream-flow 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 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 NO_3^- concentrations $< 1 \text{ mg N L}^{-1}$,

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)

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

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

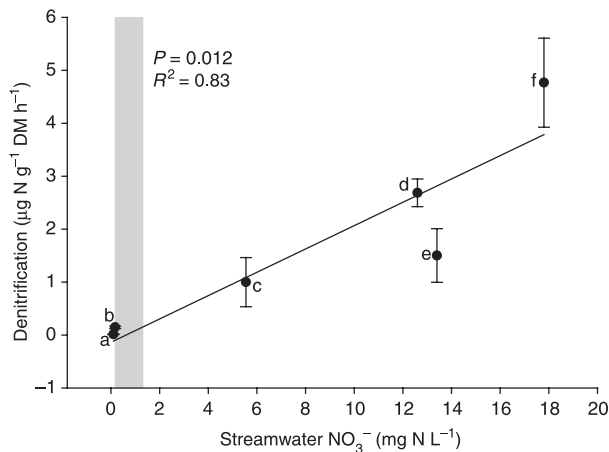


Fig. 1 The relationship between mean (\pm SE) denitrification rate and stream-water nitrate concentration in Illinois streams. Each datum, labelled a–f, is composed of 15 denitrification measures in a given stream at a given sampling period: a) LFK, autumn; b) BDO, autumn; c) BLS, autumn; d) LFK, summer; e) BLS, summer; and f) BDO, summer. The grey bar indicates lower and upper values of published half-saturation constants for denitrification with respect to NO₃⁻ [0.2–1.3 mg NO₃⁻-N L⁻¹ (Seitzinger, 1988; García-Ruiz *et al.*, 1998b)].

there was no relationship between sediment %OM and denitrification, but at NO₃⁻ concentrations >1 mg N L⁻¹ there was generally a positive effect on denitrification rate from increased sediment %OM (Fig. 2).

In the Michigan streams, denitrification rates varied significantly among substrata ($P < 0.001$), with CBOM supporting the highest rate and sand the lowest when normalised by DM (Table 3). In general in Michigan, denitrification rate increased with substratum %OM ($P < 0.001$, $R^2 = 0.77$; Fig. 3). We found a positive relationship between water-column NO₃⁻ concentrations and denitrification on CBOM (mg N m⁻² h⁻¹; $P = 0.032$, $R^2 = 0.83$, data not shown) and between water-column NO₃⁻ concentration and denitrification on biofilms (µg N g⁻¹ AFDM h⁻¹; $P = 0.023$, $R^2 = 0.86$, data not shown), but we found no such relationships with denitrification rates on sand or FBOM. Substratum-specific denitrification rates were not significantly related to water-column DOC concentration for any substratum and, although substratum-specific NO₃⁻ removal via denitrification did not vary among substrata (despite ranging widely: 10.2–118.0%; Table 3), NO₃⁻ removal rate was positively related to substratum %C ($P = 0.035$, $R^2 = 0.22$, Fig. 4). For each stream, we calculated a whole-stream denitrification rate by multiplying the substratum-

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, Pattinson & Whitton (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

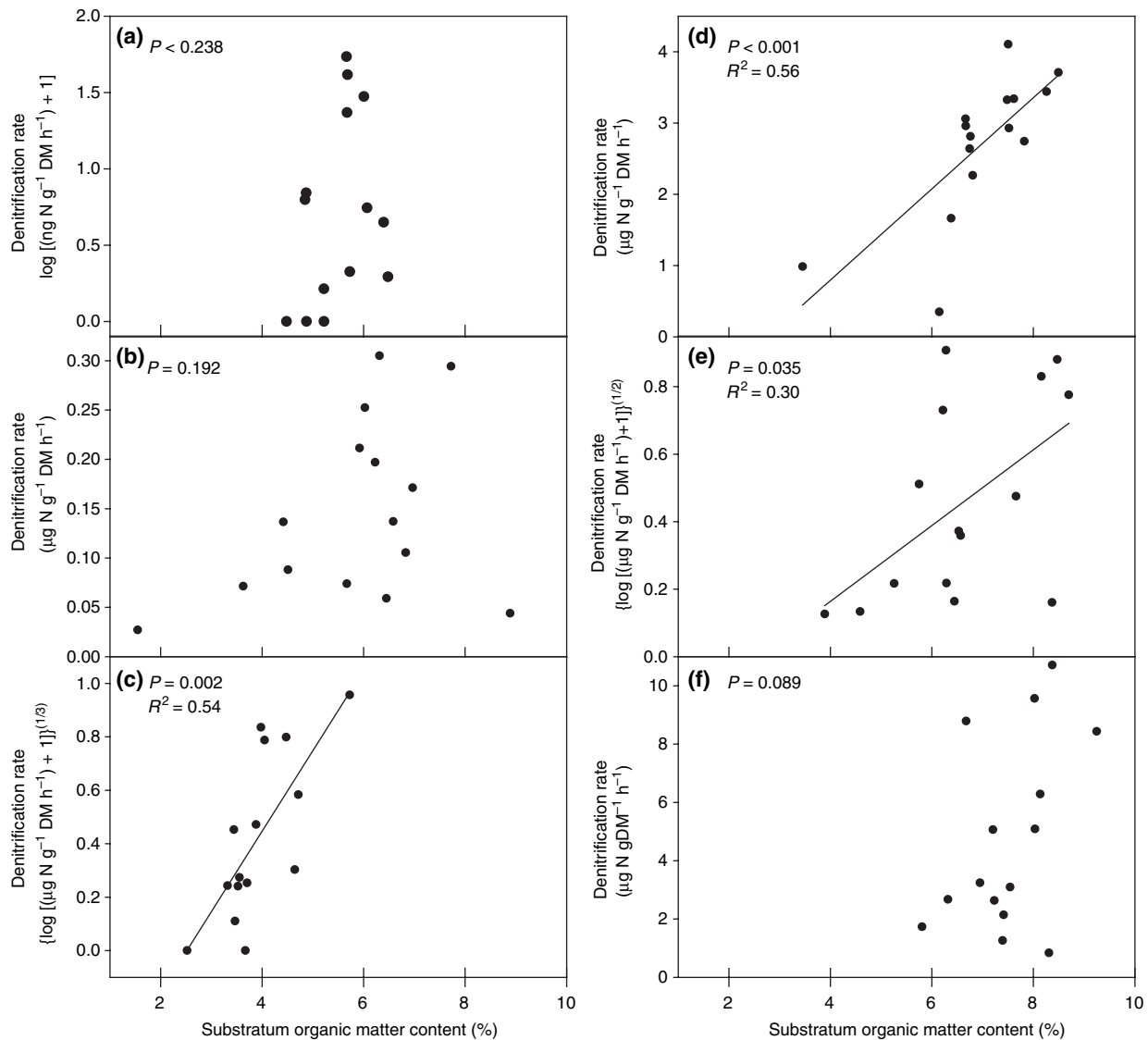


Fig. 2 The relationship between denitrification rate and the organic fraction of the sediment in Illinois streams. Plots (a)–(f) correspond to the datum with the same letter (a–f) in Fig. 1: (a) LFK, autumn; (b) BDO, autumn; (c) BLS, autumn; (d) LFK, summer; (e) BLS, summer; and (f) BDO, summer.

presence mixed with detrital material, but we measured somewhat higher rates ($2.30 \mu\text{g N g}^{-1} \text{ DM h}^{-1}$). 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 $3.8 \text{ mg N m}^{-2} \text{ h}^{-1}$ compared with 0.004 and $0.005 \text{ mg N m}^{-2} \text{ h}^{-1}$ on FBOM and CBOM, respectively). Differences between Kansas and Michigan streams may explain this disparity. Streams in Michigan had higher NO_3^- (0.42 – 6.4 mg N L^{-1}) than in Kansas (0.005 – $0.774 \text{ mg N L}^{-1}$), which would likely cause higher denitrification rates in Michigan. Also,

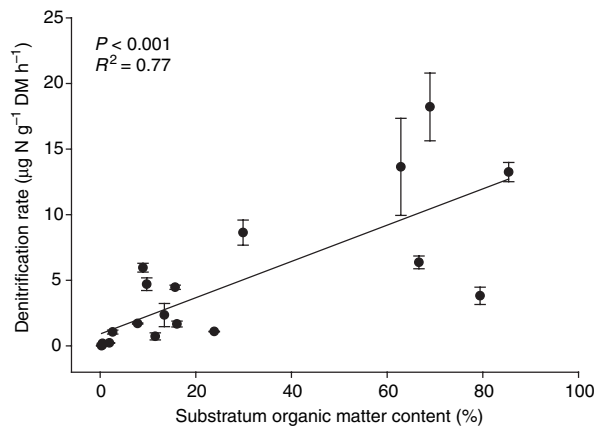


Fig. 3 Denitrification rates ($n = 3$ for each datum, \pm SE) in Michigan streams in relation to the organic fraction of the substrata.

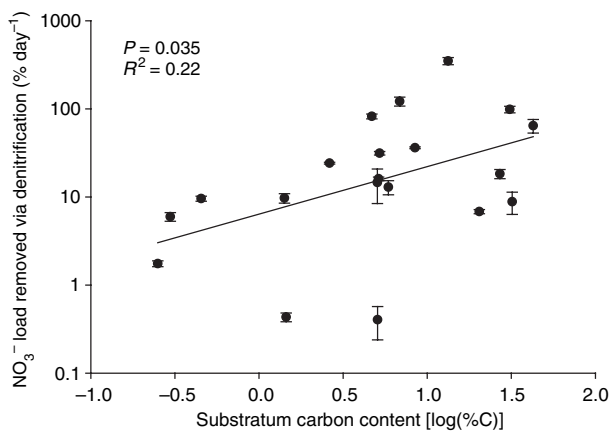


Fig. 4 The removal rate of stream-water nitrate via denitrification ($n = 3$ for each datum, \pm SE) in relation to carbon content of substrata in Michigan streams.

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 NO_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

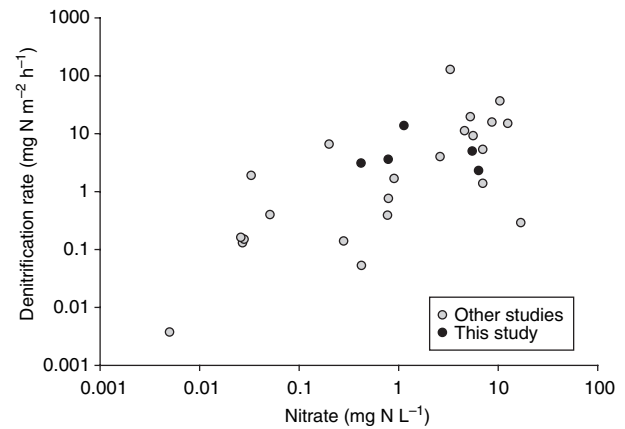


Fig. 5 Whole-stream denitrification rates calculated for the Michigan streams are consistent with denitrification rates measured in streams from diverse biomes. The denitrification rates presented here were measured using many different methods including whole-stream $^{15}\text{NO}_3^-$ releases, membrane inlet mass spectrometry (MIMS), *in situ* C_2H_2 block chambers, and laboratory C_2H_2 slurries. In general, denitrification rates are positively related to 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).

technique probably provided a reasonable whole-stream measure of *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 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 NO_3^- concentrations (Fig. 5). Denitrification is a metabolic process, however, and is subject to saturation with respect to reactants (i.e. NO_3^- and C); we can therefore analyse denitrification rate using uptake kinetics. Examined in this context, denitrification will approach saturation when NO_3^- concentrations exceed

the half-saturation constant. A study using intact cores to measure denitrification found half-saturation constants between 0.18 and 1.27 mg NO₃⁻-N L⁻¹ along a river continuum (García-Ruiz, Pattinson & Whitton, 1998b), and a study using slurries in estuarine environments found half-saturation constants between 0.38 and 0.74 mg NO₃⁻-N L⁻¹ (Seitzinger, 1988). Others have used a two-dimensional Kolmogorov–Smirnov test (Garvey, Marshall & Wright, 1998) to determine a threshold concentration beyond which factors other than NO₃⁻ control denitrification. These thresholds, 0.4 mg NO₃⁻-N L⁻¹ in small streams (Inwood *et al.*, 2005) and 0.9 mg NO₃⁻-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 C₂H₂-block slurries, they are directly comparable with our data. In our study, stream-water NO₃⁻ concentrations equalled or exceeded these threshold values (but see Big Ditch Outlet and LFK in autumn, Table 1), suggesting NO₃⁻ saturation of denitrification, but we found a positive relationship between denitrification and NO₃⁻ concentration among streams and sampling periods in Illinois (Fig. 1), suggesting NO₃⁻ 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 NO₃⁻ concentration (Fig. 1). Second, within a season at a given stream, denitrification is positively related to the sediment organic fraction only when NO₃⁻ 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 (Sørensen, Jørgensen & 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 NO₃⁻ 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 NO₃⁻ concentrations in the Michigan streams (Fig. 3), and when NO₃⁻ was high in Illinois streams (Fig. 2). We also found that stream-water NO₃⁻ load removed via denitrification was positively related to substratum %C content (Fig. 4), with FBOM and CBOM generally removing more NO₃⁻ 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 NO₃⁻-rich ground-water (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_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_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_3^- concentrations typical of these systems by limiting the denitrification potential of the stream. Engineers manipulate C levels to optimise NO_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_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.

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