Cross-stream comparison of substrate-specific denitrification potential

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Abstract Headwater streams have a demonstrated ability to denitrify a portion of their nitrate (NO_3^-) load but there has not been an extensive consideration of where in a stream this process is occurring and how various habitats contribute to total denitrification capability. As part of the Lotic Intersite Nitrogen Experiment II (LINX II) we measured denitrification potential in 65 streams spanning eight regions of the US and draining three land-use types. In each stream, potential denitrification rates were measured in common substrate types found across many streams as well as locations unique to particular streams. Overall, habitats from streams draining urban and agricultural land-uses showed higher potential rates of denitrification than reference streams draining native vegetation. This difference among streams was probably driven by higher ambient nitrate concentrations found in urban or agricultural streams. Within streams, sandy habitats and accumulations of fine benthic organic matter contributed more than half of the total denitrification capacity (mg N removed m^{-2} h^{-1}). A particular rate of potential denitrification per unit area could be achieved either by high activity per unit organic matter or lower activities associated with larger standing stocks of organic matter. We found that both small patches with high rates (hot spots) or more widespread but less active

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areas (cool matrix) contributed significantly to whole stream denitrification capacity. Denitrification estimated from scaled-up denitrification enzyme assay (DEA) potentials were not always dramatically higher than in situ rates of denitrification measured as ^{15}N gas generation following 24-h ^{15}N–NO_3 tracer additions. In general, headwater streams draining varying land-use types have significant potential to remove nitrate via denitrification and some appear to be functioning near their maximal capacity.

Introduction

Nitrogen (N) movement from terrestrial ecosystems to receiving waters is a critical environmental issue due to both increasing N inputs across the globe and frequent negative effects on aquatic ecosystems (e.g. Turner et al. 2008). Much of the N export from terrestrial ecosystems occurs via small headwater streams, and their potential for retaining or retarding N movement has significant implications for both the timing and magnitude of delivery to sensitive downstream ecosystems (Alexander et al. 2000).

Nitrate (NO_3^-) is the predominant form of fixed N in transit through many streams. This mobile anion is not subject to significant abiotic retention, although large areas of hyporheic exchange or backwaters can slow its physical transport (Gooseff et al. 2004; Lautz and Siegel 2007; Dent et al. 2007). Biological N removal can be assimilatory, with incorporation into new plant or microbial biomass, but the duration of retention depends on turnover of that biomass and whether that biomass is deposited and buried (Bernot and Dodds 2005). Nitrogen retained via assimilation can remain in the ecosystem from days to years depending on the life span of the organism and subsequent N mineralization rates. Even short-term retention of available N in a stream reach is desirable from the point of view of mitigating high N loading to downstream ecosystems. Dispersing N spikes across broader time scales or delaying release until higher flow periods may reduce acute increases in instantaneous concentrations downstream. Such smoothing of N loads may help avoid surpassing thresholds of negative responses, such as those triggering harmful algal blooms.

In contrast to biotic assimilation, microbially mediated respiratory denitrification results in the conversion of NO_3^- to relatively inert dinitrogen gas. Determining net removal of NO_3^- from streamwater and the actual pathway of removal is a significant methodological challenge (Bernot et al. 2003; Kulkarni et al. 2008) with various ^{15}N tracer approaches offering the greatest promise for quantifying processes (Mulholland et al. 2008; O'Brien et al. 2007; Peterson et al. 2001). The addition of $^{15}NO_3^-$ to a stream and subsequent collection of $^{15}N_2$ and $^{15}N_2O$ gases provides confirmation that the microbial transformation from NO_3^- to N gases is occurring, and is currently the most direct estimate of in situ whole-system denitrification (Mulholland et al. 2009). However, these reach-scale measurements cannot provide information on which particular places within the stream are active sites for this conversion. This finer-scale information is important for ecological understanding of this process, as well as for appreciating consequences of habitat manipulation during restoration.

We anticipate fine-scale variation within streams due to differences in micro-site conditions and coarse-scale variation among streams and regions due to differences in land-use or climate. Land-cover patterns are expected to influence stream denitrification for several reasons, including high NO_3^- loadings from agricultural practices, water source or wastewater discharge in streams draining populated areas (Roach and Grimm 2009). Land-use also influences riparian shading and thus light availability supporting primary production, which may be the first step in N removal from overlying water (Holmes et al. 1996). Moreover, organic-matter supply from riparian vegetation or dissolved organic matter delivered from uplands can affect stream metabolism and large inputs may increase ecosystem demand for alternate respiratory pathways. There may be substantial variability in abundance of the denitrification gene sequence even in fairly homogeneous substrate types (Knapp et al. 2009). These points suggest there will be both fine- and coarse-scale variation in denitrification potential *within* a stream, and variation *among* streams draining catchments of varying land-use.

In a recent review, Piña-Ochoa and Álvarez-Cobelas (2006) argued that we need to understand similarities or differences between habitat-scale and regional-scale controls on rates of denitrification.

The denitrification enzyme assay (DEA) is a measure of the potential for a sample to carry out denitrification and can be used to indicate where in a stream there is a large enough capacity to bring about significant NO_3^- removal and conversion to N gases. The nature of the assay (e.g., anoxic conditions along with addition of carbon and NO_3^-) is not intended to estimate in situ denitrification rates but is targeted at measuring the accumulation of enzymatic capacity for using NO_3^- as an electron acceptor. Stream habitats with high denitrification potential that can be manifest on the short time scales of the laboratory assay have presumably developed this capacity because at some point in time, in situ conditions were highly favorable for denitrifiers.

We examined potential denitrification for multiple physical substrate types sampled from 65 streams distributed across 8 regions of the US and representing 3 different land-uses (Fig. 1). Our goals were to: (1) ascertain important scales of variability including variation among substrate types within a stream and differences among land-uses; (2) determine for a wide array of streams which substrate types were potentially capable of supporting high denitrification rates; (3) assess differences between realized rates ($^{15}NO_3^- \rightarrow {}^{15}N$ gas in situ tracer approach) and potential rates derived from scaling up DEA assays.

Fig. 1 Map of study locations. *AP* Appalachia, *KS* Kansas, *MA* Massachusetts, *MI* Michigan/Indiana, *OR* Oregon, *PR* Puerto Rico, *SW* Southwest, *WY* Wyoming

Methods

Site selection and description

We obtained samples from streams in eight regions with three land-use types (visual assessment of predominant land-use (Reference [REF], Agricultural [AG], Urban [URB]) adjacent to the study reach) nested within each region (Fig. 1). A different set of streams was sampled each year (2003–2005). Out of 72 possible streams (8 regions × 3 land-use types × 3 years), we obtained samples from 65 sites. For most regions, one stream from each land-use type was sampled during baseflow in each of three consecutive years. Attributes of land-use classes varied considerably among regions; for instance, agricultural practices were row crop agriculture in Michigan compared to pasture in Wyoming. Reference streams had catchments dominated by native vegetation which varied from forest to desert. Stream size across all sites was constrained to be fairly small (range in discharge was 2–267 L s^{-1}, mean = 38 L s^{-1}) in order to have streams with fairly homogeneous land-use in their catchments with discharge that was low enough to permit the associated ^{15}N–NO_3^- tracer additions (Mulholland et al. 2008). Investigators at each site collected ancillary information on land use, riparian vegetation, water chemistry (inorganic nutrients, dissolved organic carbon and N). Samples for DEA were collected by hand from four to five different substrate types along the ∼200–1800-m study reaches within each stream with 3–4 individual replicates for each substrate type.

Standing stocks of organic matter in these substrate types were determined within the same week. All streams had substrate types comprised of fine benthic organic matter (FBOM), 36 of 65 streams yielded samples of coarse benthic organic matter (CBOM), 24 of 65 collected samples of algal mats or epilithic algae, and a few had clumps of macrophytes or masses of plant roots. Samples were shipped on ice to the Cary Institute of Ecosystems Studies, Millbrook, NY and all DEA assays were conducted within 5 days of sample collection. We examined the effect of cold storage on DEA activity and found no difference between FBOM samples from a local stream analyzed immediately versus 1, 3, or 5 days of storage on ice in the dark (data not shown).

Measurement of DEA followed Smith and Tiedje (1979) with minor modifications. Weighed samples or volumes of fine material from different substrate types were distributed into 125-mL flasks with 40 mL of incubation medium containing KNO_3 (1.4 g L^{-1}); glucose (1 g L^{-1}) and the antibiotic chloramphenicol to prevent de novo enzyme synthesis (0.25 g L^{-1}); flasks were sealed with Sigma rubber septa. There were 3–4 replicate samples of each habitat type and each field replicate was analyzed separately. Masses and volumes varied by type of sample with greater masses used for samples with larger amounts of inorganic sediment. Flasks were shaken (100–120 RPM) and sparged with N_2 three times before addition of 20 mL of acetylene to the headspace (10% total). After another 0.5 h of shaking, the headspace was sampled for initial nitrous oxide (N_2O) concentration. Assay incubations proceeded for varying lengths of time with headspace samples withdrawn after ~ 6 and ~ 24 h. After the first year, a single final time point was used for samples expected to give measurable rates in a prescribed time based on Year 1 results

N_2O was measured on a Shimadzu 8A gas chromatograph with an electron capture detector (ECD). Certified N_2O standards were analyzed with each set of headspace samples. DEA rates were calculated from rates of N_2O accumulation in the headspace and dissolved in the medium, and were standardized to organic matter in the sample (ngN g $AFDM^{-1}$ h^{-1}). Ash-free dry mass (AFDM) for each sample was determined as loss on ignition at 450°C after 4 h.

Standing stocks of organic matter (in gAFDM m^{-2}), were used to scale up the substrate-specific rates to the stream reach. Substrate types were sampled in each reach following a stratified random approach (full details at http://www.biol.vt.edu/faculty/webster/linx/linx2proto-rev5.pdf). Area of substrate types throughout the measurement reach was determined and then samples from each substrate type were collected in proportion to areal abundance. Approximately 20 samples of a given substrate type were collected throughout the reach to generate an estimate of mass representative of the reach. Coarse benthic organic matter (CBOM) and fine benthic organic matter (FBOM) were collected from a 30 cm diameter metal cylinder. CBOM was collected from the surface, then surface FBOM collected following suspension in the overlying water. After FBOM was collected the sediments were

disturbed by hand to roughly 20 cm and suspended subsurface FBOM collected. Epilithic and sediment-associated chlorophyll were estimated from hot ethanol extracts on known areas of stone surfaces or splits of the substrate samples described above. Areal DEA rates were calculated by multiplying DEA rate per unit AFDM by AFDM of a given substratum type per area (m^2) of that type in the study reach. These areal estimates are subsequently referred to as "reach-scale" rates and represent denitrification potential in a reach derived from both the activity per unit mass of each type and the standing stocks of each type in the reach.

Water chemistry samples for each stream (dissolved inorganic nitrogen, phosphate, dissolved organic carbon) were collected from six stations spaced throughout the study reach on the day before after each ^{15}N addition.
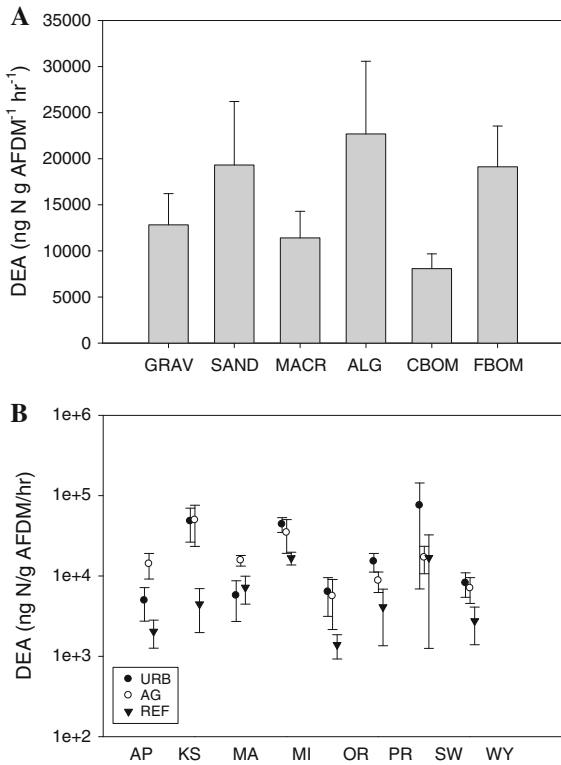
Regression analyses were used to examine relationships between variables associated with DEA (water column nitrate most obviously) and ANOVA was used to determine any differences in DEA among substrate types or land-use classes. Replicate field samples of a particular substrate type from within a stream were averaged to provide a mean, which was used in statistical analyses. All analyses were conducted using STATISTICA (Statsoft, Tulsa, OK) on log-transformed data to meet assumptions of normality.

Results

Measurable rates of N_2O production were obtained for 719 samples from 65 separate streams. Coarse sand, FBOM and CBOM, and mats of filamentous algae (ALG) were the most common substrate types analyzed. DEA rates varied by more than five orders of magnitude across individual samples with a median value of 4061 ng N g $AFDM^{-1}$ h^{-1}, and a strong right-handed skew. Rates of DEA varied almost four-fold across common substrate types (sampled in >12 streams), although differences were not significant due to high variability within streams (Fig. 2a; ANOVA, $F_{(5,57)} = 1.38$, $p = 0.24$). Sand, FBOM and epilithic algae had the highest median rates (>15,000 ng N g $AFDM^{-1}$ h^{-1}) while CBOM had the lowest (<10,000 ng N g $AFDM^{-1}$ h^{-1}).

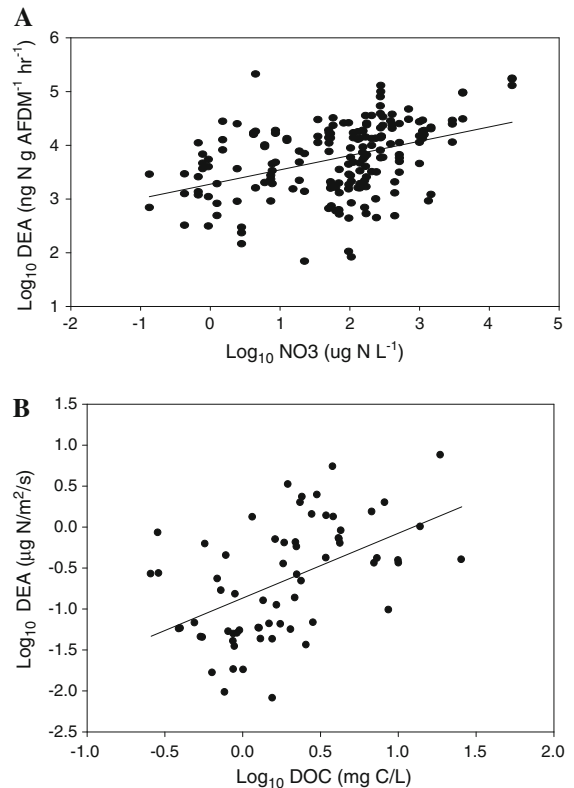To compare DEA rates across land-use and region we restricted the analysis to FBOM because it was

sampledin all 65 streams. Rates of DEA on FBOM varied significantly by land-use ($F_{(2,62)} = 9.75$; $p = 0.01$) with rates in AG and URB land-use types being almost identical and more than twice as high as in REF streams (Fig. 2b). DEA in the REF streams was consistently lower than either AG or URB streams and the land use type with highest DEA varied among regions (Fig. 2). DEA rates on FBOM varied significantly by region ($F_{(7,57)} = 7.94$, $p < 0.001$) with highest rates in KS, MI and the SW streams (Fig. 2b). Ash-free dry mass in the three most common substrate types (e.g. FBOM, algae or sand) did not vary significantly across land-use types due to high among-stream variability although the mass of FBOM in Reference streams (73 g/m^2) was only about half that of streams in Agriculture (147 g/m^2) or Urban land uses (113 g/m^2).

Potential rates of denitrification on particular substrate types were related to ambient NO_3^- concentration at the time of sample collection ($\log_{10}(DEA) = 3.277 + 0.2665*(\log_{10}NO_3^-)$; $r^2 = 0.18$, $F_{(1,62)} = 13.6$, $p < 0.001$) (Fig. 3a) suggesting that NO_3^- availability was associated with the accumulated capacity for NO_3^- removal. Total DEA at the reach scale (i.e., DEA per unit mass of patch type scaled up by standing stock in gAFDM per unit area) was weakly yet significantly ($r = 0.34$, $p < 0.05$; data not shown) associated with ambient NO_3^-, implying that other factors such as habitat extent or oxygen levels could influence reach-scale capacity for denitrification. Ambient nitrate in Reference streams was significantly lower than in either Agricultural or Urban streams which had similar concentrations (Mulholland et al. 2008). Total DEA was positively associated with stream DOC ($r = 0.5$, $p < 0.05$; Fig. 3b) suggesting ambient carbon was directly or

Fig. 2 Variation in denitrification enzyme activity (DEA) in streams by substrate type (**a**), region and land-use type (**b**). Bars in **a** represent means (+1 SE) of values for substrate types found in >12 streams. Points in **b** are means (±SE) for FBOM ($n = 65$) in (**b**). DEA did not vary significantly among substrate types (ANOVA; $F_{(5,157)} = 1.38$, $p = 0.24$), but differed significantly among land uses ($F_{(2,60)} = 4.94$; $p = 0.01$), and among regions ($F_{(7,57)} = 5.26$, $p < 0.001$)
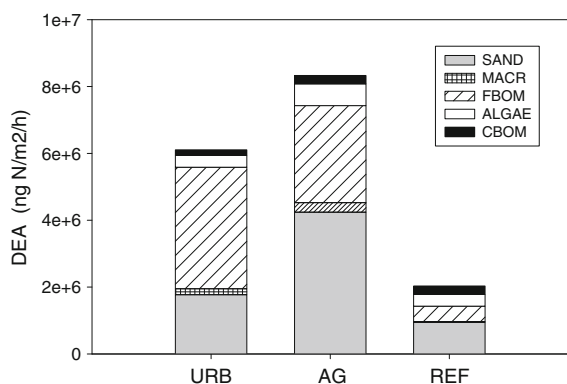

Fig. 3 **a** Relationship between ambient nitrate and DEA. Points are the means for all substrate types from all streams ($p < 0.05$, $r = 0.41$). **b** is relationship between reach-scale DEA and ambient DOC ($p < 0.05$, $r = 0.50$)

indirectly stimulating the capacity for denitrification at the reach scale. A multiple regression using NO_3 and DOC as predictors of reach-scale denitrification potential explained 31% of the variability in DEA (Log10 DEA (ug N/m^2/s) = −1.13 + 0.243 * Log10 NO_3 (ug N/L) + 0.443 * Log10 DOC (mg C/L)).

Reach-scale DEA by substrate type for each land-use was calculated using mean DEA and mean AFDM standing stocks for each substrate type in each land-use (Fig. 4). High rates of DEA per unit AFDM in samples of FBOM and sand combined with generally large reach-scale extent of these habitats resulted in these two substrate types dominating the substrate-specific contribution to reach-scale denitrification potential (values for all variables are given in Appendix Table 1). Sand was consistently the greatest contributor followed by FBOM in AG and REF streams, with algae contributing co-equally in URB streams. CBOM and macrophytes were minor contributors to denitrification potential per unit area in each class of stream.
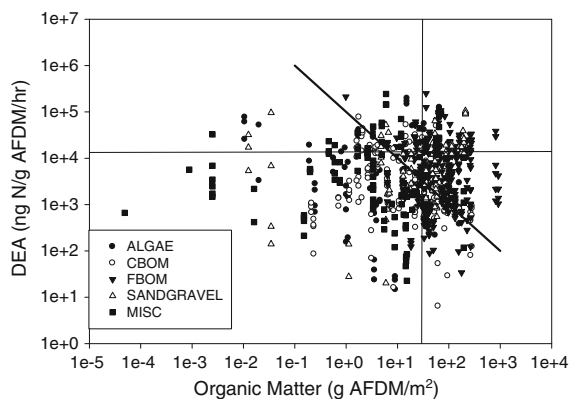
To examine the interplay between substrate-specific activity and substrate extent in contributing to denitrification potential per unit area, DEA rates per unit AFDM were plotted against mass of the substrate types (Fig. 5). This approach allows us to assess whether small but highly active pools contribute disproportionately to reach-scale denitrification potential. An isopleth of constant areal DEA (10^5 ng N m^{-2} h^{-1}) is shown for reference. Points that fall on the DEA isopleth have the same areal rate, but the relative contribution of substrate-specific rate (ng N g AFDM^{-1} h^{-1}) and quantity in terms of

AFDM (g AFDM m^{-2}) can be quite different. Points falling in the upper left quadrant (defined by the medians for each variable) would be considered "hot spots" representing high rates associated with relatively small pool sizes. In contrast, points falling in the lower right quadrant are cold patches that have low rates associated with large pools. For instance, an OM substrate with mass equal to the 25th percentile (6.25 g AFDM/m^2) matched with a DEA of 16,000 ng N/g AFDM/h (slightly above the 75th percentile) falls on the isopleths and might fairly be considered a "hot spot". Conversely, an OM pool with mass equal to the 75th percentile (73.7 g AFDM/m^2) matched with a DEA of 1357 ngN/g AFDM/h (slightly above the 25th percentile) also falls on the isopleths and would represent the cold matrix. Obviously, about one-quarter of OM substrate samples analyzed were either hotter or colder than these particular examples and so both hot and cold types are reasonably common in streams we studies. There was not a distinct aggregation of points in the upper left quadrant as would be expected if "hot spots" dominated the estimates of reach-scale DEA. Substrate types within a stream with relatively low rates of DEA can "compensate" with large mass to contribute significantly to reach-scale denitrification potential.

In these streams, FBOM is generally the substrate type with the largest pool of organic matter, as the median mass of FBOM (58 g AFDM m^{-2}) exceeds the grand median mass (34 g AFDM m^{-2}). On the activity axis however, the median activity for FBOM (4231 ng N g AFDM^{-1} h^{-1}) was almost exactly the

Fig. 4 Stacked bar showing contribution of different substrate types to reach-scale denitrification for the three land-use classes

Fig. 5 Scatterplot of DEA per unit organic matter versus quantity of organic matter per m^2. Vertical and horizontal lines mark the medians. The dashed line is an isopleth of constant areal denitrification (100,000 ng N m^{-2} h^{-1})

grand median (4061 ng N g AFDM^{-1} h^{-1}), indicating that this substrate type does not necessarily support uniformly high rates of DEA. Mass of epilithic or filamentous algae were generally low in abundance, with median mass (0.7 g AFDM m^{-2}) much lower than the grand median. Whereas the AFDM of substrate types showed clear clustering by type across the X-axis, most DEA rates were fairly evenly distributed above and below the median activity. This implies that a particular substrate type is not inherently "hot" or "cold". For example, the median activities for ALG and CBOM were 5045 ng N g AFDM^{-1} h^{-1} and 2480 ng N g AFDM^{-1} h^{-1}, which narrowly brackets the grand median of 4061 ng N g AFDM^{-1} h^{-1}. Thus, the variation along the mass axis can be separated into distinct substrate types and their respective masses. Along the activity axis however, it seems any substrate type may exhibit either high or low DEA rates. This pattern suggests the environmental conditions in which a particular substratum occurs are more important than its identity in accounting for variation in denitrification potential.

For each stream we compare the reach-scale DEA (rate/g AFDM × substrate-weighted g AFDM/m^2) with the reach-scale ^{15}N gas production from ^{15}N–NO$_3^-$ tracer additions (Mulholland et al. 2009) to see how closely streams might be approaching their maximum potential for NO$_3^-$ removal via denitrification. Reach-scale estimates of DEA [note new units to match calculation of U$_{den}$ below] ranged widely from <0.04 to nearly 14.9 ug N m^{-2} s^{-1}, with a pronounced skew and median value of 0.9 mg N m^{-2} s^{-1} (Fig. 6) As expected, measured ^{15}N gas production (uptake due to denitrification (U$_{den}$) see Mulholland et al. 2008)) had a lower median and maximum (0.6 and 9 mg N m^{-2} h^{-1} respectively). There was no correlation between potential (DEA) and realized rates for all streams or when separated by land-use. Rates of ^{15}N gas production in 23 of the 72 streams could not be estimated because of poor model fits (Mulholland et al. 2009). Nonetheless, the surprising feature of the comparison is that, with the exception of two high DEA rates (Michigan AG 7.9 and SW URB 14.9 ug N m^{-2} s^{-1}), the potential and realized reach-scale rates could be plotted at the same scale for 45 streams with valid in situ estimates and a complete set of DEA assays. The overall median of the ratio between realized and potential rates was 0.42 meaning that half of the streams with useable estimates had realized rates at least 40% as large as their potential rates.
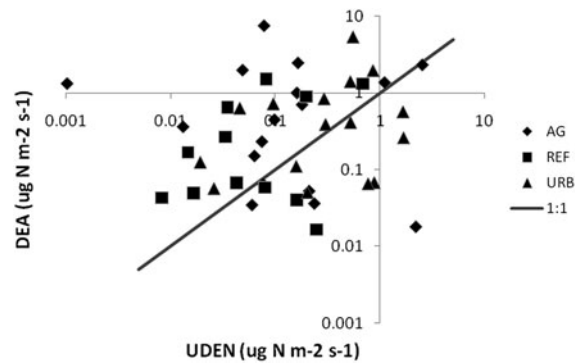
Fig. 6 Potential (DEA) and realized (UDEN) rates of denitrification across 45 streams. Two values for DEA potential fall outside the scale for this plot. *Note*—units for DEA have been changed for direct comparison with UDEN

Discussion

Capacity of streams to denitrify

Streams are well-established as important locations of transformation of materials transported from the land (Bernhardt et al. 2005; Battin et al. 2008). Most previous considerations of stream characteristics affecting their capability to alter the form or quantity of solutes delivered downstream have focused on physical attributes such as mean depth, stream order or presence of a significant hyporheic zone (e.g. Alexander et al. 2000). The land-use setting of streams, and/or all the potential factors that are associated with varying land-use can influence a stream's capacity to retain or transform materials in transport (Frost et al. 2009; Grimm et al. 2005; Johnson et al. 1997; Royer et al. 2004). Our results can address questions about which stream-specific habitats are contributing most to a particularly important transformative process, the potential for denitrification capacity.

Denitrification enzyme assays are designed to determine the potential of a given sample under ideal conditions of low oxygen, high NO$_3^-$ and abundant carbon. Chloramphenicol is added to ensure that the denitrification enzyme "status" of the original sample is contributing to the measured rates and documentation of linear time courses ensures the micro-organisms are neither proliferating nor shutting down. The "ideal" conditions under which our measurements were made suggest that the DEA approach should

dramatically over-estimate the actual rates of denitrification observed in situ yet this appears not to be the case. In a review of aquatic denitrification rates, Piña-Ochoa and Álvarez-Cobelas (2006) show (their Fig. 2) a median rate of denitrification for rivers of $\sim$100 μmol m^{-2} h^{-1} (25th and 75th quartiles of $\sim$50 and 400). The reach-scale mean DEA potentials for our three land-use types range from 140 to 700 μmol m^{-2} h^{-1} indicating our DEA rates were within or close to the literature range of estimates intended to represent denitrification under ambient conditions.

For the streams examined in this study, our reach-scale DEA rates were often comparable to, and broadly overlap, the realized rates measured from the ^{15}N-NO$_3^-$ tracer additions conducted as part of the larger LINXII study (Fig. 6). For streams below the 1:1 line, reach-scale DEA exceeds ^{15}N gas production for one of several reasons. For these streams there is some unrealized capacity that was not contributing to ^{15}N gas production at the time of field measurement. For instance, there may well be numerous substrates with potential to denitrify, but these will not all be concurrently active under a given set of environmental conditions. Secondly, the ^{15}N gas measurement will underestimate actual denitrification to some degree because any coupled denitrification (supported by in situ nitrification) is not included in these short-term measurements (see discussion in Mulholland et al. 2009). Two-thirds of the study streams have ambient NO$_3^-$ <300 ug N L^{-1}, which is low enough that coupled denitrification may be significant (Seitzinger et al. 2006); therefore, the ^{15}N gas approach could underestimate true rates, exacerbating the difference between potential and measured rates. Thirdly, the ^{15}N gas measurements were generally conducted at summer baseflow while the DEA assay represents the accumulation of denitrification capacity that may arise under conditions at other times of the year. If, for example, autotrophic-heterotrophic competition for NO$_3^-$ is intense during summer low flows, the enzymatic capability to denitrify may have accumulated during more NO$_3^-$-rich times of year but is not realized during times of high algal demand.

In contrast, streams on or about the 1:1 line (Fig. 6) are dentirifiying at rates close to their maximum potential. In streams where potential and realized rates were similar, any additional NO$_3^-$ load will not be removed via denitrification. Streams in urban land-use appear more likely to be performing at their potential compared to streams in either REF or AG settings. The median ratio of DEA:realized estimates of reach-scale denitrification for urban sites was 1.03 while the medians for AG and REF streams were 2.4 and 3.7 respectively, although not significantly different by ANOVA. Half the urban streams are performing at their potential while this proportion is lower for streams in the other two land use classes. Several measures of stream metabolism have been useful in predicting realized denitrification rates (Mulholland et al. 2008; Mulholland et al. 2009) but neither GPP nor community respiration of ambient nitrate were related to the DEA:Uden ratios. This inability to account for variation in DEA:Uden is perhaps not surprising since one of the major differences in these two estimates is the inherent time-scale. DEA captures potential activity that may have accumulated over some prior (unknown) interval while the ^{15}N approach can only measure denitrification during the time and conditions represented during the 24 h ^{15}N–NO$_3$ addition. This mismatch in time-scale allows quite different environmental factors or events to constrain the potential versus realized denitrification in a stream.

It seems incongruous that realized denitrification can exceed potential denitrification measured via DEA, but there are at least two possible explanations. The easiest (although hard to refute) is that the field sampling for DEA analysis missed some particularly important substrate type which would obviously contribute to realized denitrification but would not be included in the reach-scale DEA estimate. In general this is unlikely to have occurred for any surface substrate types, as they were selected based on measurements of their reach-scale predominance in each given stream. Each site provided data on total organic matter that had not been assayed for DEA activity. We calculated the ratio of OM mass assayed for DEA to the total OM mass and found that our assays included on average 59% of the total organic mass. Thus, we feel the worst case is that sampling missed 41% of the organic matter present in the stream reach. However, these estimates include for instance deep sediment and large wood that would be unlikely to contribute to the short-term ^{15}N gas-based estimate of reach-scale denitrification. Moreover, there was not a negative relationship between the discrepancy between realized and DEA reach-scale estimates and

mass of "unassayed" organic matter (data not shown). These facts imply our DEA analyses did not miss major substrate types capable of contributing significantly to reach scale denitrification.

Another explanation for roughly equal rates of realized and potential denitrification (particularly in urban streams) is that these streams are actually performing at peak capacity and the set of factors controlling realized denitrification are all close to optimal levels. For instance, NO_3^- concentrations in urban streams were high and may not be limiting to in situ denitrification. Perhaps carbon supply is also high enough during the sampling periods to generate demand for alternate electron acceptors and draw down oxygen levels in key sites of denitrification (Schaller et al. 2004). One implication of this explanation is that these conditions or locations of high carbon supply and low oxygen are more extensive or frequent in urban streams.

Contribution of particular substrate types

Since the process of denitrification requires the coincidence of NO_3^- supply, low O_2 and availability of organic matter, much previous attention has logically been focused on areas of high organic matter storage and limited water exchange. Streams draining areas of intensive agricultural production tend to have high NO_3^- loads and would be prime targets for management efforts to increase their denitrification capacity. Sandy sediments and accumulations of FBOM were the predominant areas contributing to reach-scale DEA capacity and Arango et al. (2007) found that the organic content of sediments was a powerful predictor of in situ denitrification activity when NO_3^- concentrations were above half-saturation. The sandy sediments apparently limit O_2 penetration while retaining sufficient organic matter to support high rates of denitrification.

Urban streams had the greatest contribution to denitrification potential by algal-dominated substrate types, which were co-equal with rates in FBOM accumulations. Algae themselves are not responsible for the DEA, but rather they represent a site of enhanced organic matter availability and potentially high heterotrophic respiratory demand by an associated microbial community. Carbon trapped by algal filaments or senescent portions of algal mats may be good microsites for supporting night-time

denitrification despite the oxygen production during times of rapid photosynthesis.

In forested headwater streams, debris accumulations have been targeted as harboring the conditions most conducive to denitrification (e.g. Steinhart et al. 2000). Our results, however, do not show high DEA potential in CBOM, nor did most reference streams have a large enough standing stock of CBOM to make a significant contribution to reach-scale denitrification potential. Even if we assumed all FBOM were associated with CBOM in retention structures (see Fig. 4) the summed rates would still be about the same as DEA contributed by the Sand habitat. Samples for our measurements were primarily conducted during summer and early autumn, which are likely times of minimal CBOM accumulation in most stream types. In streams lacking the input of large wood necessary for accumulations of CBOM to persist throughout the year, other habitats will probably be the major contributors to reach-scale denitrification. However, low ambient nitrate concentrations or lack of readily available organic carbon may limit the overall capacity of forested streams to denitrify relative to streams in agricultural or urban settings.

Our results reveal two habitats (sand and filamentous or epilithic algae) that make significant contributions to denitrification potential but probably attain necessary environmental conditions through different mechanisms. Sandy sediments allow hyporheic exchange delivering NO_3^- to shallow subsurface regions of potentially low oxygen (Triska et al. 1989; Sheibley et al. 2003; Arango and Tank 2008) and burial of new organic matter may be rapid. The balance here is between sufficiently rapid water replenishment to provide NO_3^- while maintaining low ambient oxygen levels. In algal mats or thick epilithon, the combination of heterotrophic and night-time autotrophic respiration probably depletes O_2 in microsites with abundant labile organic carbon. Since most algal-dominated habitats will have high O_2 levels during daylight hours, these have not been widely considered likely locations for denitrification (but see Kemp and Dodds 2002, Schaller et al. 2004). Our results imply there is rapid switching to NO_3^- as an electron acceptor at times or places when oxygen is drawn down. The accumulation of a significant potential for denitrification in algal mats suggests this is an important pathway of metabolism in a putatively

aerobic microenvironment. Additionally, nitrification during daytime may supply NO_3^- that supports denitrification and maintains high potentials.

Places (or times) where all three conditions conducive to denitrification co-occur have been considered to be fairly rare, leading to the presumption that denitrification will be a process dominated by "hot spots" or "hot moments" (McClain et al. 2003). In our study we found that while hot spots clearly exist, it was quite feasible to generate the same denitrification potential by the combination of much lower rates occurring in large pools of organic matter (see Groffman et al. 2009). From a practical point of view, this means that stream restoration or other management efforts intended to increase NO_3^- removal via denitrification capability must consider whether commonly manipulated units (Craig et al. 2008) are actually effective in a given setting. Stream habitats such as fine sand that are often considered poor for some larger benthic invertebrates and fishes may in fact be providing an important ecosystem service. Conversely, if debris accumulations are important in whole-system denitrification then adding debris dams to improve fish habitat will also help water quality.

Nitrate influence on DEA

We found a significant, positive relationship between denitrification potential in FBOM samples and ambient stream NO_3^- concentrations although explanatory power is fairly low. Our results suggest that streams with high NO_3^- levels have accumulated the capacity for denitrification and so there must be sufficient area or frequency of low oxygen and high carbon to support the micro-organisms capable of denitrification. The positive association also suggests sufficient demand for alternate electron acceptors such that micro-organisms with the ability to use NO_3^- comprise a significant proportion of heterotrophic metabolism. The actual shape of the relationship between capacity to denitrify and NO_3^- availability is central to understanding the overall potential of streams to moderate increased NO_3^- delivery. Mulholland et al. (2008) found a declining capacity for N removal as NO_3^- loading increased along a stream continuum. Therefore, streams have decreasing capability to control downstream delivery of NO_3^-.

The relationship we found between DEA and ambient NO_3^- does not flatten at higher ambient NO_3^- levels implying that the decreased capacity relative to load in these streams was not due to loss of potential for denitrification. The physiological capacity appears capable of increase as nitrate increases. More likely, one of the other controls on denitrification—labile carbon availability of low-O_2 sites, or hydrologic delivery of NO_3^- to sites of denitrification—was not increasing as rapidly (if at all) as external NO_3^- loadings (Garcia-Ruiz et al. 1998). We did find that ambient DOC was positively associated with denitrification potential suggesting that carbon supply may be significant. Thus, some streams may become limited by the opportunity to carry out denitrification at high rates rather than inadequate capacity. This opportunity is affected by presence of suitable low oxygen sites and/or carbon supply in excess of capacity for aerobic respiration.

Overall our findings show that land-use influences stream denitrification potential most likely through provision of high NO_3^- concentrations in urban and agricultural streams, although land use can also influence relative dominance of types and amounts of physical and organic substrata found in streams. At a finer scale, a wide array of stream substrate types contribute to reach-scale denitrification, including some types of substrates not widely considered to date (e.g., algal mats). The process is not necessarily dominated by spatially restricted spots within a stream but may be carried out by microbes at lower rates in larger substrate pools such as sand. Lastly there is a suggestion that some streams are functioning near their denitrification potential and so would be incapable of moderating a further increase in NO_3^- load. Given our findings of fairly high potential for denitrification under ideal conditions and realized rates that are often of the same magnitude, these systems are apparently functioning at some nontrivial proportion of their maximal performance at time of sampling. If we consider performance as comprised of two necessary aspects, opportunity and capacity, then our results suggest that many streams have substrate types with reasonably high capacity for denitrification. In agricultural and urban streams the opportunity for high reach-scale denitrification is realized because of their high NO_3^- availability, an abundant carbon supply and suitable locations.

Appendix

Table 1 Values for DEA (ng N g AFDM^{-1}) and organic mass associated with each of the five most common substrate types (g AFDM m^{-2}) broken out by land-use type

Land-use	Substrate	Mass (g AFDM m^{-2})	DEA (ngN g AFDM^{-1} h^{-1})	Areal DEA (ugN m^{-2} h^{-1})	% of overall
Urban	Sand	100.5	17,605	1,769	28.9
	Macro	17.2	10,554	182	3.0
	FBOM	125.9	28,870	3,634	59.5
	Algae	14.6	24,218	354	5.8
	CBOM	12.8	12,928	166	2.7
Ag	Sand	125.2	33,858	4,238	50.1
	Macro	20.3	14,105	286	3.4
	FBOM	139.9	20,752	2,903	34.9
	Algae	22.3	29,205	651	7.8
	CBOM	27.5	9,093	250	3.0
Ref	Sand	82.8	11,498	953	46.9
	Macro	14.4	325	5	0.2
	FBOM	65.2	7,219	471	23.2
	Algae	44.4	7,862	349	17.2
	CBOM	73.2	3,462	253	12.5

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