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Co-occurrence of in-stream nitrogen fixation and denitrification across a nitrogen gradient in a western U.S. watershed

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Abstract It is frequently assumed that nitrogen (N_2) fixation and denitrification do not co-occur in streams because each process should be favored under different concentrations of dissolved inorganic nitrogen (DIN), and therefore these processes are rarely quantified together. We asked if these processes could coexist by conducting a spatial survey of N_2 fixation using acetylene reduction and denitrification using acetylene block [with and without amendments of carbon (C) as glucose and nitrogen (N) as nitrate]. Rates were measured on rocks and sediment in 8 southeastern Idaho streams encompassing a DIN gradient of 26–615 μg L^{-1}. Sampling at each site was repeated in summer 2015 and 2016. We found that both denitrification and N_2 fixation occurred across the gradient of DIN concentrations, with N_2 fixation occurring primarily on rocks and denitrification occurring in sediment. N_2 fixation rates on rocks significantly decreased 100× across the DIN gradient in 1 year of the study, and amended (with N and C) denitrification rates increased 10× across the DIN gradient in both years. Multiple linear regression and partial least squares models with environmental characteristics measured at the scale of entire stream reaches showed that C and phosphorus were positive predictors of amended and unamended denitrification rates, but no significant model could explain N_2 fixation rates across all streams and years. This, coupled with the observation that detectable rates of N_2 fixation occurred primarily on rocks and denitrification occurred primarily on sediment, suggests that microhabitat scale factors may better predict the co-occurrence of these processes within stream reaches. Overlooking the potential co-occurrence of N_2 fixation and denitrification in stream ecosystems will impede understanding by oversimplifying the contribution of each process to the N cycle.

Keywords Nitrogen fixation · Denitrification · Dissolved inorganic nitrogen · Streams

Introduction

Denitrification and N_2 fixation are both important processes that control net N_2 fluxes in many aquatic

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ecosystems (Fulweiler and Heiss 2014). Despite this fact, both processes are rarely studied together in streams because different factors favor high rates of each process (An et al. 2001; Marcarelli et al. 2008, but see Dodds and Castenholz 1988). N_2 fixation is most often studied in streams with conditions suitable for photosynthetic N_2 fixers (e.g., high light availability, warm temperatures, low N and variable phosphorus (P) availability; Scott and Marcarelli 2012), while denitrification is studied in streams where sediments have high organic matter content and anoxic conditions (Groffman et al. 2009; Arango et al. 2007). The factor that differs the most between the two processes is their dissolved inorganic nitrogen (DIN) requirement. N_2 fixation is thought to occur in low DIN environments because N_2 fixation has significant energy costs to the organism, and because available DIN inhibits the production of heterocytes (Flores and Herrero 2010), such that N_2 fixation rates decrease when N availability is high (Grimm and Petrone 1997; Kunza and Hall 2013), whereas denitrification requires higher concentrations of DIN to use as an oxidant (Knowles 1982). This contrast in DIN requirements between the two processes has led to the assumption that as rates of one process increase, the other process will cease.

This assumption has led to bias in the study and understanding of the full N cycle in stream ecosystems. There have been numerous studies on denitrification because it is a critical process regulating the removal of N from natural and human-altered aquatic ecosystems (Seitzinger et al. 2006). Far less research into N_2 fixation has been performed because several studies suggested N_2 fixation rarely contributed $> 5\%$ of the N input into a stream (Marcarelli et al. 2008). Similarly, in oceans it was long thought the major component of the N cycle was denitrification occurring in oxygen-depleted waters and sediments, while N_2 fixation was only a minor part of the cycle occurring mostly in the open ocean (Capone 2001; Fernandez et al. 2011). This idea was challenged through discoveries such as nitrate and phosphate patterns in mid-oceans that pointed towards N_2 fixation (Macko et al. 1984; Capone 2001) and low ^{15}N signatures in surface waters that indicated more widespread N_2 fixation activity (Brandes et al. 1998; Capone 2001). Now research has shown that N_2 fixation can occur in marine waters where denitrification occurs despite the different requirements for each process (Fernandez et al. 2011) because the removal of N in denitrification zones can be tied to the occurrence of N_2 fixation (Deutsch et al. 2007). This revolution in the understanding of N dynamics in marine environments is an indication that a better understanding of these processes is needed in freshwaters as well.

The co-occurrence of both N_2 fixation and denitrification in streams could be affected by the loads and ratio of N and P concentrations (N:P). In lakes, when N:P were low, N_2 fixing cyanobacteria dominate an otherwise nitrogen-limited phytoplankton community and at higher N:P low proportions of N_2 fixing cyanobacteria occur (Smith 1983). In low N:P environments it was thought that the production of nitrogen by N_2 fixing cyanobacteria could offset N limitation (Schindler 1977), and some studies have suggested that N produced by N_2 fixers was sufficient to shift whole lakes to P-limitation over relatively short time scales (Schindler et al. 2008). Yet, others have argued that N produced by cyanobacterial N_2 fixers does not fully offset N deficiency in many cases (Lewis and Wurtsbaugh 2008; Scott and McCarthy 2010), because high denitrification rates remove fixed N faster than it is produced (Paerl and Scott 2010; Scott and Grantz 2013; Paerl et al. 2016). If so, this could result in co-occurrence of denitrification and N_2 fixation in lakes even when external nutrient loads are high (Scott and Grantz 2013), leading to perpetual N limitation or co-limitation by N and P, which would allow high rates of N_2 fixation to occur across a gradient of reactive N loads (Lewis and Wurtsbaugh 2008; Paerl and Scott 2010). Therefore both processes could occur in a stream even if the overall N load may appear favorable for one process over the other.

The co-occurrence of both N_2 fixation and denitrification in streams could also be facilitated by other key environmental variables. High availability of light and warm temperatures are favorable for cyanobacterial N_2 fixers (Grimm and Petrone 1997; Scott and Marcarelli 2012). Denitrifying bacteria, while not directly controlled by light, are affected by anoxia and organic matter availability (Holmes et al. 1996; Groffman et al. 2005; Arango et al. 2007). Streams may vary with respect to these factors along a reach or in habitats within reaches, potentially creating preferable habitats for both types of organisms in the same stream (Holmes et al. 1996; Dent and Grimm 1999). Thus, overall environmental heterogeneity may create variation in conditions within stream reaches that facilitate the co-occurrence of both N_2 fixation and denitrification. Despite advances in understanding how and where N_2 fixation and denitrification co-occur in other aquatic ecosystems (e.g., Deutsch et al. 2007; Newell et al. 2016), there have been only limited efforts to examine the possible co-occurrence of the two processes in stream ecosystems.

The goal of this study was to evaluate whether or how N_2 fixation and denitrification co-occur in stream ecosystems across a gradient of DIN concentrations and how the co-occurrence could be facilitated by key environmental variables. First, we hypothesized that rates of denitrification and N_2 fixation would co-occur in streams of varying DIN concentrations. Second, we hypothesized that this co-existence of processes would be facilitated by different process rates between substratum type within streams, with higher rates of N_2 fixation on rocks, which provide stable, high light habitats for photosynthetic N_2 fixers, and higher rates of denitrification in sediment, where anoxia is likely and organic matter availability should be high. Third, we hypothesized that streams with mid-range DIN concentrations would have intermediate rates of both N_2 fixation and denitrification, while streams with high DIN would have higher rates of denitrification and streams with low DIN would have higher rates of N_2 fixation. Finally, we examined whether environmental variables such as light, temperature, chlorophyll *a*, organic matter, discharge, P, dissolved organic carbon (DOC), and N:P influenced rates of both processes. We hypothesized that streams with more light, higher temperatures, and lower DIN concentrations would exhibit higher rates of N_2 fixation, whereas streams with more organic matter and higher DIN concentrations would favor higher rates of denitrification. Evaluating these hypotheses has the potential to challenge the existing paradigm that N_2 fixation and denitrification are mutually exclusive processes and therefore transform our current understanding of N cycling in streams.

Methods

Study area

This study was conducted in the Portneuf River watershed, located near Pocatello, Idaho, which drains a 3445 km^2 basin (elevation 1330–2823 m.a.s.l). The watershed is located in a semi-arid region that receives approximately 30 cm of rainfall annually, so the river is dependent on the underlying aquifer and snowmelt runoff from surrounding mountains for water (Minshall and Andrews 1973). The annual mean discharge of the Portneuf River measured at Pocatello ranged from 3.7 to 9.7 $m^3\ s^{-1}$ over the last 10 years (USGS Water Resources, Station 13075500). The Portneuf River begins as a series of mountain streams that flow down into valleys that have bedrock geology of both basalt and sedimentary rock with sediments that consist of loess, silt, and volcanic ash (Barton 2004; Hopkins et al. 2011). Sub-watersheds have $> 16\%$ of their surface area as volcanic rock with the highest being 46.5% (Table 1). The river passes through many agricultural and pasture areas that results in land use and irrigation impacts in this basin that are typical of watersheds in the western United States (Marcarelli et al. 2010; Bechtold et al. 2012). Overall watershed land use is dominated by agriculture, primarily grazing (56% of land area) and crop and pasture (22% combined). Forest cover occurs mostly at higher elevations (17%), while urban areas make up less than 4% of the watershed area (Bechtold et al. 2012). The spatial heterogeneity of geological formations and land use in this watershed cause the streams to encompass a wide range of N and P concentrations (Table 2).

Study design

We measured rates of N_2 fixation and denitrification in 8 streams that were selected in 2015 to encompass a gradient of DIN concentrations (26–581 $\mu g\ L^{-1}$ DIN) and variance in N:P by mass (1.13–102.6) based on prior studies (Bechtold et al. 2012 and Marcarelli et al. unpublished, Tables 1, 2) to determine whether N_2 fixation and denitrification co-occur. Periphyton should be strictly N limited below a N:P ratio of 9 by mass (Paerl et al. 2016). We chose 6 locations on tributary streams: Lower Mink Creek, South Fork Mink Creek, West Fork Mink Creek, Cherry Springs, Pebble Creek, and Rapid Creek, as well as one mainstem location: the Upper Portneuf River. In 2016, we added one additional site at Diggie Creek to expand the DIN gradient of streams included in our study (615 $\mu g\ L^{-1}$ DIN) and due to the high abundance and large size of the cyanobacterial colonies in this stream.

Table 1 Watershed and geological characteristics for the 8 sampling streams sorted from low to high dissolved inorganic nitrogen (DIN) concentrations measured in 2015

Stream	Drainage area (km^2)	Relief (m)	Forest cover (%)	Ag cover (%)	Mean basin slope (%)	M.A.P (cm)	Area as volcanic surficial rock (%)	Primary	Secondary	Tertiary	Age	GPS coordinates (N, W)
Pebble Creek	76.43	1183	40	0.00	30	69.3	17.4	Sandstone	Conglomerate	Siltstone, limestone	Pliocene	42.73602, – 112.02116
Cherry Springs	99.02	1146	30	0.22	30	66.3	19.3	Limestone	Dolomite	Shale, arenite	Ordovician, Cambrian	42.74916, – 112.39408
South Fork	22.97	1000	36	0.78	22	64.3	46.5	Alluvium	Alluvial fan	Alluvial terrace	Quaternary	42.70815, – 112.42272
Rapid Creek	144.62	1073	19	3.46	29	48.0	10.8	Tholeiite	Lava flow	Gravel	Late Pleistocene	42.48094, – 112.124922
West Fork	17.28	546	50	0.00	30	74.7	24.4	Limestone	Dolomite	Shale, arenite	Ordovician, Cambrian	42.72455, – 112.42553
Lower Mink	125.95	1283	24	0.61	31	63.8	16.7	Sandstone	Conglomerate	Siltstone, limestone	Pliocene	42.79824, – 122.38409
Upper Portneuf	1240.16	1244	11	31.2	16	48.5	24.2	Alluvium	Alluvial fan	Alluvial terrace	Quaternary	42.70999, – 112.01431
Diggie Creek	2.72	128	0	12.3	6	25.4	65.7	Alluvium	Alluvial terrace	Floodplain	Quaternary	43.53680, – 112.31897

Ag is drainage area that is cultivated agriculture, and M.A.P. is mean annual precipitation. The type and age of rock (columns titled Primary, Secondary, and Tertiary) found at each site was determined from the USGS mineral resources online spatial data program (http://mrdata.usgs.gov/) and is specific to each location in the stream, not the whole watershed. All other characteristics were determined from the USGS StreamStats program for the whole watershed upstream of the sampling point (http://water.usgs.gov/osw/streamstats)

Table 2 Environmental characteristics for the eight sampling streams collected from site surveys in 2015 and 2016

Year	Stream	NO_3^- ($\mu g\ L^{-1}$)	NH_4^+ ($\mu g\ L^{-1}$)	DIN ($\mu g\ L^{-1}$)	TDN ($\mu g\ L^{-1}$)	TDP ($\mu g\ L^{-1}$)	DIN:TDP	DOC (mg L^{-1})	Discharge (L/s)	Canopy cover (%)	Average temp. (°C)
2015	Pebble Creek	20.0	6.44	26.44	130.0	8.8	3.00	2.35	24.7	61.6	14.8
	Cherry Springs	20.0	6.91	26.91	120.0	19.9	1.35	1.86	80.3	78.2	17.7
	South Fork	20.0	12.50	32.50	110.0	28.8	1.13	1.70	11.6	75.8	15.0
	Rapid Creek	106.1	4.01	110.1	200.0	28.8	3.83	2.63	121.3	26.5	15.6
	West Fork	232.3	4.41	236.7	150.0	15.5	15.30	1.29	33.0	70.1	14.5
	Lower Mink	284.8	13.56	298.4	390.0	27.7	10.80	2.30	100.6	33.5	17.5
	Upper Portneuf	568.7	13.07	581.7	720.0	28.7	20.20	5.75	3254.5	0.0	17.9
2016	Pebble Creek	57.1	8.39	65.5	160.0	3.5	19.00	1.74	182.3	68.4	16.3
	Cherry Springs	92.9	4.85	97.7	210.0	12.5	7.90	2.33	107.1	97.4	19.3
	South Fork	32.6	8.31	40.9	160.0	16.2	2.52	2.41	14.6	84.7	18.3
	Rapid Creek	182.6	1.00	183.6	320.0	27.0	6.76	2.63	43.9	41.2	19.4
	West Fork	166.1	2.94	169.0	200.0	16.8	10.05	1.47	47.2	31.3	15.4
	Lower Mink	162.0	6.79	168.8	320.0	10.5	16.15	2.37	74.7	36.4	18.8
	Upper Portneuf	503.1	2.94	506.0	800.0	20.0	25.29	3.87	2929.5	0.0	20.2
	Diggie Creek	612.3	2.94	615.2	990.0	6.0	102.61	1.81	N/A	0.0	18.6

DOC dissolved organic carbon, *TDP* total dissolved phosphorus, *TDN* total dissolved nitrogen concentrations, *BDL* concentrations below the detection limits of the nutrient analysis (for NH_4^+ the average detection limit was 2.0 μg L^{-1} and for NO_3^- the average detection limit was 40 μg L^{-1} in 2015 and 1.0 μg L^{-1} in 2016)

In summer 2015, each site was visited once and rates of N_2 fixation and denitrification were measured on the same day. In 2015, N_2 fixation was only measured on rock substrata and denitrification was only measured on sediment substrata because we chose the substratum that was most likely to be favorable for each process. This sampling procedure did not encompass the full dynamic of the two processes required to test our second hypothesis, and thus we expanded in 2016 to measure both rates on both rock and sediment substrata. In 2016, each site was visited 2 days in a row, such that N_2 fixation or denitrification were measured on separate days. In 2016, we also measured rates on macrophytes at the Upper Portneuf River site only because macrophytes were a dominant substratum at this site.

N_2 fixation and denitrification rates were measured by acetylene reduction and acetylene block respectively. Chambers used for these techniques varied by substratum type. 2-L polycarbonate food storage containers were used for rocks and macrophytes (Gettel et al. 2007). The chamber lids were sealed airtight with a Viton o-ring, and lids were fit with a 13 × 20 mm septa for sample collection. For sediment, chambers were made from quart size glass mason jars in 2015 and pint size glass mason jars in 2016, and lids were similarly fit with an airtight sampling septa.

Rock substratum was collected by haphazardly sampling rocks from the study area and placing them in the polycarbonate chamber until its bottom was covered. Sediment substratum was collected haphazardly from sediment patches within each stream using a 7 cm diameter suction corer to collect ~ 200 mL of sediment that was then placed into the mason jars. Macrophytes were collected using the 2-L polycarbonate chamber lid to approximate surface area of macrophyte to sample, then pulling from the root and placing in chambers. N_2 fixation and denitrification rates were measured mid-day during peak hours of sunlight.

N_2 fixation

N_2 fixation rates were measured using acetylene reduction (Capone 1993; Dodds et al. 2017). An acetylene-filled balloon was added to the 6 sample chambers and 3 blank chambers. The 3 blanks were set up to simulate an environment with no possible N_2 fixing or denitrifying taxa to control for chamber effects. Materials used for the blanks were selected based on their relative specific heats to mimic the specific heats of incubated substrata in order to correct for changes in temperature. Rocks found on the shore near the stream were used for blanks for stream rocks, and streamwater was used as a blank for sediment and macrophyte substrata. The sample chambers had stream rock, sediment, or macrophyte placed in them. Chambers were filled with streamwater and sealed underwater, then balloons were popped with a needle through the sampling septum to introduce an acetylene headspace equal to 20% of the total chamber volume. Chambers were then shaken for approximately 20 s to equilibrate the gas dissolved in the water with that in the headspace. Initial gas samples were collected within 5 min of sealing the chambers. Chambers were placed in the stream for a 2-h incubation to maintain ambient stream temperatures. Chambers were shaken again to equilibrate and then final samples were collected. All gas samples were placed into evacuated 9-mL serum vials and kept in the dark until analyzed. Ethylene concentrations were measured using a SRI 8610C gas chromatograph equipped with a Hayesep T column, He carrier gas, and a flame ionization detector. The column oven was set to 40 °C. To obtain N_2 fixation rates, ethylene concentrations in the chambers were compared to 100 ppm ethylene standards (Matheson Tri Gas). N_2 fixation rates were calculated following Capone (1993) and Dodds et al. (2017), then converted to µg of N assuming a ratio of 3 mol of ethylene produced for every 1 mol of N_2 gas potentially fixed (Capone 1993).

Denitrification

Denitrification rates were measured using the acetylene block method (Groffman et al. 2006). Chloramphenicol was used to suppress additional protein synthesis during the incubation in all chambers. We measured rates with and without amendments of N and C to capture rates when optimal conditions were present and to insure comparability to many other studies in streams that have used nutrient amendments. Moreover, the acetylene block method also inhibits nitrification, which produces nitrate, so measuring without amendments of limiting nutrients can underestimate denitrification rates (Dodds et al. 2017). However, these measurements are not true estimates of potential denitrification since the chambers were not sparged to create a complete anoxic environment prior to sampling. We also measured unamended rates to determine if high denitrification rates could occur under suboptimal conditions. Three sample chambers were randomly chosen to remain unamended and received chloramphenicol only (2 g L^{-1}), and 3 chambers were chosen to be amended and received 0.62 g L^{-1} Glucose as a C source and 0.62 g L^{-1} $NaNO_3$ as an N source, plus chloramphenicol. After the amendment, acetylene was introduced, chambers were incubated, and initial and final gas samples were collected as described previously for N_2 fixation. Nitrous oxide (N_2O) concentrations were measured using a SRI 8610C gas chromatograph equipped with a Hayesep D column, He carrier gas, and an electron capture detector. The column oven was set to 80 °C. N_2O concentrations in chambers were compared to standard concentrations of 1000 ppm N_2O (Matheson

Tri Gas). Denitrification rates were calculated following Dodds et al. (2017).

Substratum analysis

All substratum material (sediment and algal material from rocks) was collected and analyzed after incubations to scale process rates by substratum area and/or biomass. Algal material on rocks was analyzed for chlorophyll *a* to provide an estimate of photosynthetically active algal biomass. The algal material was collected by scrubbing the substratum in water and filtering the produced slurry through pre-ashed GF/F filters (0.7 µm) and then freezing. Laboratory analysis of chlorophyll *a* followed standard methods using a spectrophotometer and methanol extraction (APHA 2005). Sediment and algal material were analyzed for ash free dry mass (AFDM), which provides an estimate of the total organic material present in a sample and is measured as the difference between the mass of the oxidized samples and the initial dry samples. AFDM samples were dried at 50 °C, weighed for dry mass and then oxidized in a muffle furnace at 550 °C, rewetted, and dried before a final weighing. Surface area and volume of all substrata were also measured for use in scaling process rates for biomass and surface area. Surface area of rocks was determined by calculating planar area, the two-dimensional area enclosed by an object's perimeter, by tracing the outline of rocks on paper and weighing these cut-out tracings on an analytical balance. A standard equation for the weight of paper cut-outs of known area was used to convert rock cut-out weight to area (Bergey and Getty 2006). Sediment surface area was calculated as the diameter of the corer. Rock volume was determined using displacement and sediment volume was determined by multiplying the surface area by average sediment core depth. Scaling per unit area or biomass for all N_2 fixation and denitrification revealed similar patterns. Therefore, we only present the area-scaled rates here.

Environmental characteristics

Streamwater was collected for nutrient analysis upstream of each incubation site to test the third hypothesis regarding DIN relationships with N_2 fixation and denitrification. The water was filtered using Millipore 0.45 µm nitrocellulose membrane filters into 60 mL bottles. Samples were frozen until later laboratory analysis for nitrate (NO_3^-) and ammonium (NH_4^+). NH_4^+ was analyzed using a fluorometric method (Holmes et al. 1999; Taylor et al. 2007) on a Turner Aquafluor (Turner Designs, Palo Alto California). NO_3^- samples from 2015 were analyzed on a Dionex ICS-900 Ion Chromatograph (Dionex, Sunnyvale California), and samples from 2016 were analyzed via the cadmium reduction method on an auto analyzer by the University of Michigan Biological Station Analytical Lab. DIN concentration was then calculated by adding concentrations of NH_4^+ and NO_3^-. Sample concentrations that were below the detection limit were set to a concentration of half the detection limit for analysis.

To test our final hypothesis regarding environmental variables as predictors of N_2 fixation and denitrification, we measured canopy cover (%) using a spherical densiometer (Lemmon 1956). Discharge (L s^{-1}) was measured using a Marsh McBirney Flomate attached to a wading rod to measure velocity (m s^{-1}) at 0.6× stream depth at each point along a 10 point transect. A YSI 6920 sonde was used to measure stream water temperature (°C), conductivity (mS cm^{-1}), pH, turbidity (NTU), optical dissolved oxygen (ODO) saturation (%), and ODO concentration (mg L^{-1}) upstream of the incubation site for the duration of the incubations. Water samples were filtered using Millipore 0.45 µm nitrocellulose membrane filters into 60 mL bottles and were kept frozen until lab analysis for DOC, total dissolved nitrogen (TDN), soluble reactive phosphorus (SRP), and total dissolved phosphorus (TDP). DOC and TDN samples were acidified with hydrochloric acid and quantified using a Shimadzu TOC-V_{CSN} with a total N module TNM-1 (Shimadzu Scientific Instruments, Columbia, Maryland). SRP and TDP samples were analyzed on a Thermo Scientific 10 s UV–Vis spectrophotometer using the ascorbic acid method and molybdenum antimony colorimetric determination methods (APHA 2005). An ammonium persulfate digestion was used prior to this analysis for TDP samples.

Statistical analysis

We plotted and visually compared rates of both processes across all streams to evaluate our first hypothesis that rates of denitrification and N_2 fixation would co-occur in streams of varying DIN concentrations. If measured rates were negative and had negative standard error they were considered to be 0 μg m^{-2} h^{-1}. If rates were above 0 μg m^{-2} h^{-1} they were considered detectable. Since this hypothesis was simply a detection of whether both rates were occurring, no statistical test was applied.

We used a paired two sample *t* test to test the second hypothesis that rates of N_2 fixation and denitrification (both amended and unamended) would be different depending on stream substratum. We could only compare rates between substrata for 2016, because in 2015 we did not measure both rates on all substratum types. N_2 fixation rates failed to meet normality and equal variance assumptions so they were log transformed for all analyses. Amended and unamended denitrification rates were further analyzed with a paired *t*-test to determine if the rates were significantly different. Rates of N_2 fixation were compared to amended and unamended denitrification rates using separate paired *t*-tests to determine if the rates were significantly different. The *t*-tests were performed in R (version 3.2.2, R Foundation for Statistical Computing).

We used simple linear regression to evaluate DIN concentrations as a predictor of rates of N_2 fixation and denitrification to test our third hypothesis. Regressions were performed separately for both years because rates of each process were not measured on both sediment and rock substratum in both years. Simple linear regression analyses were also performed in R.

Multiple linear regression was used to identify significant predictors of rates of N_2 fixation and denitrification for all streams to test our final hypothesis that a combination of environmental variables may better predict process rates than DIN alone. We compared models with DIN, TDP, DIN:TDP, DOC, canopy cover, temperature, organic matter availability, and chlorophyll *a* content as predictors. Prior to model selection, we removed the predictors NH_4^+, NO_3^-, TDN, and SRP due to significant correlations with other predictor variables ($R^2 \geq 0.50$; see Online Resource 1). Predictors were also tested against the normality, noncollinearity, and homoscedasticity assumptions of multiple linear regression models and removed if they failed to meet the assumptions. We identified the best model based on the smallest Akaike's information criteria (AIC, Burnham and Anderson 2002). Multiple regression analyses were also performed in R.

A multivariate partial least squares analysis (PLS) was used to further assess the final hypothesis. In PLS analysis, variance in the predictors is used to explain variance in the response. This is more advantageous than the multiple linear regression because the PLS is less sensitive to correlation among predictor variables and deviations from normality because PLS performs simultaneous decompositions of predictor and response variables (Carrascal et al. 2009). Therefore, PLS is best informed with the inclusion of all individual predictors, and we did not exclude any correlated predictors from this analysis. Because of the limited number of streams included in this study, we could not use analyses that identify indirect effects caused by multiple factors such as structural equation modeling (Grace et al. 2010; Benjamin et al. 2013). PLS analyses were conducted with N_2 fixation, amended denitrification, and unamended denitrification rates as response variables and 12 environmental factors as predictor variables: NH_4^+, NO_3^-, DIN, SRP, TDP, DIN:TDP, DOC, TDN, canopy cover, average temperature, discharge, and average sediment organic matter. The importance of an environmental predictor to the overall model was determined by the variable importance on the projection scores (VIP). VIP scores greater than 2 were considered highly influential, those between 1 and 2 were considered moderately influential, and less than 1 as not influential on the overall model (Kothawala et al. 2014). K fold cross validation was performed to test the repeatability and validate the model. When plotted, the location of the variables in relation to the origin and one another indicates the correlation. Variables closer to each other are positively correlated and variables further from one another are negatively correlated. Those variables closer to the origin are less influential to the overall model and those situated further from the origin have greater influence on the overall model. The PLS was performed using JMP Pro (version 13.0.0, SAS Institute, Inc.).

Results

Process co-occurrence and rate comparison by substratum

We found that both N_2 fixation and denitrification co-occurred in several streams and that N_2 fixation primarily occurred on rock substratum and denitrification primarily on sediment substratum when testing our first hypothesis. We found detectable rates of both N_2 fixation and denitrification in Pebble Creek, South Fork Mink Creek, Rapid Creek, Lower Mink Creek and the Upper Portneuf, which encompassed a range of 26–581 µg L^{-1} DIN, suggesting that these processes co-occur across the full gradient of DIN concentrations included in our study (Fig. 1). N_2 fixation rates differed between substratum types (range of 0–160 µg m^{-2} h^{-1}) with the highest rates two orders of magnitude higher on rocks than on sediment substratum (Fig. 1). However high N_2 fixation rates on rocks only occurred in one stream, therefore log transformed N_2 fixation rates were not significantly different between sediment and rock substratum ($t = 1.72$, df = 7, $p = 0.13$). Unamended denitrification rates on sediment were 100 times higher than on rock ($t = -4.76$, df = 7, $p < 0.01$), and amended rates on sediment were 1000 times higher than on rock ($t = -3.68$, df = 7, $p = < 0.01$; Fig. 1). Amended and unamended denitrification rates were statistically different from one another across substrata and years ($t = -4.64$, df = 23, $p = < 0.01$), with amended rates being 5–9 times higher than unamended denitrification rates. Both amended and unamended denitrification rates were significantly higher than N_2 fixation rates ($t = 4.65$, df = 23, $p = < 0.01$; $t = 3.93$, df = 23, $p = < 0.01$, respectively), with amended rates being roughly 1000 times higher and unamended rates being roughly 100 times higher.

DIN as a predictor of process rates

We found that DIN concentration was negatively related to N_2 fixation rates on rocks in 2016 and positively related to amended denitrification rates on sediments in 2015 and 2016 when testing our third hypothesis that streams with varying DIN concentrations would have different rates of both N_2 fixation and denitrification. We further tested these relationships by using the components of DIN, NH_4^+, and NO_3^- as single predictors and found one significant relationship with NH_4^+ concentrations to amended denitrification rates on rocks in 2016. We also observed the same significant relationships with NO_3^- as with DIN, which suggests NO_3^- is the main component driving these relationships (see Online Resource 1). In 2015,

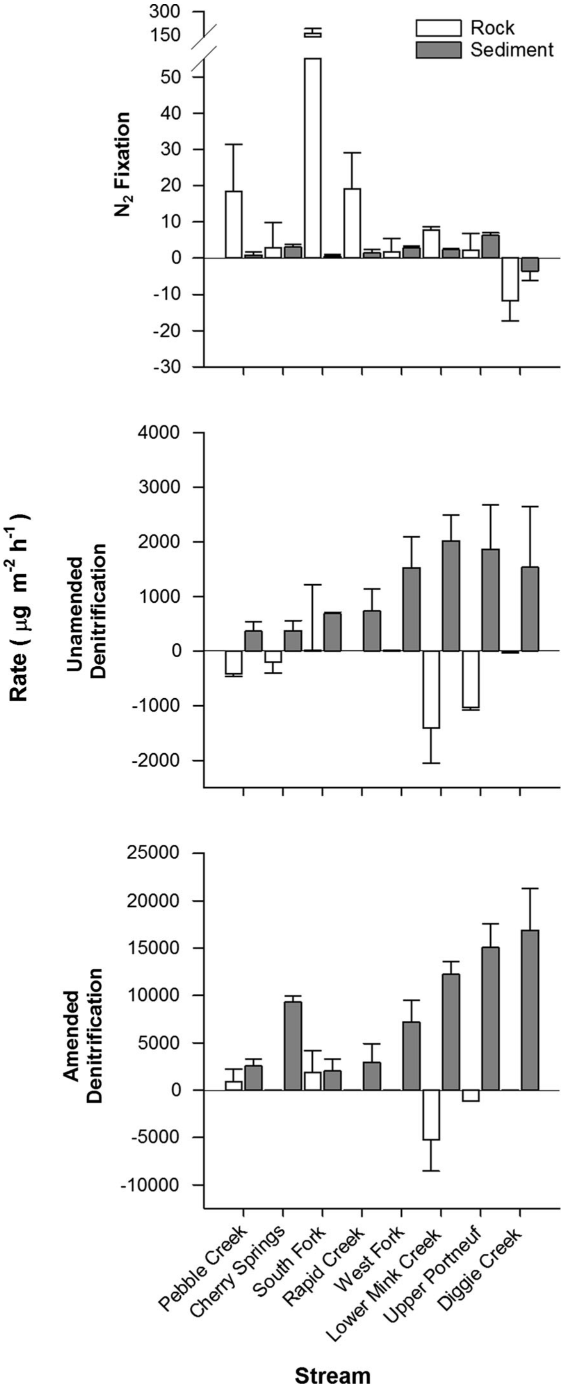

Fig. 1 N_2 fixation rates (n = 6) and denitrification rates (amended and unamended, n = 3) on rock and sediment substrata in 2016; streams are arranged from low to high DIN concentrations from left to right on the x axis. Error bars are standard error. Note that Y axis for unamended denitrification rates is 6.25 times lower than that of the amended denitrification rates, and the Y axis for N_2 fixation is 83.3 times lower than that of the amended denitrification rates

the highest N_2 fixation rate on rocks was observed in one of the streams with low DIN concentration (32.5 μg L^{-1}, Fig. 2). In 2016, the highest N_2 fixation rate on rocks occurred in the same stream, although the DIN concentration was higher in 2016 than 2015 (32.5 μg L^{-1} in 2015 and 40.9 μg L^{-1} in 2016), almost certainly due to our use of a more sensitive analysis for NO_3^- in 2016. Contrary to our hypothesis, streams with higher DIN concentrations (> 350 μg L^{-1}) did not have the lowest N_2 fixation rates; instead, streams with both high and more intermediate DIN concentrations (~ 100–300 μg L^{-1}) had some of the lowest N_2 fixation rates in both years (Fig. 2). DIN concentration was a significant predictor of N_2 fixation rates on rocks in 2016, but not 2015 (Table 3). The stream with the highest DIN concentration (615 μg L^{-1}) had the lowest N_2 fixation rate on sediments, but the stream with the second highest DIN concentration in 2016 (506 μg L^{-1}) had the highest N_2 fixation rate on sediments. Consequently, DIN concentration was not a significant predictor of N_2 fixation rates on sediment (Table 3). Rates of N_2 fixation on macrophytes were high in the single stream where they were measured, which was one of the streams with high DIN concentration (506 μg L^{-1}, Fig. 2). In both years, the highest unamended denitrification rate occurred on sediments in Lower Mink Creek, which had intermediate DIN concentrations (168–298 μg L^{-1}). DIN concentration was a significant predictor of amended denitrification rates on sediments, but not unamended denitrification rates on sediments or rocks for either year (Table 3). In both years the lowest amended denitrification rate occurred in the same stream with a low DIN concentration (32.5 and 40.9 μg L^{-1}, respectively) and the highest rate occurred in the stream with the highest DIN concentration in that year (581 and 615 μg L^{-1}, respectively).

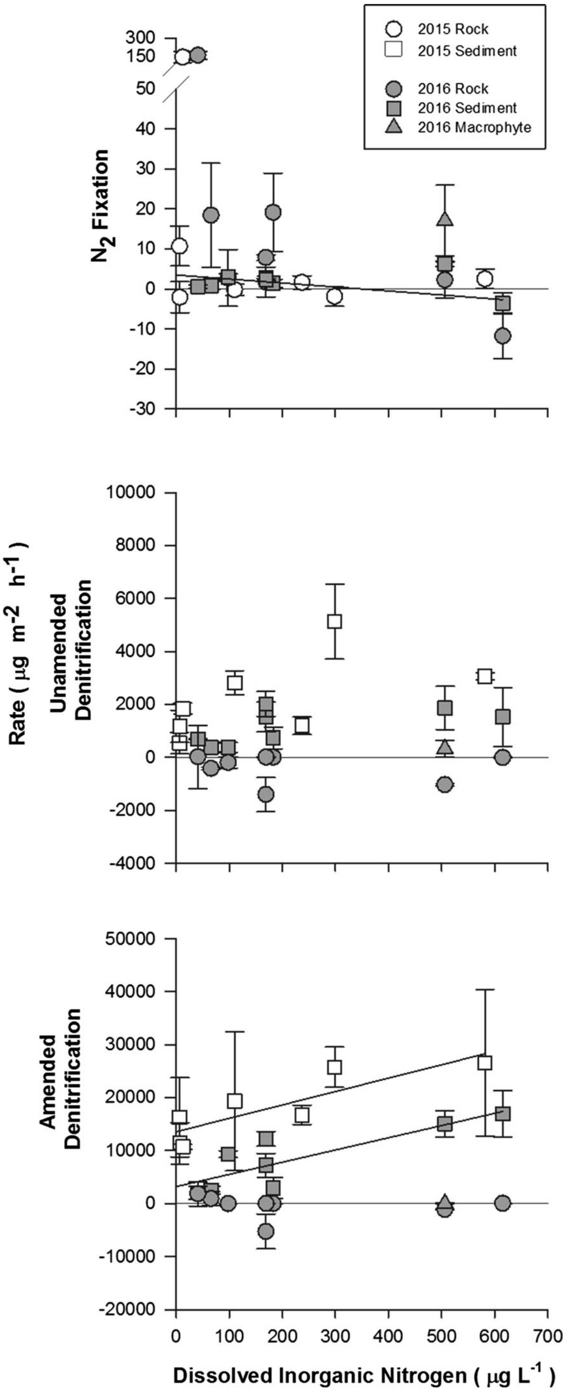

Fig. 2 N_2 fixation (n = 6) and denitrification rates (amended and unamended, n = 3), from both 2015 and 2016 versus DIN concentrations with standard error bars. Note the Y axis for amended denitrification rates is five times that of unamended denitrification, and the Y axis for N_2 fixation is 167 times less than that of amended denitrification rates

Other environmental factors as predictors

Testing of our fourth hypothesis, that a combination of environmental variables and DIN would be a better predictor of rates of each process than DIN alone, revealed that P and organic matter availability were important predictors of denitrification rates. For amended denitrification, there were 4 significant multiple regression models produced with environmental variables, including the full model. The best model explained 75% of the variance and included

Table 3 Simple linear regression results for process rates versus dissolved inorganic nitrogen as a single predictor variable

Process rate		R^2	F	p	S.E.E.	Y-intercept	Slope
N_2 fixation (log transformed)	2015 Rock*	0.02	0.12	0.74	3.03	0.63	0.00
	2016 Rock	**0.62**	**9.89**	**0.02**	**1.44**	**3.50**	**− 0.01**
	2016 Sed	0.08	0.51	0.50	1.33	0.66	0.00
Amended denitrification	**2015 Sed***	**0.73**	**13.70**	**0.01**	**3563.00**	**13,574.30**	**25.30**
	2016 Sed	**0.70**	**14.24**	**0.01**	**3427.00**	**3207.50**	**23.06**
	2016 Rock	0.23	1.84	0.22	651.70	715.80	− 1.58
Unamended denitrification	2015 Sed*	0.32	2.32	0.19	1417.00	1506.94	4.14
	2016 Sed	0.37	3.53	0.11	576.00	691.93	1.93
	2016 Rock	0.15	1.08	0.34	7.03	6.06	− 0.01

Degrees of freedom are 1 and 6 for all except those denoted by a * which have 1 and 5 degrees of freedom. S.E.E. stands for standard error of the estimate, otherwise known as residual standard error. Bold values are significant models at $p \leq 0.05$

DIN:TDP, DOC, and organic matter content as positive predictors, and average temperature as a negative predictor (Table 4). For unamended denitrification, there were 5 significant models with environmental variables (Table 4). The best model explained 72% of the variance and included TDP and organic matter content as positive predictors and canopy cover as a negative predictor (Table 4). No significant multiple regression models were found for N_2 fixation rates.

To further assess environmental variables as predictors, the PLS model for amended denitrification rates identified 2 latent variables (LVs), which collectively explained 67.8% of the variance in

Table 4 Stepwise multiple linear regression models for rates of N_2 fixation and denitrification (both amended and unamended)

Process rate	Models	AIC	p	R^2	ΔAIC
N_2 fixation (log transformed)	+ DIN:TDP	34.74	0.30	0.05	0
	+ TDP + DIN:TDP	36.10	0.52	0.06	1.36
	+ TDP + DIN:TDP − CC	36.40	0.74	0.06	1.66
	+ TDP + DIN:TDP − CC − OM	37.80	0.71	0.1	3.06
	+ TDP + DIN:TDP − CC + TEMP − OM	39.30	0.82	0.11	4.56
	+ TDP + DIN:TDP + DOC − CC + TEMP − OM	41.20	0.82	0.13	6.46
	− DIN + TDP + DIN:TDP + DOC − CC + TEMP − OM	43.15	0.52	0.28	8.41
	Original model	45.14	0.63	0.29	10.40
Amended denitrification	**+ DIN:TDP + DOC − TEMP + OM**	**410.64**	**< 0.01**	**0.75**	**0**
	− DIN + DIN:TDP + DOC − TEMP + OM	**411.46**	**< 0.01**	**0.76**	**0.82**
	− DIN + TDP + DIN:TDP + DOC − TEMP + OM	**413.07**	**< 0.01**	**0.77**	**2.43**
	Original model	**414.95**	**< 0.01**	**0.77**	**4.31**
Unamended denitrification	**+ TDP − CC + OM**	**319.96**	**< 0.01**	**0.72**	**0**
	+ TDP − CC + DOC + OM	**321.33**	**< 0.01**	**0.72**	**1.37**
	− DIN + TDP − CC + DOC + OM	**322.95**	**< 0.01**	**0.73**	**2.99**
	− DIN + TDP + DIN:TDP − CC + DOC + OM	**323.51**	**< 0.01**	**0.74**	**3.55**
	Original model	**325.25**	**< 0.01**	**0.75**	**5.29**

Original models included DIN (dissolved inorganic nitrogen), TDP (total dissolved phosphorus), DIN:TDP, DOC (dissolved organic carbon), TEMP (temperature), CC (canopy cover), OM (organic matter content), and Chl-*a* (chlorophyll *a* content). Bold values are significant models at $p \leq 0.05$

amended denitrification rates (R^2Y) and 62.3% of the variance in the predictor variables (R^2X, Fig. 3a). Organic matter was a highly influential predictor (VIP > 2), NH_4^+, TDP, and temperature were moderately influential (2 > VIP > 1) and the remaining variables were less influential predictors. The first axis (LV1) explained 22.7% of the variability in the X variables and 58.8% in the Y variable. LV1 had positive loadings from NH_4^+ and TDP and negative loadings from temperature. The second axis (LV2) explained 39.6% of the variability in X and 8.9% of the variability in Y. LV2 had positive loadings from TDP and temperature and negative loadings from organic matter and NH_4^+. For unamended denitrification rates the PLS model identified 2 LVs. Collectively the LVs explained 74.6% of the variance in unamended denitrification rates (R^2Y) and 61.2% of the variance in the predictor variables (R^2X, Fig. 3b). Organic matter was a highly influential predictor, NH_4^+ and TDP were moderately influential and the remaining variables were less influential predictors. LV1 explained 21.6% of the variability in the X variables and 66.0% in the Y variable. LV1 had positive loadings from organic matter, NH_4^+, and TDP and all negative loadings were from less influential predictors. LV2 explained 39.6% of the variability in X and 8.6% of the variability in Y. LV2 had positive loadings from TDP, and negative loadings from NH_4^+ and organic matter. No predictive PLS models were produced for N_2 fixation rates.

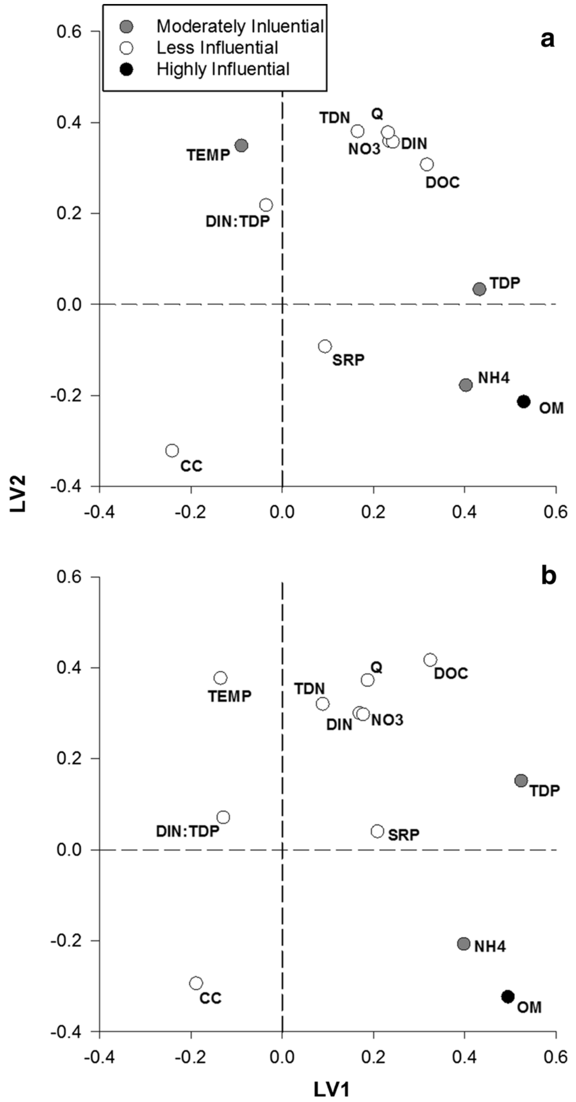

Fig. 3 Partial least squares loading plots for amended denitrification rates (**a**) and unamended denitrification rates (**b**) with the predictor variables NH_4^+ (NH4), NO_3^- (NO_3), DIN, SRP, TDP, DIN:TDP, DOC, TDN, canopy cover (CC), average temperature (TEMP), discharge (Q), and average sediment organic matter (OM). Highly influential predictors (VIP > 2) are depicted in black circles, moderately influential predictors (2 > VIP > 1) in grey, and less influential predictors (VIP < 1) in white

Discussion

N_2 fixation and denitrification co-occurred in streams encompassing the full range of DIN concentrations in our study (26–615 µg L^{-1} DIN). Across streams, N_2 fixation rates were on average approximately 1000 times lower than amended denitrification rates, but in streams where both processes co-occurred N_2 fixation rates were approximately 10 times lower than amended denitrification rates. Our results suggest that the rates of N_2 fixation and denitrification in these stream ecosystems cannot be predicted by DIN concentrations alone. DIN concentrations were significantly related to amended denitrification rates on sediment in both years and N_2 fixation on rock in 2016, but not unamended denitrification rates on either substratum in either year. NH_4^+, temperature, organic matter content, DIN:TDP, and TDP were part of significant multiple regression and PLS models explaining variance in denitrification rates when other environmental factors were included as predictors. No significant environmental models predicted N_2 fixation rates across all substrata, streams, and study dates.

Our observations of both N_2 fixation and denitrification co-occurring across the full N gradient encompassed by our study and the fact that environmental characteristics at the stream-reach scale were not consistently able to predict rates of these processes suggests differences in environmental variables on the sub-reach scale may control the co-occurrence of these processes.

It has been hypothesized that above a certain concentration of DIN, rates of N_2 fixation will drop off dramatically due to inhibition (Marcarelli and Wurtsbaugh 2007; Kunza and Hall 2013). In one study, rates of N_2 fixation were high only when nitrate concentrations were < 20 μg L^{-1}, indicating a nutrient threshold-like pattern for N_2 fixation activity (Kunza and Hall 2014). Similarly, Hiatt et al. (2017) studied the effects of alder cover on in-stream N_2 fixation rates and observed a similar threshold pattern, such that above 20–40 μg L^{-1} DIN N_2 fixation rates dropped sharply. These results are not unlike what we observed for N_2 fixation on rock, in that high rates dropped off above ~ 45 μg L^{-1}. However, our low number of study streams precluded determination of whether there was truly a threshold. Moreover, we observed low N_2 fixation rates in streams with low DIN concentration, indicating other environmental variables like light availability, temperature, or trace metal availability may constrain or limit the process rates in these low DIN streams (Kunza and Hall 2013; Finlay et al. 2011; Welter et al. 2015), even though none of the multiple linear regression or PLS models that included these factors significantly explained observed rates of N_2 fixation. P availability can be an important limiting factor, particularly for N_2-fixing bacteria (Elwood et al. 1981; Marcarelli and Wurtsbaugh 2007), and the stream with highest N_2 fixation rates had high TDP concentrations compared to other low DIN streams. Light availability and temperature can also be important factors limiting N_2 fixation rates (Finlay et al. 2011; Welter et al. 2015), however our study sites did not vary much in temperature (± 3 °C) and most streams had relatively high light input, so we may not have encompassed enough variation in these factors for them to explain variation in the N_2 fixation rates we measured.

Amended, but not unamended denitrification rates were positively and linearly related to DIN concentrations, which is consistent with previous observations of increasing denitrification rates with increasing NO_3^- concentrations (Seitzinger 1988; Holmes et al. 1996; Seitzinger et al. 2006). The different relationships between DIN concentrations in background streamwater and amended versus unamended denitrification point to C as an important additional control of denitrification rates. Unamended denitrification rates did not have an additional C source and showed no linear response to DIN concentrations, but when additional C was added for amended denitrification the rates did respond linearly to changes in DIN concentration. This suggests the C source available only to amended denitrification rates helped overcome C limitation. Thus, C was the important limiting factor for denitrification in our amended assays. Similarly, multiple linear regression and PLS models for both amended and unamended denitrification rates included predictors related to C sources (DOC and organic matter content). Organic matter as a source of C can often be a limiting factor for denitrification rates as C is an electron donor in the denitrification process (Knowles 1982; Holmes et al. 1996; Arango et al. 2007), and our findings corroborate this.

Our analysis also suggests that P availability is an important predictor of denitrification rates. Multiple linear regression and PLS models for both amended and unamended denitrification rates included predictors related to relative and absolute P availability (DIN:TDP and TDP). While increases in TDP concentration lead to increases in unamended rates, increases in DIN:TDP lead to increases in amended denitrification rates. This relationship suggests that more P facilitates higher denitrification rates in streams where P is limited relative to N. In lake ecosystems, studies have shown that N:P greater than 9 by mass are associated with co-limitation of primary producers by N and P, while N:P > 23 can lead to strict P limitation (Paerl et al. 2016). Studies in streams have suggested that such co-limitation by N and P should occur along a gradient of N:P in streams as well (Dodds et al. 2002). In our study, about half of the streams in each year had N:P > 9, suggesting that P may limit or co-limit productivity in these streams. The mechanism behind the observed positive relationship between P and denitrification in our study merits further study, but could be similar to that observed by Finlay et al. (2013) in P-limited lake ecosystems, which have increased rates of N removal after lake P inputs were increased. The mechanism proposed behind this phenomenon in lakes is that additional P stimulates algal production and N uptake and when algae die they end up in the sediments, delivering N and organic matter, which increase denitrification rates (Finlay et al. 2013).

A more complete comparison of the relative biogeochemical roles of denitrification and N_2 fixation can be achieved in the context of a nutrient budget. The sampling design for this study did not allow us to calculate N budgets for each stream since we did not measure the total area covered by each substratum in all stream reaches. However, we have quantified substratum areas as part of a different study in South Fork Mink Creek, where we detected the highest N_2 fixation rates and co-occurrence of denitrification in the current study. Using estimates of 76% cover of rock substratum and 22.5% of cover as sediment for an 80 m-long stream reach with total area of 571.75 m^2, we estimated the reach-scale N_2 fixation rate to be 67 mg h^{-1}, and the amended denitrification rate to be 1630 mg h^{-1}. Even though the sediment area in the stream was 1/4 that of the rock area, amended denitrification rates were high enough that reach-level inputs of N via N_2 fixation equaled 4% of the output via denitrification. For comparison, Bechtold et al. (2012) found that NO_3-N uptake for South Fork Mink Creek was 21,360 μg m^{-2} h^{-1}, which scaled up to the 80-m reach equals 12,213 mg h^{-1}. This suggests that denitrification equals about 13% of total NO_3-N uptake and that the remainder is likely due to assimilation. All together, these results suggest that even when N_2 fixation rates were high in this stream, they are still low relative to uptake of DIN from the water column and removal via denitrification which is in contrast with findings of a different study where N_2 fixation rates equaled uptake rates in a stream (Kunza and Hall 2014). In the other streams in this study we would expect denitrification to be of more importance to the overall N balance because these streams had higher rates of amended denitrification and more sediment cover as well as lower N_2 fixation rates. Yet, it is likely that that the relative balance between N_2 fixation and denitrification may be temporally dynamic in a way that is not encompassed by our 1–2 days of observation. For example, coastal estuarine sediments vary from an N sink (denitrification higher than N_2 fixation) to an N source on certain dates and under certain conditions (Fulweiler and Heiss 2014; Newell et al. 2016). Moreover, N_2 fixation delivers biologically available N directly to organisms, even if the overall fluxes are small, which could be important to the diversity and distribution of organisms in these streams. Small differences in nutrient availability can allow for more diverse groups of organisms to occupy the same space and to alter the distribution of types of organisms in streams across multiple spatial scales (Pringle et al. 1988; Henry and Fisher 2003).

Even though we did measure rates on multiple substrata, this study did not specifically address heterogeneity in environmental characteristics within stream reaches, which could be important in explaining the co-occurrence of N_2 fixation and denitrification that we observed. Stream ecosystems are characterized by spatial and temporal habitat heterogeneity at multiple, nested scales (Frissell et al. 1986) that, in turn, influences heterogeneity in streamwater chemistry, organisms, and ecosystem processes across scales (e.g., Dent and Grimm 1999; McGuire et al. 2014). Patches, or spatially-related areas that control ecosystem structure and function, are created by this heterogeneity (Pringle et al. 1988). Spatial heterogeneity in DIN and NO_3^- concentrations can affect the distribution of N_2-fixing organisms in stream reaches (Dent and Grimm 1999; Henry and Fisher 2003). Denitrification rates can vary spatially with organic matter availability and temperature at the sub-reach scale (Holmes et al. 1996; Groffman et al. 2005). Both N_2 fixation and denitrification rates can also vary on the microhabitat scale among substratum types, with higher rates of N_2 fixation on rocks and higher rates of denitrification on fine benthic organic matter (Kemp and Dodds 2002; Marcarelli and Wurtsbaugh 2009), which agrees with our findings. Spatial heterogeneity in oxygen availability on the scale of centimeters effects rates of nitrification (Kemp and Dodds 2001), indicating heterogeneity in resources at very small scales can influence related biogeochemical processes. Such small-scale differences in resources could explain why we observed relatively high rates of denitrification on sediment substratum in our current study of South Fork Mink Creek where DIN concentrations are low. The substratum in these ecosystems may have been located in patches where local conditions were favorable for these processes compared to unfavorable conditions at the scale of the entire reach (McClain et al. 2003). Such patches create hotspots for particular nutrient transformations that can make disproportionate contributions to ecosystem nutrient fluxes, even when average conditions are unfavorable to that process (McClain et al. 2003), thereby permitting co-existence of both processes. A multi-scale approach may more accurately capture differences and characterize environmental factors that control rates of these processes when examining the effect of environmental variables on the co-occurrence of N_2 fixation and denitrification in streams.

In conclusion, we found that N_2 fixation and denitrification co-occur in stream ecosystems across a gradient of DIN concentrations in a western U.S. watershed, although N_2 fixation rates were lower than denitrification, and that rates of both processes were related to a variety of environmental variables and only occasionally to DIN alone. Our observation of N_2 fixation and denitrification co-occurring in streams is similar to recent findings in coastal marine ecosystems where both processes contribute to N_2 fluxes, therefore understanding both processes simultaneously is required to accurately capture the balance between the two (Fulweiler and Heiss 2014; Newell et al. 2016). Denitrification is typically thought of as the primary process relevant to N management because it removes N from ecosystems (Seitzinger 1988), and our work did show that losses via denitrification were much higher than inputs from N_2 fixation in these streams on our study dates. Yet, there are other streams where N_2 fixation inputs can rival whole-stream N uptake rates, losses via denitrification, and/or are large contributors to the N budget at daily to annual time scales (Dodds and Castenholz 1988; Grimm and Petrone 1997; Kunza and Hall 2014). Although inputs from N_2 fixation may be small at the reach scale, they may be key to controlling biodiversity and heterogeneity at smaller temporal or spatial scales. There are also other understudied pathways by which N may be removed, such as anaerobic ammonium oxidation (“anammox”), by which bacteria remove N through the transformation of nitrite to N_2 gas, or dissimilatory nitrate reduction to ammonium, which can co-occur with denitrification but actually results in more biologically reactive N rather than removal (Burgin and Hamilton 2007). The potential, within a stream reach, for simultaneous input from N_2 fixation and removal by denitrification as well as other understudied N transformations suggests that the management of N in stream ecosystems is currently operating based on assumptions that do not consider the importance of small fluxes and requires improved understanding of the complexity of N cycling in these ecosystems.

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References

American Public Health Administration (2005) Standard Methods for examination of water and wastewater. APHA, Washington DC

An S, Gardner WS, Kana T (2001) Simultaneous measurement of denitrification and nitrogen fixation using isotope pairing with inlet mass spectrometry analysis. Appl Environ Microbiol 67:1171–1178

Arango CP, Tank JL, Schaller JL et al (2007) Benthic organic carbon influences denitrification in streams with high nitrate concentration. Freshw Biol 52:1210–1222

Barton GJ (2004) Surface and ground-water relations on the Portneuf River, and temporal changes in ground-water levels in the Portneuf Valley, Caribou and Bannock Counties, Idaho, 2001–2002. USGS Scientific Investigations Report 2004-5170

Bechtold HA, Marcarelli AM, Baxter CV et al (2012) Effects of N, P, and organic carbon on stream biofilm nutrient limitation and uptake in a semi-arid watershed. Limnol Oceanogr 57:1544–1554

Benjamin JR, Lepori F, Baxter CV et al (2013) Can replacement of native by non-native trout alter stream-riparian food webs? Freshw Biol 58:1694–1709

Bergey EA, Getty GM (2006) A review of methods for measuring the surface area of stream substrates. Hydrobiologia 556:7–16

Brandes J, Devol A, Yoshinari T et al (1998) Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: a tracer for mixing and nitrogen cycles. Limnol Oceanogr 44:106–115

Burgin AJ, Hamilton SK (2007) Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front Ecol Environ 5:89–96

Burnham KP, Anderson DR (2002) Model selection and multimodel inference: a practical information-theoretic approach, 2nd edn. Springer, New York

Capone DG (1993) Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. In: Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds) Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton

Capone DG (2001) Marine nitrogen fixation: what’s the fuss? Curr Opin Microbiol 4:341–348

Carrascal LM, Galvan I, Gordo O (2009) Partial least squares regression as an alternative to current regression methods uses in ecology. Oikos 118:681–690

Dent CL, Grimm NB (1999) Spatial heterogeneity of stream water nutrient concentrations over successional time. Ecology 80:2283–2298

Deutsch C, Sarmiento JL, Sigman DM et al (2007) Spatial coupling of nitrogen inputs and losses in the ocean. Nature 445:163–167

Dodds WK, Castenholz RW (1988) The nitrogen budget of an oligotrophic cold water pond. Arch Hydrobiol 79(Suppl):343–362

Dodds WK, Smth VH, Lohman K (2002) Nitrogen and phosphorus relationship to benthic algal biomass in temperate streams. Can J Fish Aquat Sci 59:865–874

Dodds WK, Burgin AJ, Marcarelli AM et al (2017) Nitrogen transformations. In: Lamberti GA, Hauer FR (eds) Methods in stream ecology, vol 2. ecosystem function. Elsevier/Academic Press, Amsterdam/New York, pp 143–196

Elwood JW, Newbold JD, Trimble AF et al (1981) The limiting role of phosphorus in a woodland stream ecosystem: effects of P enrichment on leaf decomposition and primary producers. Ecology 62:146–158

Fernandez C, Farias L, Ulloa O (2011) Nitrogen fixation in denitrified marine waters. PLoS ONE 6:e20539

Finlay JC, Hood JM, Limm MP et al (2011) Light-mediated thresholds in stream water nutrient composition in a river network. Ecology 92:140–150

Finlay JC, Small GE, Sterner RW (2013) Human influences on nitrogen removal in lakes. Science 342:247–250

Flores E, Herrero A (2010) Compartmentalized function through cell differentiation in filamentous cyanobacteria. Nat Rev Microbiol 8:39–50

Frissell CA, Liss WJ, Warren CE et al (1986) A hierarchical framework for stream habitat classification: viewing streams in a watershed context. Environ Manag 10:199–214

Fulweiler RW, Heiss EM (2014) (Nearly) a decade of directly measured sediment N2 fluxes: what can Narganset Bay tell us about the global ocean nitrogen budget? Oceanography 27:184–195

Gettel GM, Giblin AE, Howarth RW (2007) The effects of grazing by the snail, Lymnaea elodes, on benthic N_2 fixation and primary production in oligotrophic arctic lakes. Limnol Oceanogr 52:2398–2409

Grace JB, Anderson TM, Olff H et al (2010) On the specification of structural equation models for ecological systems. Ecol Monogr 56:1001–1014

Grimm NB, Petrone K (1997) Nitrogen fixation in a desert stream ecosystem. Biogeochemistry 37:33–61

Groffman PM, Dorsey AM, Mayer PM (2005) N processing within geomorphic structures in urban streams. J N Am Benthol Soc 24:613–625

Groffman PM, Altabet MA, Bohlke JK et al (2006) Methods for measuring denitrification: diverse approaches to a difficult problem. Ecol Appl 16:2091–2122

Groffman PM, Butterbach-Bahl K, Fulweiler RW et al (2009) Challenges to incorporating spatially and temporally explicit phenomena (hot spots and hot moments) in denitrification models. Biogeochemistry 93:49–77

Henry JC, Fisher SG (2003) Spatial segregation of periphyton communities in a desert stream: causes and consequences for N cycling. J N Am Benthol Soc 22:511–527

Hiatt DL, Robbins CJ, Back JA et al (2017) Catchment-scale alder cover controls nitrogen fixation in boreal headwater streams. Freshw Sci. https://doi.org/10.1086/692944

Holmes RM, Jones JB, Fisher SG et al (1996) Denitrification in a nitrogen-limited stream ecosystem. Biogeochemistry 33:125–146

Holmes RM, Aminot A, Kerouel R et al (1999) A simple and precise method for measuring ammonium in marine and freshwater ecosystems. Can J Fish Aquat Sci 56:1801–1808

Hopkins JM, Marcarelli AM, Bechtold HA (2011) Ecosystem structure and function are complementary measures of water quality in a polluted, spring-influenced river. Water Air Soil Pollut 214:409–421

Kemp MJ, Dodds WK (2001) Centimeter-scale patterns in dissolved oxygen and nitrification rates in a prairie stream. J N Am Benthol Soc 20:347–357

Kemp MJ, Dodds WK (2002) The influence of ammonium, nitrate, and dissolved oxygen concentrations on uptake, nitrification, and denitrification rates associated with prairie stream substrata. Limnol Oceanogr 47:1380–1393

Knowles R (1982) Denitrification. Microbiological Rev 46:43

Kothawala DN, Stedmon CA, Muller RA et al (2014) Controls of dissolved organic matter quality: evidence from a large-scale boreal lake survey. Glob Change Biol 20:1101–1114

Kunza LA, Hall RO Jr (2013) Demographic and mutualistic responses of stream nitrogen fixers to nutrients. Freshw Sci 32:991–1004

Kunza LA, Hall RO Jr (2014) Nitrogen fixation can exceed inorganic nitrogen uptake fluxes in oligotrophic streams. Biogeochemistry 121:537–549

Lemmon PE (1956) A spherical densitometer for estimating forest overstory density. For Sci 2:314–320

Lewis WM, Wurtsbaugh WA (2008) Control of lacutrine phytoplankton by nutrients: erosion of the phosphorus paradigm. Internat Rev Hydrobiol 93:446–465

Macko SA, Enzeroth L, Parker PL (1984) Regional differences in nitrogen and carbon isotopes on the continental shelf of the Gulf of Mexico. Naturwissenschaften 71:374–375

Marcarelli AM, Wurtsbaugh WA (2007) Effects of upstream lakes and nutrient limitation on periphytic biomass and nitrogen fixation in oligotrophic, subalpine streams. Freshw Biol 52:2211–2225

Marcarelli AM, Wurtsbaugh WA (2009) Nitrogen fixation varies spatially and seasonally in linked stream-lake ecosystems. Biogeochemistry 94:95–110

Marcarelli AM, Baker MA, Wurtsbaugh WA (2008) Is in-stream N_2 fixation an important N source for benthic communities and stream ecosystems? J N Am Benthol Soc 27:186–211

Marcarelli AM, Van Kirk RW, Baxter CV (2010) Predicting effects of hydrologic alteration and climate change on ecosystem metabolism in a western U.S. river. Ecol Appl 20:2081–2088

McClain ME, Boyer EW, Dent L et al (2003) Biogeochemical hot spots and hot moments at the interface of terrestrial and aquatic ecosystems. Ecosystems 6:301–312

McGuire KJ, Torgersen CE, Likens GE et al (2014) Network analysis reveals multiscale controls on streamwater chemistry. Proc Natl Acad Sci USA 111(19):7030–7035

Minshall GW, Andrews DA (1973) An ecological investigation of the Portneuf River, Idaho: a semiarid-land stream subjected to pollution. Freshw Biol 3:1–30

Newell SE, McCarthy MJ, Gardner WS et al (2016) Sediment nitrogen fixation: a call for re-evaluating coastal N budgets. Estuar Coast. https://doi.org/10.1007/s12237-016-0116-y

Paerl HW, Scott JT (2010) Throwing fuel on the fire: synergistic effects of excessive nitrogen inputs and global warming on harmful algal blooms. Environ Sci Technol 44:7756–7758

Paerl HW, Scott JT, McCarthy MJ et al (2016) It takes two to tango: when and where dual nutrient (N & P) reductions are needed to protect lakes and downstream ecosystems. Envrion Sci Technol 50(20):10805–10813

Pringle CM, Naiman RJ, Bretschko G et al (1988) Patch dynamics in lotic systems: the stream as a mosaic. J N Am Benthol Soc 7:503–524

Schindler DW (1977) Evolution of phosphorus limitation in lakes. Science 195:260–262

Schindler DW, Hecky RE, Findlay DL et al (2008) Eutrophication of lakes cannot be controlled be reducing nitrogen input: results of a 37-year whole-ecosystem experiment. Proc Natl Acad Sci USA 105:11254–11258

Scott JT, Grantz EM (2013) N2 fixation exceeds internal nitrogen loading as a phytoplankton nutrient source in perpetually nitrogen-limited reservoirs. Freshwater Sci 32:849–861

Scott JT, Marcarelli AM (2012) Cyanobacteria in freshwater benthic environments. In: Whitton BA (ed) Ecology of Cyanobacteria II: their diversity in space and time. Springer, Dordrecht

Scott JT, McCarthy MJ (2010) Nitrogen fixation may not balance the nitrogen pool in lakes over timescales relevant to eutrophication management. Limnol Oceanogr 55:1265–1270

Seitzinger SP (1988) Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. Limnol Oceanogr 33:702–724

Seitzinger SP, Harrison JA, Bohlke JK et al (2006) Denitrification across landscapes and waterscapes: a synthesis. Ecol Appl 16:2064–2090

Smith VH (1983) Low nitrogen and phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. Science 221:669–671

Taylor BW, Keep CF, Hall RO Jr (2007) Improving the fluorometric ammonium method: matrix effects, background fluorescence, and standard additions. J N Am Benthol Soc 26:167–177

Welter JR, Benstead JP, Cross WF et al (2015) Does N_2 fixation amplify the temperature dependence of ecosystem metabolism? Ecology 96:603–610