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CO-OCCURRENCE OF NITROGEN FIXATION AND DENITRIFICATION ACROSS A STREAM NITROGEN GRADIENT IN A WESTERN WATERSHED

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By

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Preface

This thesis has been written as an article that will be submitted for publication in the scientific journal *Biogeochemistry*. For this article I primarily led the research (study design, protocol development, field data collection, laboratory analysis, data analysis and interpretation) and wrote the manuscript. This article was written in collaboration with Amy M. Marcarelli and Colden V. Baxter of the Stream Ecology Center at Idaho State Univeristy. Dr. Amy M. Marcarelli acquired the funding, designed the study, assisted with field data collection and data analysis, and edited the manuscript. Dr. Colden V. Baxter provided access to lab resources at Idaho State University, assisted with study design and field data collection.

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ABSTRACT[1]

It is frequently assumed that N_2 fixation and denitrification do not co-occur in streams because each process should be favored under different concentrations of reactive nitrogen. Yet, both N_2 fixation and denitrification have been found to co-occur in marine and coastal ecosystems despite their differences in nitrogen requirements, and we cannot evaluate this assumption for streams because both processes are rarely quantified together. We asked if these processes could co-exist by measuring rates of N_2 fixation using acetylene reduction, denitrification using acetylene block, and N_2 flux using membrane inlet mass spectrometry on rocks and sediment in 8 southeastern Idaho streams encompassing a dissolved inorganic nitrogen (DIN) gradient of 6-615 μg/L. N_2 flux rates on rocks had a mean of -12,000 ± 4,900 $\mu g/m^2/h$ and on sediment of -2,400 ± 12,000 $\mu g/m^2/h$, which were significantly different. N_2 fixation rates were not significantly different among rock and sediment substrate with means of 22.9 ± 54.4 and 2.2 ± 2.0 $\mu g/m^2/h$, respectively. Unamended denitrification rates were significantly different among rock and sediment substrates with means of 3 ± 7 and 2248 ± 1565 $\mu g/m^2/h$, respectively. Amended denitrification rates were also significantly different among substrates with a mean of 352 ± 690 $\mu g/m^2/h$ on rocks and 18,100 ± 6287 $\mu g/m^2/h$ on sediment. DIN concentration was not a significant predictor of unamended denitrification rates, but was a significant predictor of N_2 flux and N_2 fixation rates on rocks in 2016, and amended denitrification rates on sediments in 2015 and 2016, indicating that DIN concentration alone cannot predict occurrence of processes on all

[1] The material contained in this chapter is in preparation for submission to *Biogeochemistry*

substrates at all times. Multiple linear regression models relating environmental variables to measured rates showed that carbon and phosphorus availability were important predictors of denitrification rates and phosphorus, carbon, and light availability were important predictors of N_2 flux rates across all sites. No significant model was produced for N_2 fixation rates. Environmental characteristics measured at the scale of entire stream-reaches may not be at a fine enough spatial scale to characterize and predict the co-occurrence of these processes within stream reaches. N_2 flux is balanced by the rates of N_2 fixation and denitrification, and in order to better understand the fluxes and cycling of N through stream ecosystems we need to examine the co-occurrence of these processes.

INTRODUCTION

Denitrification and nitrogen (N) fixation are both important nitrogen cycle processes in streams, yet the occurrences of both processes are rarely studied together in these ecosystems (An et al. 2001, Marcarelli et al. 2008). Denitrification is the microbial conversion of nitrate (NO_3^-) into N_2 gas, while N_2 fixation is the microbial conversion of N_2 gas into biologically usable N. Together, both processes control net N_2 fluxes in many aquatic 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 (Marcarelli et al. 2008). 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 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 has been observed to decrease when N availability is high (Grimm and Petrone 1997, Kunza and Hall 2013), while 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.

The assumption that increased N concentrations will cause N_2 fixation to cease while denitrification increases 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 anthropogenic-altered aquatic ecosystems (Seitzinger et al. 2006). In contrast there has been far less research into N_2 fixation 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 like 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 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 we need a better understanding of these processes in freshwater ecosystems, particularly through application of new technology.

In coastal regions, research into both N_2 fixation and denitrification has increased with the improvements of technology for measuring rates of each process as well as N_2 flux. Membrane inlet mass spectrometry (MIMS), a high precision technique that allows for the use of small sample sizes (An et al. 2001, Kana et al. 1994), created a way to collect large quantitates of accurate data on gas ratios in water samples in a short time period. In river channels the water column has been found to contribute to overall denitrification rates in addition to fluxes from sediment through MIMS analysis (Reisinger et al. 2016). In lake ecosystems epilimentic sediments switched from net denitrification to net N_2 fixation in response to the cycle of nitrate availability indicating that both processes are important to N_2 flux (Grantz et al. 2012). The use of MIMS in Texas estuaries demonstrated that the sources and sinks of N_2 are nearly balanced (Gardner et al. 2006). Other studies in coastal and marine areas have shown that both N_2 fixation and denitrification play a part in the balance of N_2 flux with sediment switching from a net sink to a net source of N over an annual cycle (Fulweiler et al. 2007, Fulweiler et al. 2013). These studies in ocean, lake, and coastal ecosystems in particular have shown that both processes play an important role in N cycling and the balance of N_2 flux. Despite these discoveries in other aquatic ecosystems, stream ecosystem research still

tends to favor studying one process over another, ignoring the possibility of co-occurrence.

The co-occurrence of both N_2 fixation and denitrification in streams could be affected by the loads and ratio of N and phosphorus (P) concentrations. In lakes, it has been observed that when N:P ratios were low, N_2 fixing cyanobacteria would dominate an otherwise nitrogen-limited phytoplankton community and at higher N:P ratios lakes would exhibit low proportions of N_2 fixing cyanobacteria (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 the whole lake 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 from reduced N loads in many cases (Lewis and Wurstbaugh 2008, Scott and McCarthy 2010), potentially because high denitrification rates may remove fixed N faster than it is produced via N fixation (Paerl and Scott 2010, Scott and Grantz 2013). This can result in co-occurrence of denitrification and N_2 fixation in lakes even when external nutrient loads are high (Scott and Grantz 2013), and lead 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 Wartsbaugh 2008, Paerl and Scott 2010). Therefore the N:P ratio could allow both processes to occur in a stream even if the overall N load may appear to be favorable for one process over the other.

The co-occurrence of both N_2 fixation and denitrification could also be facilitated by other key environmental variables. High availability of light and warm temperatures are favorable for cyanobacterial N_2 fixers (Scott and Marcarelli 2012, Grimm and Petrone 1997). 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 will have differing quantities of these variables along their reach, potentially creating preferable habitats for both types of organisms in the reach (Holmes et al. 1996, Dent and Grimm 1999). The overall differences in environmental variables of each stream then may create variation in conditions within 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, 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 dissolved inorganic nitrogen (DIN) concentrations. We hypothesized first that rates of denitrification and N_2 fixation would differ by substrate type, with higher rates of N_2 fixation on rocks, which provide stable, high light habitats for photosynthetic N_2 fixers, while denitrification rates would be higher on sediment, where anoxia is likely and organic matter availability should be high. We then 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 high rates of denitrification and low N_2 fixation and streams with low DIN would have

high rates of N_2 fixation and low denitrification. We also examined whether environmental variables such as light, temperature, chlorophyll a, organic matter, discharge, phosphorus, dissolved organic carbon, and N:P ratios interacted to control 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, while streams with more organic matter and higher DIN concentrations would favor higher rates of denitrification. Understanding whether these processes co-occur will 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.

STUDY AREA

This study was conducted in the Portneuf River watershed, located near Pocatello, Idaho, which drains a 3,445 km^2 basin (elevation 1,330 to 2,823 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 – 9.7 m^3/s over the last ten years (USGS Water Resources). Land use and irrigation impacts in this basin are typical of watersheds in the western United States (Marcarelli et al. 2010). 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). Bedrock geology

includes both basalt and sedimentary rock in the form of loess, silt, and volcanic ash (Hopkins et al. 2011, Barton 2004). Sub-watersheds have >16% of their surface area as volcanic rock with the highest being 46.5% (Table 1). The spatial heterogeneity of land and geological formations in this watershed cause the streams to encompass a wide range of N and P concentrations (Table 2).

STUDY DESIGN

To determine whether N_2 fixation and denitrification co-occur in streams we measured rates of N_2 fixation, denitrification, and N_2 flux. 8 streams were selected in 2015 to encompass a gradient of DIN concentrations (0.06 to 0.58 mg/L DIN) and variance in N:P ratios (0.60 to 18.13) based on prior studies (Bechtold et al. 2012 and Marcarelli et al. unpublished), and differences in land use and bedrock geology (Tables 1, 2). 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: Portneuf at Upper Sportsman's Access. In 2016, we added one additional tributary site at Diggie Creek to expand the DIN gradient of streams included in our study (0.62 mg/L DIN) and due to the high abundance and large size of the cyanobacterial colonies in this stream (Figure 1).

In summer 2015, each site was visited once and rates of N_2 fixation, denitrification, and N_2 flux were all measured on the same day. In 2015, N_2 fixation was only measured on rock substrate and denitrification was only measured on sediment substrate because we chose the substrate 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 first hypothesis and thus we expanded in 2016 to measure both rates on both rock and sediment substrates. In 2016, each site was visited two days in a row, where N_2 flux was measured both days and N_2 fixation or denitrification were measured on separate days. In 2016, we also measured rates on macrophytes at the Upper Portneuf site only because macrophyte was a dominant substrate at this site.

N_2 fixation, denitrification, and N_2 flux rates were measured by acetylene reduction, acetylene block, and MIMS techniques, respectively. Chambers used for these techniques varied by substrate type. 2-L polycarbonate Cambro food storage containers were used for rock and macrophyte substrate (Gettel et al. 2007, Figure 2A). The chamber lids were sealed airtight with a Viton o-ring, and lids were fit with a 13x20 mm septa for sample collection. For sediment substrate, chambers were made from quart size glass mason jars in 2015 and pint size glass mason jars in 2016 (Figure 2B), and lids were similarly fit with an airtight sampling septa.

Rock substrate was collected by haphazardly sampling rocks from the study area until the bottom of the polycarbonate chamber was covered (Figure 3A). Sediment substrate 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 (Figure 3B). Macrophyte substrate was collected using the 2-L polycarbonate chamber lid to approximate surface area of macrophyte to sample. Macrophytes were pulled from the root and placed in chambers. On each day in both years, N_2 flux was measured first and then chambers were kept with the same substrate to measure N_2 fixation or denitrification rates. This allowed N_2 fixation and denitrification rates to be

measured mid-day during peak hours of activity and potentially to estimate N_2 fixation or denitrification contributions to N_2 flux.

N_2 Flux

N_2 flux measurements were used to examine the overall rate of N_2 production or consumption as driven by denitrification and N_2 fixation together. N_2 flux measurements were determined using MIMS and the N_2/Ar technique (Kana et al. 1994, An et al. 2001). Measuring changes in ambient N_2 concentrations can be difficult because deviations from equilibrium concentrations are affected by both biological and physical processes and the changes in flux can be very small (<1% deviations), so in order to capture changes it is necessary to measure dissolved gases at high precision such as with the N_2/Ar technique (Kana et al. 1994). Ar is affected only by physical processes, so using this as a tracer allows for the separation of physical and biological driven processes contributing to the flux. A total of 12 chambers were used per substrate (rock, sediment, and macrophyte). All 12 chambers were randomly assigned into categories: 3 were blanks, 3 were initials, and 6 were samples. 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 substrates and to correct for a change in temperature due to physical processes. 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 substrates. The initial and sample chambers had stream rock, sediment, or macrophyte placed in them.

Chambers were filled with substrate and streamwater then sealed underwater without the presence of air bubbles. Initial water samples were collected at time 0 in triplicate by siphoning from the 3 assigned initial chambers. The 9 remaining chambers were then incubated in the stream for 2-hours to maintain ambient stream temperatures. Final water samples were collected in triplicate from the remaining chambers at the end of the incubation period. All water samples were collected in 12-mL exetainers, preserved by the addition of 0.16 mL of 50 g/100 mL zinc chloride, and later analyzed in the lab using MIMS to determine ambient N_2/Ar ratios. The change in N_2 concentration over the incubation period was determined as: (Equations 1, 2, and 3, Kana et al. 1994).

$$(1)\ N_2 = \frac{N_2}{Ar} \times Ar_{sat\ (Temp., BP)}$$

$$(2)\ \Delta N_2 = \frac{F - I}{T}$$

$$(3)\ N_2\ Flux = \left(\frac{\Delta N_2}{area} \times A\right)$$

Where Ar_{sat} is the predicted Ar concentration at air saturation from Colt (2012) for specific temperature and barometric pressure (mg/L), F is the N_2 concentration of final samples (mg), I is the sample N_2 concentration of initial samples (mg), T is incubation time (h), ΔN_2 is change in concentration in sample or blank chamber (mg/h), A is sample water volume (L), and area is the surface area of the substrate (m^2).

N_2 flux rates are of positive and negative magnitude. Rates that are positive are indicative of denitrification because this process releases N_2 into the atmosphere. N_2 flux rates that are negative are indicative of N_2 fixation because this process removes N_2 from

the atmosphere. Though the positive and negative N_2 flux rates can be attributed to one net process, they do not tell you the actual magnitude of each individual process.

N_2 Fixation

N_2 fixation rates were measured using acetylene reduction (Capone 1993). After collection of the MIMS samples, an acetylene-filled balloon was added to the 6 sample chambers and 3 blank chambers to achieve a 20% acetylene headspace. Chambers were filled with streamwater and sealed underwater, then balloons were popped with a needle through the sampling septum to introduce a headspace. Chambers were then shaken for approximately 20 seconds to equilibrate the gas dissolved in the water with that in the headspace. Initial gas samples were collected within 10 minutes of sealing the chambers. Chambers were placed in the stream for a 2-hour 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 known standard concentrations of 100 ppm ethylene (Matheson Tri Gas). N_2 fixation rates were calculated as: (Equation 4 & 5, Capone 1993).

$$(4)\ SC = 1 + (\beta \times \tfrac{A}{B})$$

$$(5)\ \text{Sample} = \frac{\text{Peak Height}_{\text{sample}}}{\text{Peak Height}_{\text{standard}}} \times C_{\text{standard}} \times B \times SC$$

Where SC is the solubility correction, β is the saturation concentration of gas of interest, A is total water volume (mL), B is headspace volume (mL), Sample is the concentration of the gas of interest in a given sample (nmol), $\frac{\text{Peak Height}_{sample}}{\text{Peak Height}_{standard}}$ is the ratio of peak heights of the gas of interest from the sample and standard, and $C_{standard}$ is the concentration of the gas of interest standard (nmol/mL). The rates were then converted to μg of N assuming a ratio of 3 mols 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). The rates of denitrification were measured as unamended and amended rates. After conclusion of the MIMS incubation, 3 sample chambers were randomly chosen as unamended and received chloramphenicol only (2 g/L), and 3 chambers were chosen as amended and received nutrient amendment (Glucose (0.62 g/L), $NaNO_3$ (0.62 g/L)) plus chloramphenicol. Chloramphenicol was used to suppress additional protein synthesis during the incubation and nutrient amendments were used to measure the potential for denitrification in the absence of nutrient limitation. We measured potential rates because most previous stream studies measured nutrient-amended denitrification rates and we wanted to compare these studies (Marcarelli et al. 2008). The acetylene block method also inhibits nitrification, which produces nitrate, so measuring without amendment solutions can underestimate denitrification rates (Dodds et al. in press). 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 ECD. The column oven was set to 40 °C. N_2O concentrations in chambers were compared to standard concentrations of 1000 ppm N_2O (Matheson Tri Gas). Denitrification rates were calculated using equations 4 and 5 above (Capone 1993).

Substrate Analysis

To scale process rates by substrate area and/or biomass, all substrate material (sediment and algal material from rocks) was collected and analyzed after incubations. Algal material on rocks was analyzed for chlorophyll a to provide an estimate of algal biomass. The algal material was collected by scrubbing the substrate and filtering the produced filtrate through pre-ashed GF/F filters and then freezing for laboratory analysis following 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 then oxidized in a muffle furnace at 550°C, rewetted, and dried before weighing. Surface area and volume of all substrates was also measured to scale process rates for biomass and surface area. Surface area for rocks was determined from tracings of the rocks that were weighed. The weights were then compared to a standard curve to calculate area. Sediment surface area was calculated as the uppermost exposed layer by using 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 in the jar.

Environmental Characteristics

To test the second hypothesis of DIN relationships with N_2 fixation, denitrification, and N_2 flux rates, streamwater was collected for nutrient analysis upstream of each incubation site. The water was filtered using 0.45 μm HA filters into 60 mL Nalgene bottles. Samples were frozen until later laboratory analysis for nitrate (NO_3^-) and ammonium (NH_4^+). NH_4^+ was analyzed using a fluorometeric method (Holmes et al. 1999, Taylor et al. 2007) on a Turner Aquafluor (Turner Designs, Palo Alto California). NO_3^- samples from 2016 were analyzed via the cadmium reduction method on an auto analyzer by the University of Michigan Biological Station Analytical Lab and in 2015 they were analyzed on a Dionex ICS-900 Ion Chromatograph (Dionex, Sunnyvale California). DIN concentration was then calculated by adding the concentrations of NH_4^+ and NO_3^-.

To test our final hypothesis of environmental variables as predictors of N_2 fixation, denitrification, and N_2 flux, we measured canopy cover (%) using a spherical densitometer (Lemmon 1956). Discharge (L/s) was measured using a Marsh McBirney Flo-mate attached to a wading rod to measure velocity (m/s) 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), pH, turbidity (NTU), ODO saturation (%), and ODO concentration (mg/L) upstream of the incubation site for the duration of the incubations. Water samples were filtered using 0.45 μm HA filters into 60 mL Nalgene

bottles and were kept frozen until lab analysis for dissolved organic carbon (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 10s UV-Vis spectrophotometer using the ascorbic acid method and molybdenum antimony colorimetric determination methods (APHA 2005). For TDP samples, an ammonium persulfate digestion was used prior to this analysis.

Statistical Analysis

To test the first hypothesis that rates of N_2 fixation, denitrification, and N_2 flux would be different depending on stream substrate, we performed two-way ANOVA or t-tests. First, to examine whether N_2 flux differed between blanks and sample chambers in the same stream, both blank and sample N_2 fluxes were plotted and analyzed using a paired two sample t-test. We then performed a two-way ANOVA for N_2 flux rates including both 2015 and 2016 data. The N_2 flux rates used in the two-way ANOVA and later analysis were the difference in N_2 flux between sample and blank chambers. A paired two sample t-test was also used to evaluate if the mean rates of N_2 fixation and denitrification (both amended and unamended) were significantly different by rock and sediment substrate only for the year 2016, because in 2015 we did not measure both rates on all substrate types. N_2 fixation rates failed to meet normality and equal variance assumptions so they were log transformed for all analyses. The ANOVA and t-test analyses were performed in R (version 3.2.2, R Foundation for Statistical Computing).

To test our second hypothesis we used simple linear regression to evaluate DIN concentrations as a predictor of rates of N_2 fixation, denitrification, and N_2 flux. Simple linear regression analyses were performed in R (version 3.2.2, R Foundation for Statistical Computing).

To test our third hypothesis that a combination of environmental variables may better predict process rates than DIN alone, we first performed a principal components analysis (PCA) to compare environmental characteristics among streams and to create new variables to be used in later analyses. The 14 environmental factors included in this analysis were NH_4^+, NO_3^-, DIN, SRP, TDP, the ratio of DIN:TDP, DOC, TDN, canopy cover (CC), average temperature (TEMP), discharge (Q), average biofilm organic matter (BM), average sediment organic matter (OM), and average chlorophyll a (ChlA). Strong loadings for each PCA axis were determined from loading values that described the correlation between the specific variable and the specific PCA axis. Loading values farther from 0 were considered to have stronger loadings. The PCA was performed using JMP Pro (version 13.0.0, SAS Institute, Inc.).

Following the PCA, multiple linear regression was used to identify significant predictors of rates of N_2 fixation, denitrification, and N_2 flux for all streams. We ran two separate models: (1) with only environmental variables as predictors and (2) with only PC axes as predictors. Prior to model selection, we removed some predictors due to significant correlations with other predictor variables ($p < 0.05$). Predictors were also tested against the 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 performed in R (version 3.2.2, R Foundation for Statistical Computing).

RESULTS

Rate Comparison by Substrate

To evaluate our hypothesis that higher rates of denitrification would occur on sediments and higher rates of N_2 fixation would occur on rocks, we compared rates of N_2 flux, N_2 fixation, and denitrification by substrate type. In 2015, N_2 flux rates on rocks ranged from -18,682 to -7,297 $\mu g/m^2/h$ with a mean $\pm$ standard deviation (s.d.) of -11,999 $\pm$ 4,959 $\mu g/m^2/h$ and in 2016 they ranged from -42,245 to -2,971 $\mu g/m^2/h$ with a mean $\pm$ s.d. of -13,858 $\pm$ 13,772 $\mu g/m^2/h$ (Figure 4a, 4b). In 2015, N_2 flux rates on sediments ranged from -18,682 to 15,157 $\mu g/m^2/h$ with a mean $\pm$ s.d of -2,410 $\pm$ 11,748 $\mu g/m^2/h$ and in 2016 they ranged from -1,269 to 4,208 $\mu g/m^2/h$ with a mean $\pm$ s.d. of 1,753 $\pm$ 2,110 $\mu g/m^2/h$. In 2016, N_2 flux rate on macrophytes was -30,203 $\mu g/m^2/h$ at the only site it was measured. In 2015, when comparing N_2 flux rates from blanks and the respective paired samples there was a significant difference between blank and sample N_2 flux rates on rocks ($t = 6.40$, $df = 6$, $p = < 0.01$), but not on sediment ($t = 0.54$, $df = 6$, $p = 0.61$). In 2016 this difference was significant on rocks ($t = 2.85$, $df = 7$, $p = 0.02$) and on sediment ($t = -2.35$, $df = 7$, $p = 0.05$). N_2 flux rates did differ significantly by substrate type ($p < 0.01$, $F_{1, 26} = 13.69$), but not by year ($p = 0.74$, $F_{1, 26} = 0.11$) or the interaction between substrate and year ($p = 0.39$, $F_{1, 26} = 0.75$).

N_2 fixation rates measured via acetylene reduction differed between substrate types, but those differences were not significant (Figure 5a, 5b). In 2015, N_2 fixation rates on rocks ranged from 0 to 198 μg N/m^2/h with a mean ± s.d. of 22.9 ± 54.4 and in 2016 they ranged from 0 to 218 μg N/m^2/h with a mean ± s.d. of 26.6 ± 54.7 μg N/m^2/h. In contrast in 2016, N_2 fixation rates on sediments ranged from 0 to 9 μg N/m^2/h with a mean ± s.d. of 2.2 ± 2.0 μg N/m^2/h, which was considerably lower than those measured on rocks. Also in 2016, N_2 fixation on macrophytes was 23 μg N/m^2/h at the only site where it was measured, which was similar to the average N_2 fixation rates measured on rocks across all sites. In 2016, log transformed N_2 fixation rates were not significantly different between sediment and rock substrate ($t = 1.72$, $df = 7$, $p = 0.13$). This is most likely because besides the one site with high N_2 fixation rates, rates on sediment and rock substrate were of a similar magnitude. Scaling per unit biomass for all N_2 fixation rates did not change patterns (Figure 6, Figure 7).

Both amended and unamended denitrification rates measured via acetylene block differed significantly by substrate type (Figure 5c-f). In 2016, amended denitrification rates on rocks ranged from 0 to 1864 μg N/m^2/h with a mean ± s.d. of 352 ± 690 μg N/m^2/h, while in 2016 unamended denitrification rates ranged from 0 to 20 μg N/m^2/h with a mean ± s.d. of 3 ± 7 μg N/m^2/h (Figure 5e, 5c). In 2015, unamended denitrification rates on sediments ranged from 531 to 5130 μg N/m^2/h with a mean ± s.d. of 2248 ± 1565 μg N/m^2/h and in 2016 they ranged 367 to 2020 μg N/m^2/h with a mean ± s.d. of 1137 ± 672 μg N/m^2/h (Figure 5d). In 2015, amended denitrification rates on sediments ranged from 10697 to 26570 μg N/m^2/h with a mean ± s.d. of 18100 ± 6287

μg N/m^2/hr, and in 2016 they ranged 2046 to 16909 μg N/m^2/h with a mean ± s.d. of 8527 ± 5828 μg N/m^2/hr (Figure 5f). Also in 2016, macrophyte denitrification at the only site where it was measured was 22.9 and 329 μg N/m^2/h for amended denitrification and unamended denitrification rates, respectively. In 2016, unamended denitrification rates did differ significantly between rock and sediment ($t = -4.76$, $df = 7$, $p < 0.01$). Amended denitrification rates differed significantly as well between rock and sediment ($t = -3.68$, $df = 7$, $p = < 0.01$). Scaling per unit biomass for all denitrification rates did not change patterns (Figure 6, Figure 7).

DIN as a Predictor of Process Rates

To test our hypothesis 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 high rates of denitrification and low N_2 fixation and streams with low DIN would have high rates of N_2 fixation and low denitrification, we compared rates of N_2 flux, N_2 fixation, and denitrification to DIN concentrations using linear regression. In 2015 the highest positive N_2 flux rate on sediment was observed in a stream with moderate DIN concentration (~ 110 μg/L, Figure 8). In 2016, the highest positive N_2 flux on sediments occurred in the stream with the highest DIN concentration (615 μg/L). For both years negative N_2 flux was observed on rocks, suggesting net N_2 fixation and some negative N_2 flux was observed on sediments. As a predictor of N_2 flux rates on sediments, DIN concentration was not significant (Table 3). As a predictor of N_2 flux rates on rocks, DIN was only a significant predictor in 2016 (Table 3).

The highest 2015 N_2 fixation rate on rocks was observed in one of the streams with the lowest DIN concentration (12.5 μg/L, Figure 8). The highest 2016 N_2 fixation rate on rocks also occurred in the same stream, although the DIN concentration was higher in 2016 than 2015 (40.9 μg/L). In both years streams with higher DIN concentrations (> 350 μg/L) did not have the lowest N_2 fixation rates and streams with more intermediate DIN concentrations (~ 100 – 300 μg/L) had some of the lowest N_2 fixation rates observed. DIN concentration was a significant predictor of N_2 fixation rates on rocks in 2016, but the slope of the relationship was near zero, suggesting rates were not changing much in response to changes in DIN concentrations (Table 3). The stream with the highest DIN concentration (615 μg/L) had the lowest N_2 fixation rate on sediments, but the stream with the second highest DIN concentration in 2016 (506 μg/L) had the highest N_2 fixation rate on sediments. DIN concentration was not a significant predictor of N_2 fixation rates on sediment (Table 3).

The highest amended and unamended denitrification rates on rocks occurred in South Fork, a stream with low DIN concentration (40.9 μg/L), which also had the highest rates of N_2 fixation. DIN concentration was not a significant predictor of unamended or amended denitrification rates on rocks. In both years, the highest unamended denitrification rate on sediments occurred in Lower Mink Creek, which had intermediate DIN concentrations (170 – 298 μg/L). DIN concentration was not a significant predictor of unamended denitrification rates on sediments for both years (Table 3). However, DIN concentration was a significant predictor of amended denitrification rates on sediments (Table 3). In both years the lowest amended denitrification rate occurred in the same low

DIN concentration stream (< 50 µg/L) and the highest rate occurred in the stream with the highest DIN concentration in that year (505 and 615 µg/L, respectively).

Other Environmental Factors as Predictors

The PCA model of environmental factors identified four principal components (PCs) that explained 86% of the variation in the model. The first PC axis explained 43% of the variability in the model and had strong positive loadings (> 37%) from NO_3^-, DIN, DIN:TDP, DOC, TDN, discharge, average temperature, biofilm organic matter, and chlorophyll a, and had strong negative loadings from canopy cover (Figure 9, Table 4). The second PC axis explained 21.2% of the variability in the model and had strong positive loadings from NH_4^+, TDP, organic matter content, and DOC, and had strong negative loadings from biofilm organic matter and chlorophyll a (Figure 9, Table 4). PC 3 explained 11.9% of the variation in the model and had strong positive loadings from SRP, TDP, and average temperature and strong negative loading from NH_4^+ and organic matter content (Table 4). PC 4 explained 9.8% of the variation in the model and had strong positive loadings from NH_4^+, average temperature, average biofilm organic matter, average chlorophyll a, and average organic matter content and, had strong negative loading from DIN:TDP (Table 4).

To test our hypothesis that a combination of environmental variables and DIN would be a better predictor of rates of each process than DIN alone, we performed stepwise multiple linear regression. To decide the best variables to use in the models we examined a correlation matrix of all variables including the PC axes (Table 5). Based on these results, we selectively removed NO_3^-, NH_4^+, SRP, TDN, and Q (discharge L/s), so

the original input model without PC axes included: DIN, TDP, DIN:TDP, DOC, canopy cover, temperature, sediment organic matter content, average chlorophyll a, and average biofilm organic matter. The original input model for PC axes included PC1, PC2, and PC3. PC4 was not included because it explained <10% of the variation in the model.

Stepwise multiple linear regression models did provide significant predictors for N_2 flux and denitrification rates. For N_2 flux, there was 1 significant stepwise multiple linear regression model with environmental variables and no significant models with PC axes (Table 6, 7). The best model based on environmental variables explained 35% of the variance and included TDP, DIN:TDP, DOC, canopy over, and organic matter content (Table 6). The best model based on PC axes explained 11% of the variance and included PC1, which had strong positive loadings from NO_3^-, DIN, and TDN (Table 7). For N_2 fixation, stepwise multiple linear regression resulted in no significant models that predicted N_2 fixation rates (Table 6, 7). For amended denitrification, there were 4 significant multiple regression models with environmental variables including the full model, and 3 significant models with PC axes. The best model based on environmental variables explained 75% of the variance and included DIN:TDP, DOC, average temperature, and organic matter content (Table 6). The best model based on PC axes for amended denitrification rates explained 32% of the variance and included PC2, which had strong positive loadings from DOC and organic matter content (Table 7). For unamended denitrification, there were 5 significant models with environmental variables and 3 significant models with PC axes (Table 6, 7). The best model of environmental variables explained 72% of the variance and included TDP, canopy cover, and organic

matter content (Table 6). The best PC axes model explained 47% of the variance and included PC2, which has strong positive associations with carbon sources (Table 7).

DISCUSSION

N_2 fixation and denitrification co-occurred across all of our study streams. N_2 flux rates ranged from positive to negative, indicating both denitrification and N_2 fixation contributed to N_2 flux in these streams. N_2 fixation rates from acetylene reduction were approximately 10 to 100 times lower than denitrification rates from acetylene block and 100 times lower than N_2 flux rates. DIN concentrations were significantly related to amended denitrification rates on sediment in both years and N_2 flux and N_2 fixation on rock in 2016, but not unamended denitrification rates on either substrate in either year. When other environmental factors were included as predictors, organic matter content, either alone or as part of PC2, and phosphorus concentrations were part of significant models predicting denitrification rates. For N_2 flux rates, the significant model included phosphorus concentrations, organic matter content, and canopy cover as significant predictors. No significant environmental models predicted N_2 fixation rates across all substrates, streams, and study dates. Our observations of both N_2 fixation and denitrification co-occurring across all streams 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.

N_2 flux rates for our study streams were relatively similar or higher than the reported N_2 flux values in other aquatic ecosystems. One study in Waquoit Bay observed

ranges of net denitrification from 0 to 784 μg/m^2/hr (Newell et al. 2016). In freshwater ecosystems, N_2 flux in sediments ranged from -7560 to 5152 μg/m^2/hr in a wetland and from non-detectable to 1800 μg/m^2/hr in a river system (Scott et al. 2008, Reisinger et al. 2016). In estuaries, rates of N_2 flux have been reported to range from -700 to 14,840 μg/m^2/hr and 1960 to 2800 μg/m^2/hr on sediment cores (Fulweiler et al. 2007, Gardner et al. 2006), which were somewhat similar to the range of N_2 flux rates in our study streams. N_2 flux rates for stream ecosystems have not been estimated previously, but even our high-nutrient study streams have low nutrient concentrations when compared to many eutrophic systems, so it may be expected that the N_2 flux rates from this study should be of lower magnitude than those published previously from eutrophic systems, which contradicts what we measured. Statistical analysis shows that the N_2 flux rates of our samples and blanks were significantly different for both rock and sediment in 2016, but only rock in 2015. This suggests that we are detecting biologically-driven N_2 fluxes in most of our incubations.

Because both N_2 fixation and denitrification contribute to overall N_2 flux, and because the unamended denitrification rates from acetylene block were overall higher than N_2 fixation rates from acetylene reduction, we would expect positive N_2 flux on most sites and dates. This does not match our observations, where we saw mostly negative N_2 fluxes. The N_2 fluxes were similar in order of magnitude to amended denitrification rates, but there were few positive fluxes. This difference in direction suggests a large discrepancy between indirect acetylene based assays and direct measurements of N_2 flux using MIMS. This contrasts with a previous study comparing

denitrification estimates from MIMS and acetylene inhibition methods where no significant differences between methods was detected (Bernot et al. 2003). Our N_2 flux rates may be so much higher than the acetylene rates because of air bubble formation (mostly observed with rock substrate during the N_2 flux incubation period), or due to possible issues sealing the incubation chambers. Due to the discrepancy in rates between the methods, we are not currently able to partition the N_2 flux into N_2 fixation and denitrification.

Our results suggest that the rates of N_2 fixation and denitrification in these stream ecosystems cannot be predicted by DIN concentrations alone. N_2 flux and N_2 fixation rates were significantly related to DIN concentrations in 2016 on rocks, but not in 2015, suggesting that the observed linear pattern may not consistently capture the relationship between N_2 fixation rates and DIN. 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 Wurstbaugh 2007, Kunza and Hall 2013). In one study, rates of N_2 fixation were high only when nitrate concentrations were < 20 μg/L, indicating a nutrient threshold for N_2 fixation activity (Kunza and Hall 2014). This is not unlike what we observed for N_2 fixation on rock, where high rates dropped off above ~ 45 μg/L. However, low N_2 fixation rates were observed below this threshold as well, indicating other environmental variables may be constraining or limiting the process rates. This has been observed in the Great Salt Lake, where high rates of N_2 fixation occurred below a salinity threshold, but below the threshold phosphorus concentrations further limited N_2 fixation (Marcarelli et al. 2006). Amended denitrification rates were positively and

linearly related to DIN concentrations, which is similar to previous observations where increasing nitrate concentrations have been shown to increase denitrification rates (Seitzinger 1988, Holmes et al. 1996, Seitzinger et al. 2006). In contrast, unamended denitrification rates were not linearly related to DIN concentration. The different responses of amended and unamended denitrification to increases in DIN concentration in streamwater point to carbon as an important control of denitrification rates. Without amended carbon and nitrogen, denitrification rates did not respond to streamwater DIN concentrations. Since both amended and unamended denitrification samples were exposed to the same concentrations of streamwater DIN, but only amended denitrification rates increased as DIN increased, this suggests that the amended carbon source was the important limiting factor for denitrification.

Similarly, environmental factors other than DIN appeared to be important for explaining denitrification rates across sites. Multiple linear regression models for both amended and unamended denitrification rates included predictors related to carbon sources (DOC, organic matter content, and PC2), and phosphorus availability (DIN:TDP and TDP). Organic matter as a source of carbon has been shown to be a limiting factor for denitrification rates (Holmes et al. 1996, Arango et al. 2007), and our findings similarly implicate that denitrification rates are limited by carbon availability. Phosphorus availability also appeared to be important for denitrification rates, with increases in TDP concentration leading to increases in unamended rates and increases in DIN:TDP leading to increases in amended denitrification rates. The relationship of phosphorus availability to denitrification rates suggests that more phosphorus facilitates

higher denitrification rates in streams where phosphorus is limited relative to nitrogen. Similarly, phosphorus-limited lake ecosystems have been shown to have increased rates of nitrogen removal after lake phosphorus inputs were increased (Finlay et al. 2013). The mechanism proposed behind this phenomenon in lakes is that additional phosphorus stimulates algal production and N uptake and when the algae die they end up in the sediments delivering N and organic matter, which increase denitrification rates (Finlay et al. 2013). Multiple linear regression models for N_2 flux rates also included predictors related to light availability (canopy cover), carbon sources (DOC, organic matter content) and phosphorus availability (DIN:TDP and TDP), variables known to effect both denitrification and N_2 fixation. No significant multiple linear regression models were found for N_2 fixation rates. It has been shown that phosphorus availability can be an important limiting factor, particularly for N_2-fixing bacteria (Elwood et al. 1981, Marcarelli and Wurtsbaugh 2007), along with light availability and temperature (Finlay et al. 2011, Welter et al. 2015). In this study, however these environmental variables were not found to be good predictors of N_2 fixation rates, which could be because our stream-reach scale measurements of environmental variables did not adequately capture the sub-reach variability in resources predicting rates of these processes.

Our study did not address fine scale differences in environmental characteristics, which could have been important in explaining the environmental variables that facilitate the co-occurrence of N_2 fixation and denitrification that we observed in our study streams. Stream ecosystems are characterized by high degrees of spatial and temporal heterogeneity (Dent and Grimm 1999). Patches, or spatially-related areas that control

ecosystem structure and function, are created by this heterogeneity (Pringle et al. 1988). In past studies, spatial heterogeneity in DIN and nitrate concentrations have been shown to affect the spatial distribution of N_2-fixing organisms (Henry and Fisher 2003, Dent and Grimm 1999). Denitrification rates have been shown to vary spatially with organic matter availability and temperature (Holmes et al. 1996, Groffman et al. 2005). Both N_2 fixation and denitrification rates have also been shown to vary among substrate 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 agree with our findings. Spatial heterogeneity in oxygen availability on a centimeter scale effects rates of nitrification (Kemp and Dodds 2001), indicating heterogeneity in resources on the finest of scales can influence biogeochemical processes. These patch-scale differences in resources could explain why we saw relatively high rates of N_2 fixation on macrophyte and rock substrate in streams with relatively high DIN concentrations. The substrates in these systems 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, creating hotspots of N_2 fixation in an otherwise high denitrification stream (McClain et al. 2003). These patches or hotspots where local conditions are favorable can have disproportionate contributions to ecosystem nutrient fluxes in unfavorable average conditions (McClain et al. 2003), thereby permitting co-existence of both processes. When examining the effect of environmental variables on the co-occurrence of N_2 fixation and denitrification in streams, a patch scale approach may more accurately

capture differences and characterize environmental factors that control rates of these processes.

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, and that rates are related to a number of environmental variables and only occasionally to DIN alone. This finding of N_2 fixation and denitrification co-occurring in streams is similar to recent findings in coastal marine ecosystems where it has also been shown that both processes contribute to N_2 flux (Fulweiler and Heiss 2014). Furthermore, wider recognition of the occurrence of N_2 fixation in oceans has transformed the paradigm that this process was negligible in relation to denitrification and has shown both processes are important to N cycling (Capone 2001). Therefore, understanding overall N_2 flux in stream ecosystems requires knowledge of both N_2 fixation and denitrification, and examining both processes simultaneously is required to accurately capture the balance between the two over time and space (Fulweiler et al. 2007, Newell et al. 2016). Furthermore, both N_2 fixation and denitrification are needed to understand the overall N cycle in streams, which is important when approaching management of aquatic ecosystems. Denitrification is typically thought of as the primary process in N management because it removes N from the system (Seitzinger 1988). In order to accurately understand the removal of N, though, one needs to also understand the relative input of N into the system from processes such as N_2 fixation. There are also other understudied pathways through which N may be removed, such as anammox, which removes N through the production of N_2 gas, or through dissimilatory nitrate reduction to

ammonium, which actually introduces more biologically reactive N into the system (Burgin and Hamilton 2007). The simultaneous input from N_2 fixation and removal by denitrification as well as potential contributions of understudied N cycling processes all suggest that the management of N in stream ecosystems is much more complex than just focusing on the removal by denitrification. Continuing to overlook the potential for co-occurrence of denitrification and N_2 fixation will impede our understanding of overall N cycling in stream ecosystems.

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Table 1. Watershed and geological characteristics for the 8 sampling streams sorted from low to high dissolved inorganic nitrogen (DIN). Ag is drainage area that is cultivated agriculture, and M.A.P. is mean annual precipitation. Primary, Secondary, and Tertiary group the type of rock found at each site. The type and age of rock found at each site came from the USGS mineral resources online spatial data program (http://mrdata.usgs.gov/). The data from the online spatial data program is specific to each coordinate for the stream, not the whole watershed. All other characteristics were from the USGS stream stats program for the whole watershed (http://water.usgs.gov/osw/streamstats).

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
Pebble Creek	76.43	1183	43	0.00	30	69.3	17.4	sandstone	conglomerate	siltstone, limestone	Pliocene
Cherry Springs	99.02	1146	30	0.22	30	66.3	19.3	limestone	dolomite	shale, arenite	Ordovician and Cambrian
South Fork	27.97	1000	36	0.78	22	64.3	46.5	alluvium	alluvial fan	alluvial terrace	Quaternary
Rapid Creek	144.62	1073	19	3.46	29	48.0	10.8	tholeiite	lava flow	gravel	Late Pleistocene
West Fork	17.28	546	50	0.00	30	74.7	24.4	limestone	dolomite	shale, arentie	Ordovician and Cambrian
Lower Mink	125.95	1283	24	0.61	31	63.8	16.7	sandstone	conglomerate	siltstone, limestone	Pliocene
Upper Portneuf	1240.16	1244	11	31.2	16	48.5	24.2	alluvium	alluvial fan	alluvial terrace	Quaternary
Diggie Creek	2.72	128	0	12.30	6	25.4	65.7	alluvium	alluvial terrace	floodplain	Quaternary

Table 2. Environmental characteristics for the 8 sampling streams. Nutrient and discharge data collected from site surveys in 2015 and 2016. Streams are arranged from low to high 2015 dissolved inorganic nitrogen (DIN) concentrations. DOC stands for dissolved organic carbon, TDP is total dissolved phosphorus, and TDN is total dissolved nitrogen concentrations. BDL stands for concentrations below the detection limits of the nutrient analysis (For NH_4 the average detection limit was 0.002 mg/L and for NO_3^- the average detection limit was 0.04 mg/L in 2015 and 0.001 mg/L in 2016).

Year	Stream	NO_3 (mg/L)	NH_4 (mg/L)	DIN (mg/L)	TDN (mg/L)	TDP (mg/L)	DIN:TDP	DOC (mg/L)	Discharge (L/s)	Canopy Cover (%)	Average Temp. (°C)
2015	Pebble Creek	BDL	0.006	0.01	0.13	0.01	0.60	2.35	24.70	61.6	14.8
	Cherry Springs	BDL	0.007	0.01	0.12	0.04	0.18	1.86	80.32	78.2	17.7
	South Fork	BDL	0.010	0.01	0.11	0.04	0.25	1.70	11.69	75.8	15.0
	Rapid Creek	0.11	0.004	0.11	0.20	0.06	1.83	2.63	121.36	26.5	15.6
	West Fork	0.23	0.004	0.24	0.15	0.02	12.00	1.29	33.07	70.1	14.5
	Lower Mink	0.28	0.010	0.29	0.39	0.03	9.67	2.30	100.68	33.5	17.5
	Upper Portneuf	0.57	BDL	0.58	0.72	0.03	19.33	5.75	3254.59	0.00	17.9
2016	Pebble Creek	0.06	0.008	0.07	0.16	0.00	20.30	1.74	182.37	68.4	16.3
	Cherry Springs	0.09	0.006	0.10	0.21	0.01	8.09	2.33	107.12	97.4	19.3
	South Fork	0.03	0.008	0.04	0.16	0.02	2.47	2.41	14.62	84.7	18.3
	Rapid Creek	0.18	BDL	0.18	0.32	0.03	6.66	2.63	43.92	41.2	19.4
	West Fork	0.17	0.002	0.17	0.20	0.02	10.10	1.47	47.22	31.3	15.4
	Lower Mink	0.16	0.008	0.17	0.32	0.01	16.26	2.37	74.77	36.4	18.8
	Upper Portneuf	0.51	BDL	0.51	0.80	0.02	25.49	3.87	2929.55	0.00	20.2
	Diggie Creek	0.62	0.002	0.62	0.99	0.01	103.41	1.81	N/A	0.00	18.6

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

Process Rate		R^2	F	P	S.E.E	Y-intercept	Slope
N_2 Flux	2015 Rock*	0.03	0.20	0.67	5327.00	-11182.67	-4.56
	2016 Rock	**0.53**	**6.88**	**0.04**	**10150.00**	**-2903.26**	**-47.48**
	2015 Sed*	0.17	0.99	0.36	11750.00	1614.16	-22.49
	2016 Sed	0.03	0.19	0.68	2243.00	1350.68	1.74
N Fixation	2015 Rock*	0.02	0.12	0.74	3.03	0.63	0.00
(log tranformed)	**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**	**13574.30**	**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

Table 4. Loading matrix of the four principal components for the PCA model of environmental characteristics. Loading values are the correlation between the variable and the principal component. Numbers in bold indicate strong positive or negative loadings i.e. their distance from 0.

	PC 1	PC 2	PC 3	PC 4
Ammonium	-0.14	**0.58**	**-0.47**	**0.52**
Nitrate	**0.94**	0.22	-0.11	-0.11
Dissolved Inogranic Nitrogen	**0.94**	0.23	-0.12	-0.10
Soluble Reactive Phosphorus	-0.37	0.28	**0.71**	-0.23
Total Dissolved Phosphorus	-0.08	**0.78**	**0.44**	0.17
ratio of N:P	**0.75**	-0.24	-0.35	**-0.37**
Dissolved Organic Carbon	**0.59**	**0.46**	0.35	0.34
Total Dissolved Nitrogen	**0.98**	0.09	-0.06	-0.07
Canopy Cover	**-0.77**	-0.38	-0.08	0.24
Temperature	**0.64**	-0.25	**0.41**	**0.40**
Discharge	**0.90**	0.24	0.03	0.01
Bioflim Organic Matter	**0.41**	**-0.77**	-0.02	**0.40**
Sediment Organic Matter	-0.19	**0.48**	**-0.45**	**0.45**
Chlorophyll a	0.44	**-0.69**	0.30	**0.41**

Table 5. Correlation and probability matrix of all possible predictors for multiple linear regression. Numbers in bold indicate statistically significant correlations. The R^2 values are on top and p-values are below and italicized for each variable row. NH4 (ammonium), NO3 (nitrate), DIN (dissolved inorganic nitrogen), SRP (soluble reactive phosphorus), TDP (total dissolved phosphorus), DIN:TDP (ratio N:P), DOC (dissolved organic carbon), TDN (total dissolved nitrogen), TEMP (temperature), CC (canopy cover), Q (discharge), BM (biofilm organic matter), OM (sediment organic matter content), ChlA (chlorophyll a), and PC # (PC axis).

	NH4	NO3	DIN	SRP	TDP	DIN:TDP	DOC	TDN	CC	TEMP	Q	BM	OM	ChlA	PC 1	PC 2	PC 3
NH4		-0.07	-0.05	-0.20	0.28	-0.24	0.26	-0.11	0.03	-0.22	0.04	-0.24	**0.59**	-0.45	-0.14	**0.58**	-0.47
		0.81	*0.86*	*0.47*	*0.30*	*0.39*	*0.36*	*0.70*	*0.91*	*0.44*	*0.88*	*0.38*	***0.02***	*0.09*	*0.61*	***0.02***	*0.08*
NO3			**1.00**	-0.32	0.07	**0.73**	**0.55**	**0.95**	**-0.77**	0.43	**0.88**	0.18	0.00	0.22	**0.94**	0.22	-0.11
			<0.01	*0.24*	*0.80*	***<0.01***	***0.03***	***<0.01***	***<0.01***	*0.11*	***<0.01***	*0.53*	*0.99*	*0.43*	***<0.01***	*0.43*	*0.70*
DIN				-0.33	0.08	**0.73**	**0.55**	**0.95**	**-0.77**	0.43	**0.88**	0.17	0.01	0.21	**0.94**	0.23	-0.12
				0.24	*0.78*	***<0.01***	***0.03***	***<0.01***	***<0.01***	*0.11*	***<0.01***	*0.54*	*0.97*	*0.45*	***<0.01***	*0.40*	*0.67*
SRP					**0.52**	-0.40	-0.01	-0.33	0.10	-0.09	-0.26	-0.40	-0.07	-0.23	-0.08	0.28	**0.71**
					0.04	*0.14*	*0.96*	*0.23*	*0.72*	*0.75*	*0.34*	*0.14*	*0.80*	*0.41*	*0.17*	*0.31*	***<0.01***
TDP						-0.41	0.41	-0.01	-0.19	-0.01	0.09	**-0.58**	0.34	-0.30	0.75	**0.78**	0.44
						0.13	*0.13*	*0.96*	*0.50*	*0.99*	*0.75*	***0.02***	*0.21*	*0.28*	*0.78*	***<0.01***	*0.10*
DIN:TDP							-0.01	**0.79**	**-0.52**	0.29	**0.56**	0.37	-0.20	0.27	**0.59**	-0.24	-0.35
							0.98	***<0.01***	***0.05***	*0.30*	***0.03***	*0.17*	*0.48*	*0.34*	***<0.01***	*0.39*	*0.20*
DOC								**0.53**	**-0.59**	0.43	**0.75**	0.02	-0.09	0.15	**0.98**	0.46	0.35
								0.04	***0.02***	*0.11*	***<0.01***	*0.93*	*0.75*	*0.60*	***0.02***	*0.08*	*0.20*
TDN									**-0.79**	**0.56**	**0.90**	0.31	-0.10	0.35	**0.99**	0.09	-0.06
									<0.01	***0.03***	***<0.01***	*0.26*	*0.73*	*0.20*	***<0.01***	*0.75*	*0.83*
CC										-0.40	**-0.72**	0.05	0.12	0.04	**-0.77**	-0.38	-0.08
										0.14	***<0.01***	*0.85*	*0.68*	*0.90*	***<0.01***	*0.16*	*0.76*
TEMP											0.44	**0.55**	-0.19	**0.69**	**0.63**	-0.25	0.41
											0.10	***0.03***	*0.50*	***<0.01***	***0.01***	*0.36*	*0.13*
Q												0.21	-0.17	0.23	**0.90**	0.25	0.03
												0.46	*0.55*	*0.41*	***<0.01***	*0.38*	*0.92*
BM													-0.26	**0.86**	0.41	**-0.77**	-0.02
													0.36	***<0.01***	*0.12*	***<0.01***	*0.94*
OM														-0.28	-0.19	0.48	-0.45
														0.32	*0.49*	*0.07*	*0.09*
ChlA															0.44	**-0.69**	0.30
															0.09	***<0.01***	*0.28*
PC 1																0.00	0.00
																1.00	*1.00*
PC 2																	0.00
																	1.00
PC 3																	

Table 6. Stepwise multiple linear regression models for rates of N_2 fixation, denitrification (both amended and unamended), and N_2 flux. DIN (dissolved inorganic nitrogen), TDP (total dissolved phosphorus), DIN:TDP (ratio N:P), DOC (dissolved organic carbon, TEMP (temperature), CC (canopy cover), and OM (organic matter content). Original models included all variables DIN, TDP, DIN:TDP, DOC, CC, TEMP, OM, and Chla.

Process Rate	Models	AIC	p	R^2	ΔAIC
N_2 Flux	-TDP-DIN:TDP-DOC-CC+OM	557.48	0.05	0.35	0
	-TDP-DIN:TDP-DOC-CC+TEMP+OM	558.88	0.08	0.36	1.40
	Orignial Model	560.71	0.13	0.37	3.23
N_2 Fixation	+DIN:TDP	34.74	0.30	0.05	0
(Log transformed)	+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
	Orginal 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

Table 7. Stepwise multiple linear regression models for rates of N_2 fixation, denitrification (both amended and unamended), and N_2 flux. PC # refers to the axis from our principal components analysis.

Process Rate	Models	AIC	p	R^2	ΔAIC
N_2 Flux	-PC1	558.97	0.07	0.11	0
	-PC1+PC3	559.81	0.12	0.14	0.84
	Original Model	561.81	0.25	0.14	2.84
N_2 Fixation	1	37.43	-	-	0
(Log transformed)	-PC1	38.55	0.21	0.07	1.12
	-PC1-PC3	40.10	0.47	0.07	2.67
	Original Model	41.94	0.73	0.06	4.51
Amended Denitrification	+PC2	429.11	<0.01	0.32	0
	+PC2-PC3	429.65	0.01	0.35	0.54
	Original Model	431.53	0.03	0.36	2.42
Unamended Denitrification	+PC2	330.80	<0.01	0.47	0
	+PC2-PC3	331.31	<0.01	0.50	0.51
	Original Model	333.32	<0.01	0.51	2.52

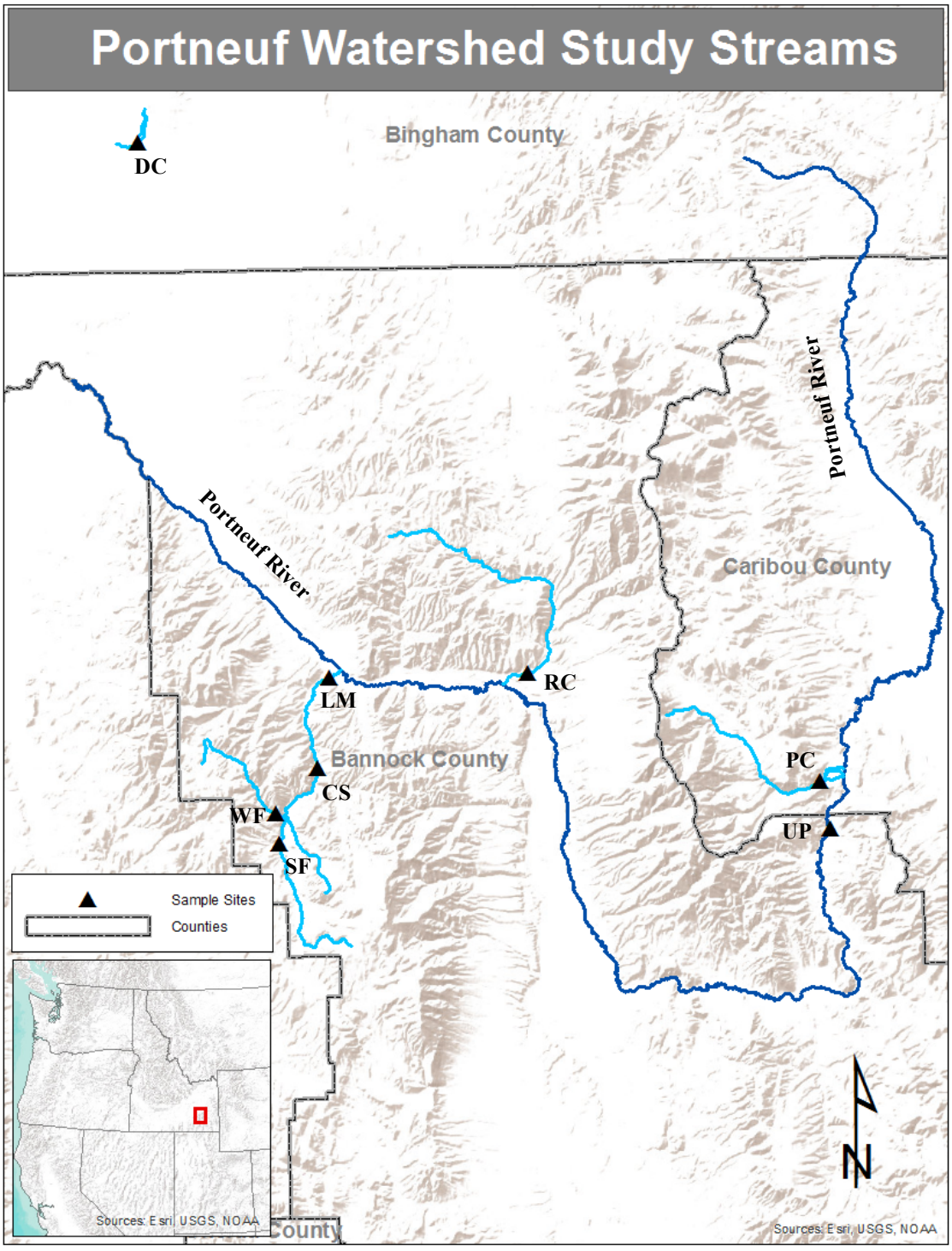

Figure 1. Location of the 8 study streams in southeastern Idaho. The Portneuf River is depicted in dark blue and tributaries are in light blue. Sites were abbreviated as follows: Pebble Creek (PC), Cherry Springs (CS), South Fork Mink Creek (SF), Rapid Creek (RC), West Fork Mink Creek (WF), Lower Mink Creek (LM), Upper Portneuf (UP) and Diggie Creek (DC).

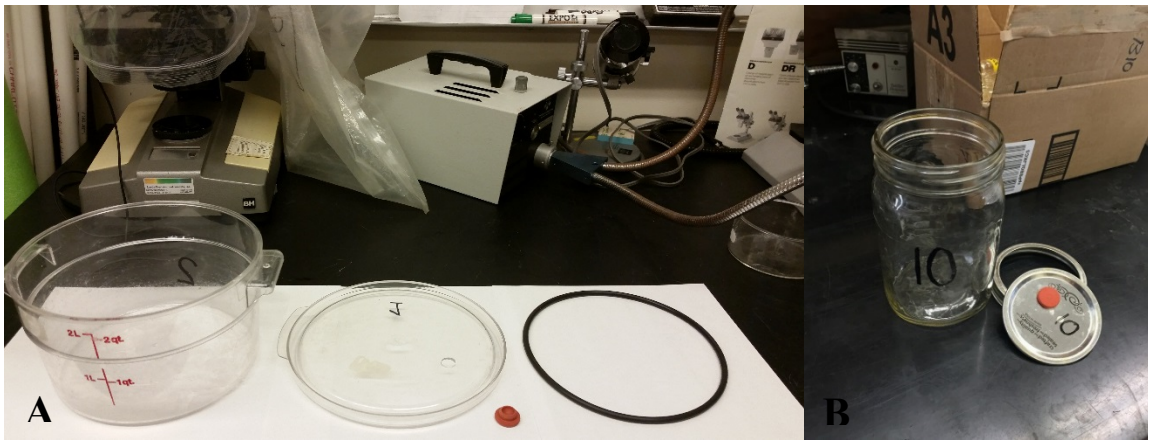

Figure 2. (A) From left to right, the 2-L polycarbonate chamber, lid with hole for septa, the 13x20mm sampling septa, and the Viton o-ring needed to seal the chamber. Chambers were used for rock and macrophyte substrate. (B) Mason jar and lid with septa inserted into hole in lid. Jars were used for sediment substrate.

Figure 3. (A) Rock substrate placed on the bottom of a 2-L polycarbonate chamber. (B) Sediment substrate placed in glass mason jar after suction coring. Both images represent how substrate was collected and placed in chambers for analysis.

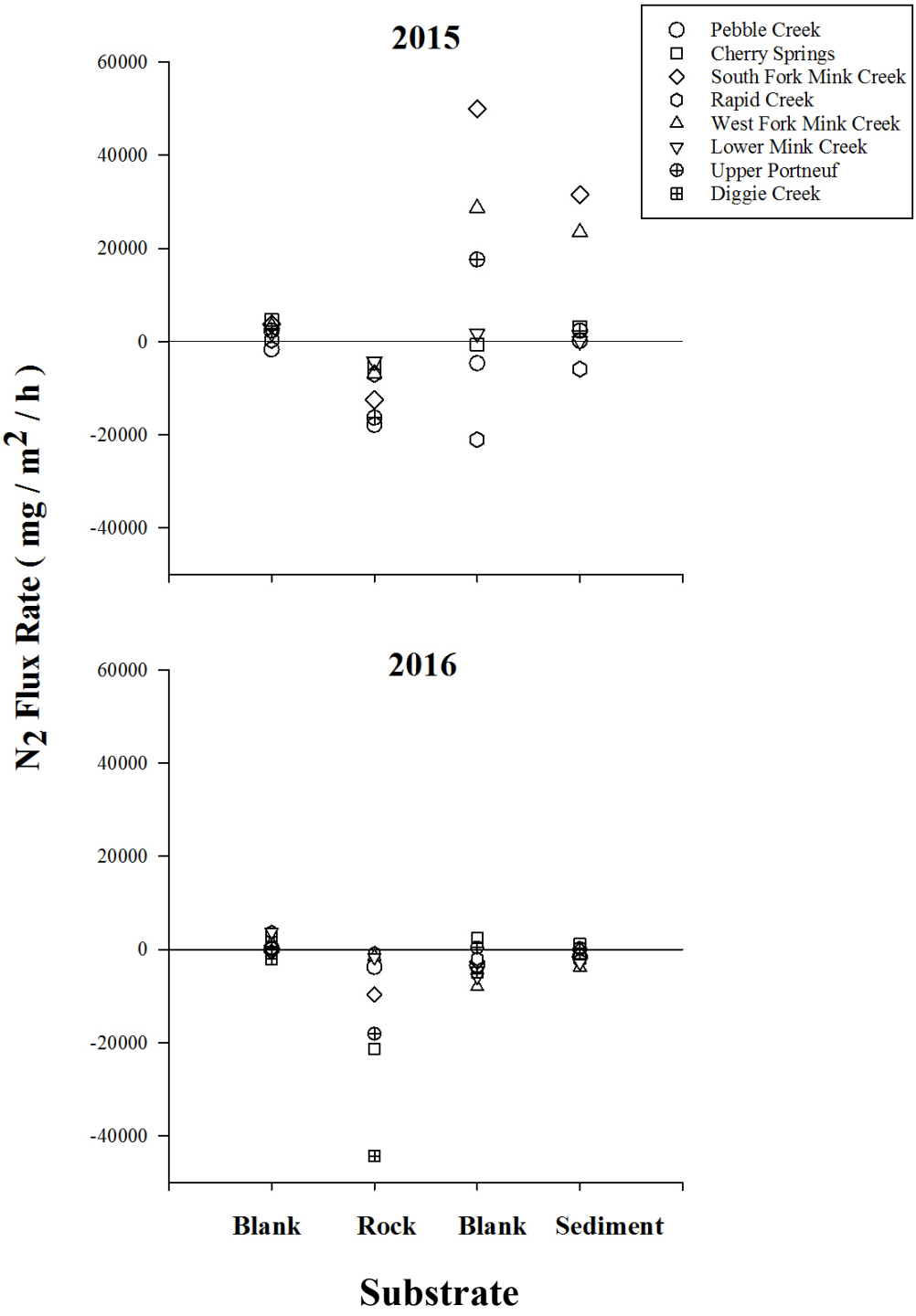

Figure 4. N_2 flux rates on rock and sediment substrate in all streams (n = 6 for each data point in 2015, n = 12 for each data point in 2016). The first blank values are rock substrate blanks and the second blank values are sediment substrate blanks. Symbols visually link substrates to specific streams.

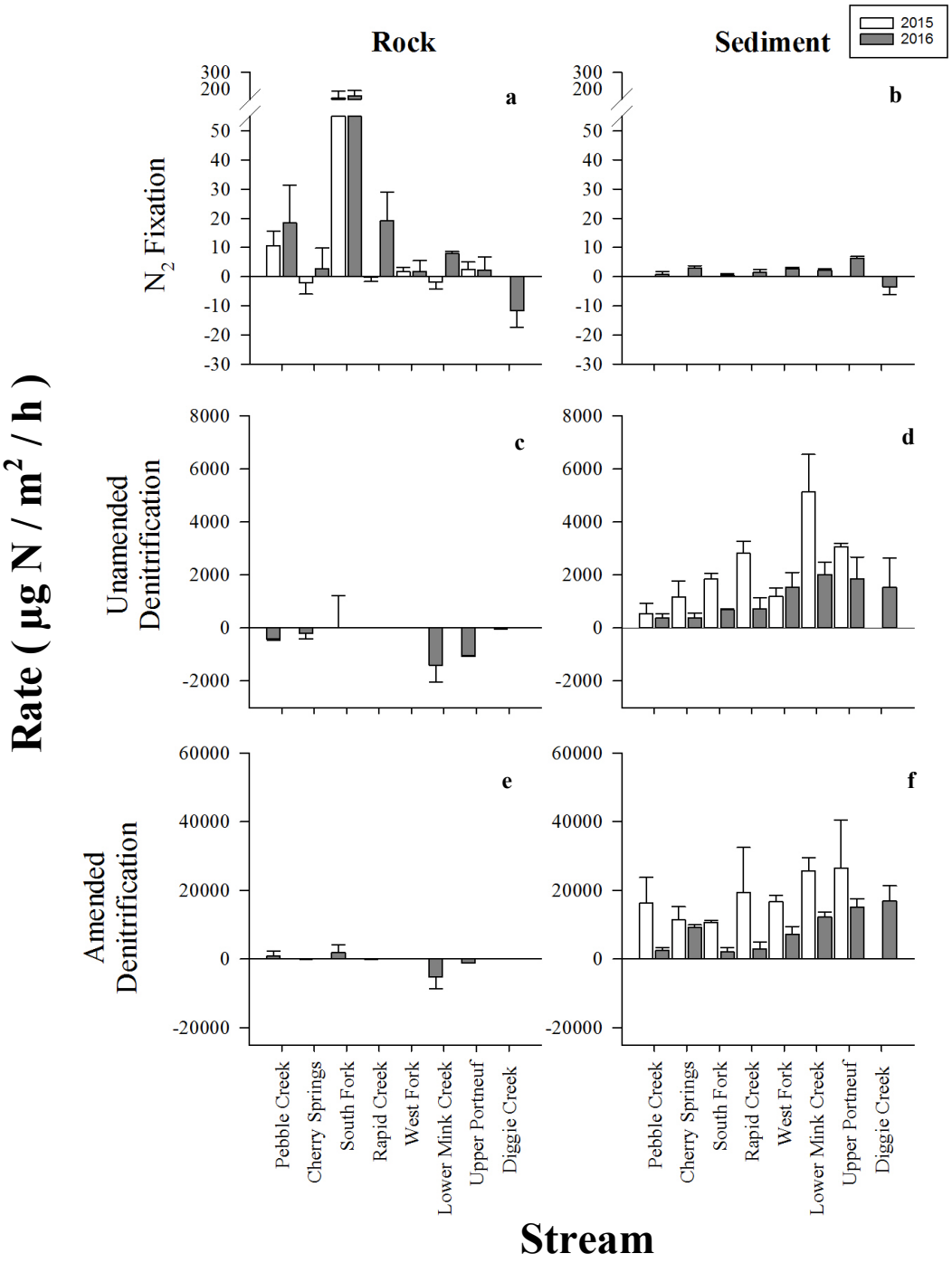

Figure 5. N_2 fixation rates (n = 6) and denitrification rates (amended and unamended, n = 3); arranged from low to high 2015 DIN concentrations. Error bars are standard error. Panels a, c, and e represent rates on rocks and panels b, d, and f are rates on sediments. In 2015, denitrification was only measured on sediment and N_2 fixation was only measured on rock substrate. The study location Diggie Creek was added in 2016. Y axes for unamended denitrification rates are 7.5 times lower than that of the amended denitrification rates and the Y axes for N_2 fixation are 200 times lower than that of the amended denitrification rates.

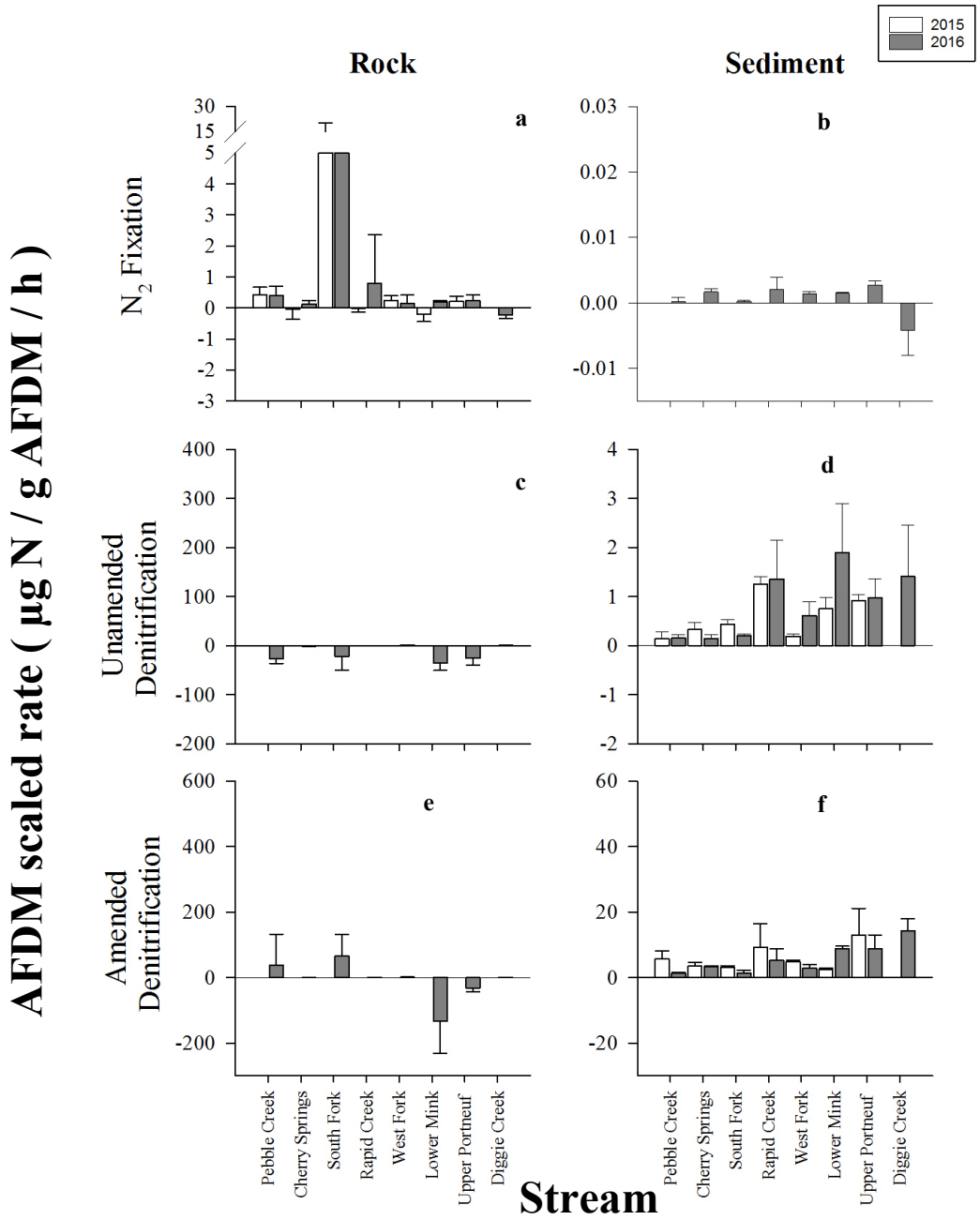

Figure 6. Bar graph of ash free dry mass (AFDM) scaled N_2 fixation rates (n = 6) and denitrification rates (amended and unamended, n = 3) vs. stream with standard error bars. Panels a, c, and e represent rates on rocks and panels b, d, and f are rates on sediments. Streams are arranged in order of low to high 2015 DIN concentrations. In 2015 denitrification was only measured on sediment and N_2 fixation was only measured on rock substrate. The study location Diggie Creek was added in 2016 and therefore was not measured in 2015. Y axes for amended denitrification rates are 1.5 times that of unamended rates. The Y axes for N_2 fixation on rock substrate is 1000 times that for sediment.

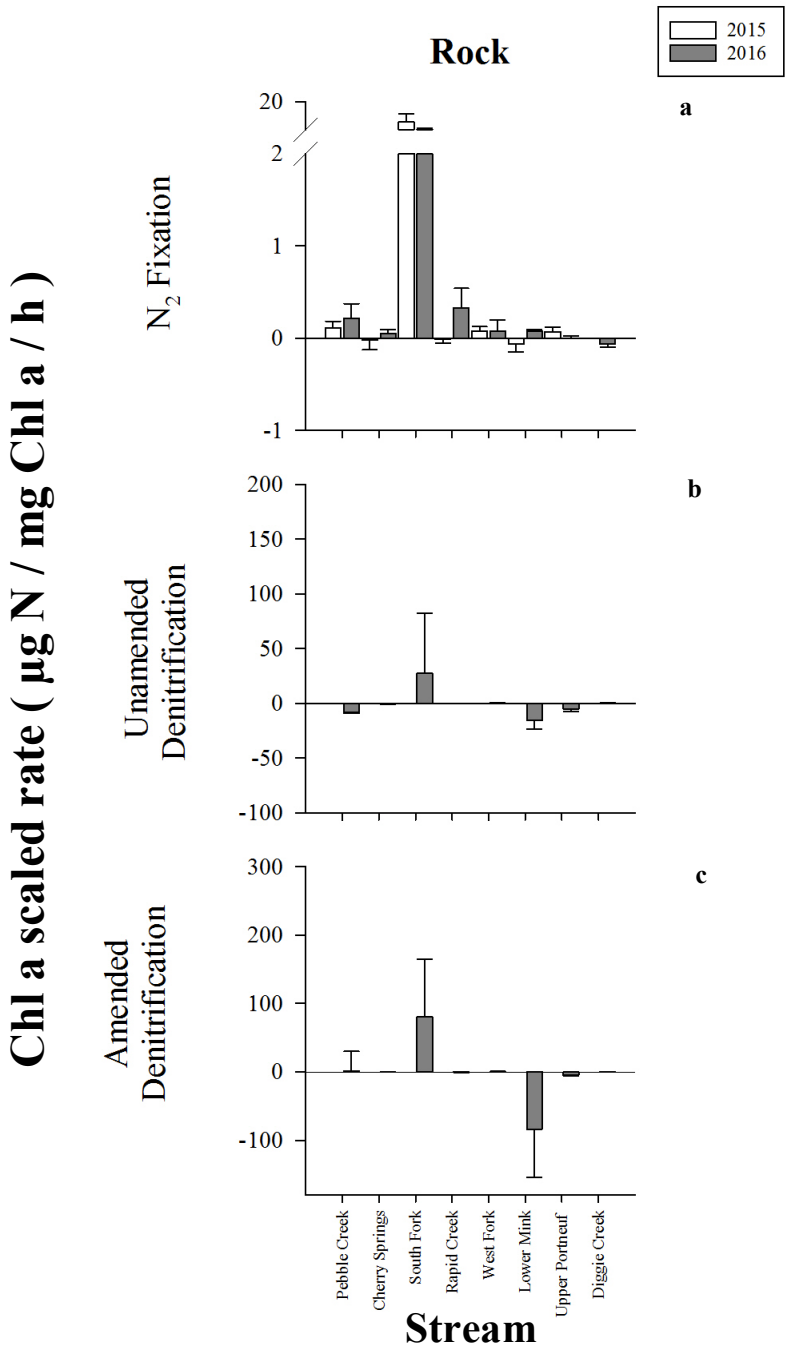

Figure 7. Bar graph of Chlorophyll a scaled N_2 fixation rates (N = 6) and denitrification rates (amended and unamended, N = 3) vs. stream with standard error bars. Panels a, b, and c represent rates on rock substrate. Streams are arranged in order of low to high 2015 DIN concentrations. In 2015 denitrification was only measured on sediment and N_2 fixation was only measured on rock substrate. The Y axis for N_2 fixation is 10 times less than that of the Y axes for both unamended denitrification rates and 15 times less than that of the Y axes of amended denitrification rates.

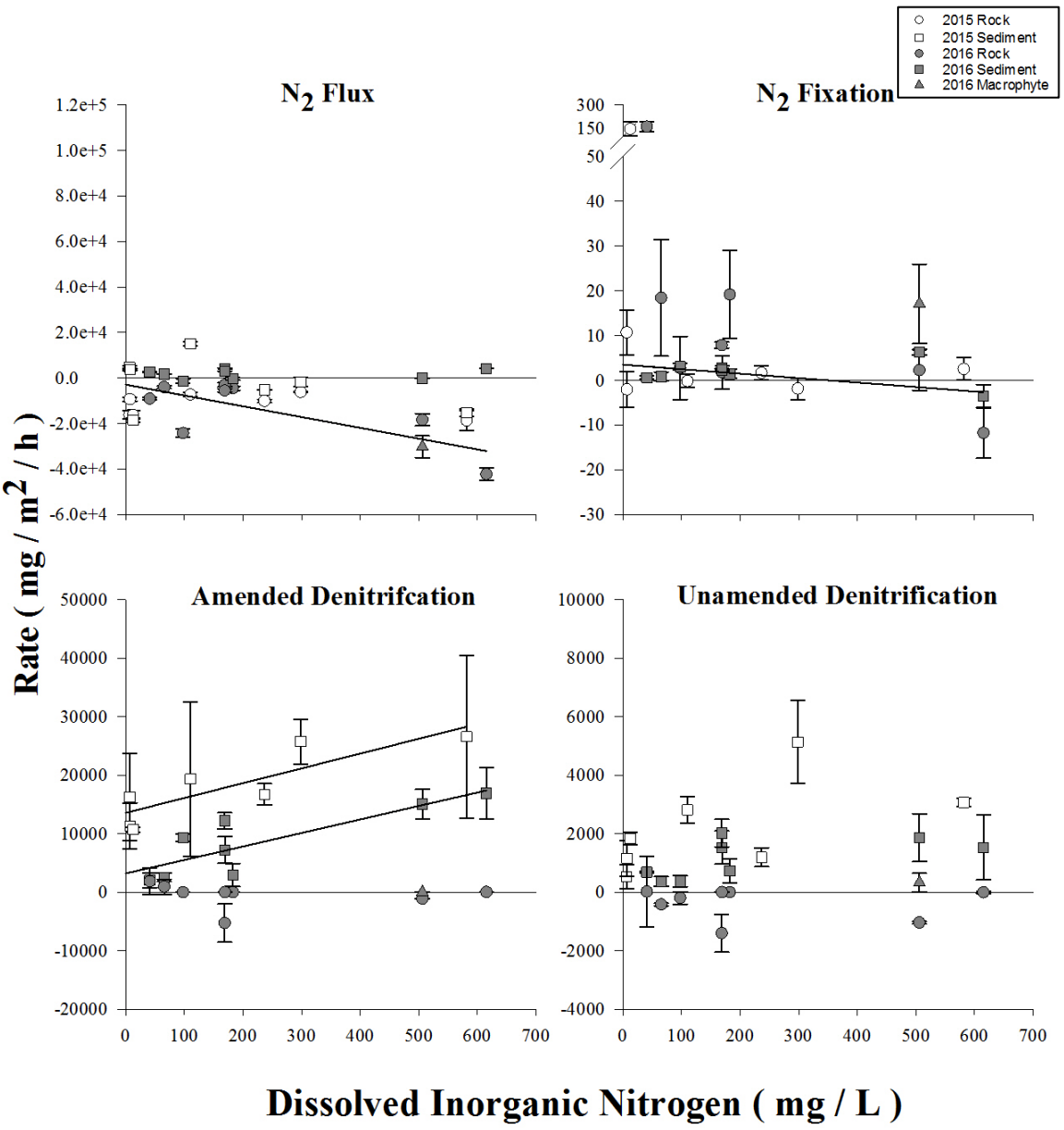

Figure 8. N_2 fixation (N = 6), denitrification (amended and unamended, N = 3), and N_2 flux rates (N = 6 for 1025, n = 12 for 2016) from both 2015 and 2016 vs. DIN concentrations with standard error bars. Y axis for amended denitrification rates is 5 times that of unamended denitrification. The y axis for N_2 fixation is 200 times less than that of amended denitrification rates and 400 times less than that of N_2 flux.

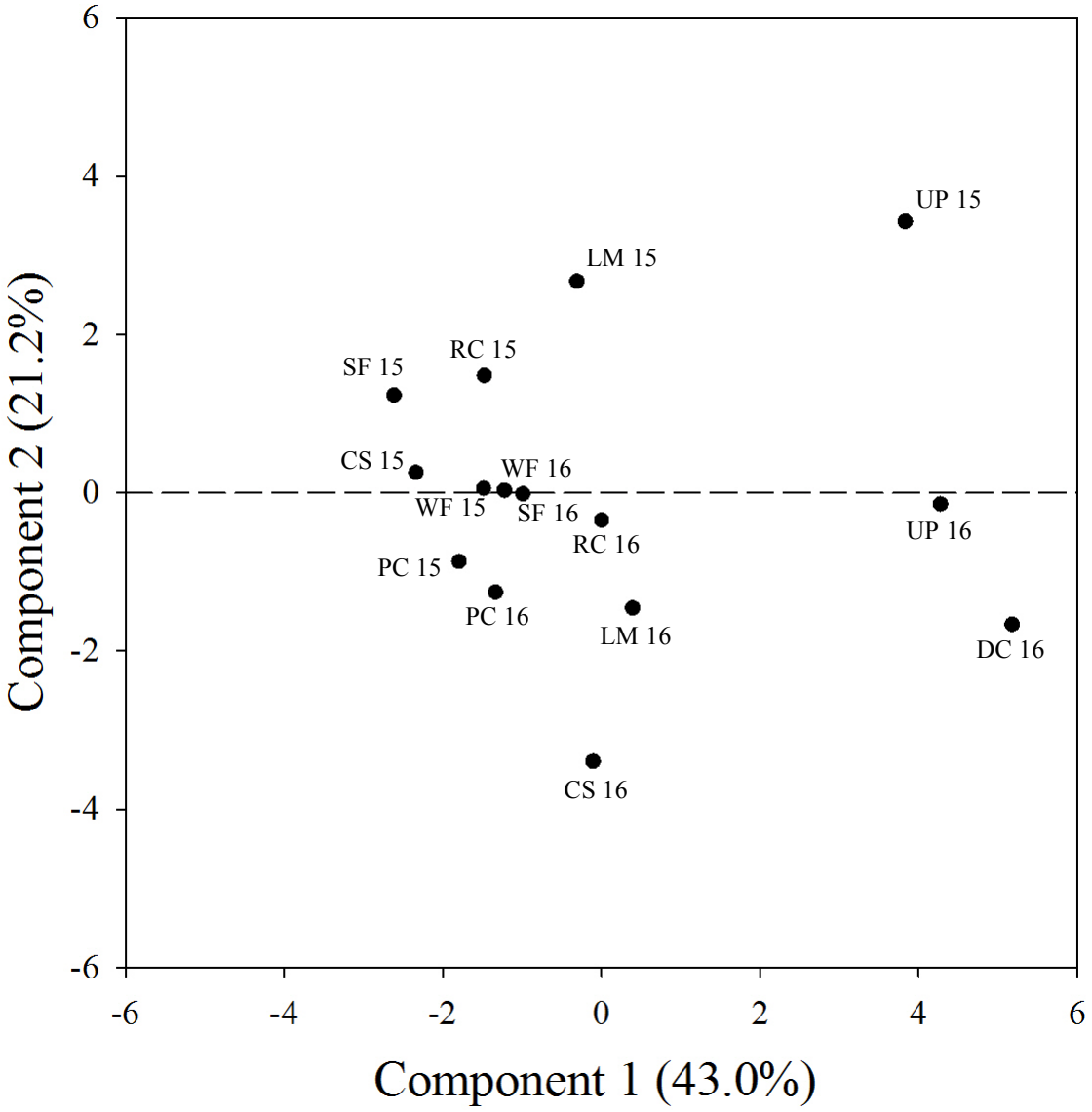

Figure 9. Principal component analysis plot of the first two principal components axes displaying sites. Sites were abbreviated as follows: Pebble Creek (PC), Cherry Springs (CS), South Fork Mink Creek (SF), Rapid Creek (RC), West Fork Mink Creek (WF), Lower Mink Creek (LM), Upper Portneuf (UP) and Diggie Creek (DC). Years are abbreviated as 15 for 2015 and 16 for 2016.