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DENITRIFICATION AND NITROGEN FIXATION COMMONLY CO-OCCUR BUT RATES VARY THROUGHOUT THE YEAR AND IN DIFFERENT ENVIRONMENTS

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DENITRIFICATION AND NITROGEN FIXATION COMMONLY CO-OCCUR BUT RATES VARY THROUGHOUT THE YEAR AND IN DIFFERENT ENVIRONMENTS

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Abstract

Denitrification and nitrogen fixation are nitrogen cycling processes that can occur simultaneously in streams but are rarely studied together. The overarching goal of this dissertation was to quantify temporal and spatial variation of these processes in streams across the USA, characterize the environmental drivers of that variation, and determine the role that denitrification plays in the carbon cycle. To characterize temporal variation in these processes, a 2-year study in the Pilgrim River in Michigan's Upper Peninsula found no difference in rates among seasons but high day-to-day variation in rates of both processes (maximum daily change 4,390 μg $N/m^2/hr$ for denitrification and 39 μg $N/m^2/hr$ for nitrogen fixation) that was related to dissolved nitrogen concentrations. A second study characterizing spatial variation in rates in 12 streams distributed across 9 ecoregions found that denitrification ranged from 0 ± 0 to 10,355.8 ± 3,054.8 μg $N/m^2/hr$ and nitrogen fixation ranged from 0 ± 0 to 155.4 ± 120.6 μg $N/m^2/hr$ (mean ± 95% CI) and co-occurred in 9 streams. Finally, we incorporated organic carbon removal via denitrification with carbon removal estimates from aerobic respiration in 23 streams across 12 ecoregions. In 13 stream/substrate combinations 100% of the carbon removal was due to denitrification. Overall, these studies show that denitrification and nitrogen fixation commonly cooccur in streams, that rates are more variable spatially and temporally than expected, and that this variation is not simply explained by environmental characteristics as commonly assumed.

Chapter 1: General Introduction

Denitrification and nitrogen fixation not only regulate nitrogen (N) concentration in streams but can also play a role in other biogeochemical cycles, influencing processes such as stream metabolism. Denitrification is a form of anaerobic respiration, which breaks down organic carbon for energy and removes reactive nitrogen from ecosystems, using NO_3^- (nitrate) in the place of O_2 (oxygen) as an electron acceptor and releasing CO_2 (carbon dioxide) and N_2 (di-nitrogen) gas (Wall et al., 2005). Nitrogen fixation increases the bioavailable N in an ecosystem by converting N_2 into NH_4^+ (ammonium) or organic N via an energetically expensive process (Bandyopadhyay et al., 2013; Burris and Roberts, 1993). Nitrogen fixation is rarely studied in streams as streams often have high reactive N loads, and it is assumed that organisms will not expend energy to fix nitrogen when it is readily available. Even fewer studies have quantified nitrogen fixation and denitrification in the same stream (Marcarelli, Baker, & Wurtsbaugh, 2008). In the past, these 2 processes were believed to be mutually exclusive, with denitrification occurring in high nitrogen environments and nitrogen fixation occurring in low nitrogen environments. However, recent studies in ocean, lake and stream habitats have shown that these two processes can and do occur together (Eberhard, Marcarelli, & Baxter, 2018; Emerson, Mecking, & Abell, 2001; Scott & Grantz, 2013). Yet, how common it is for these processes co-occur, and the mechanisms that allow them to co-occur in streams, are not well known.

Although nitrogen availability is a key controlling factor for both nitrogen fixation and denitrification, there are many other environmental factors which could influence the rate of these processes. The availability of other nutrients such as carbon (C) and phosphorus (P) alter nitrogen fixation and denitrification rates. Denitrification breaks down organic carbon molecules to release energy, and as such requires a supply of organic carbon (Cornwell, Kemp, & Kana, 1999). Nitrogen fixation may be affected more by the availability of nitrogen relative to phosphorus (described in the N:P ratio) than by nitrogen concentrations alone, with lower N:P promoting nitrogen fixation (Smith 1990; Paerl et al., 2016). Stream size including depth, discharge, and water velocity can impact nutrient retention (Horner, Welch, Seele, & Jacoby, 1990; Judy L. Meyer & Likens, 1979; Stottlemyer & Toczydlowski, 1999a), and runoff delivers nutrients to streams (Grimm & Fisher, 1989; Grimm & Petrone, 1997; Howarth, Marino, & Cole, 1988). Light availability due to variation in solar inputs and/or canopy cover can be important for nitrogen fixation as it is often tied to photosynthesis due to its high energy demand (Scott & Marcarelli, 2012; Śpiewla, 1995). Additionally, increased light availability could lead to higher rates of in-stream nutrient uptake due primary productivity (Nelson & Shearer, 2005; Roberts & Mulholland, 2007a). Microbial communities performing nitrogen fixation and denitrification are also temperature sensitive, with nitrogen-fixing microbes typically being more active at warmer temperatures (Christensen, et al., 1990; Kim, Lee, & Keller, 2006). O_2 concentration can impact denitrification as it is an anaerobic process (Cornwell et al., 1999). These environmental variables

can vary throughout the year and in different locations among and within streams, which may both explain variability in rate processes and provide a mechanism for their coexistence in time and space.

Other instream processes such as aerobic respiration and photosynthesis can also affect nitrogen fixation and denitrification. High aerobic respiration rates linked to decomposition of allochthonous organic matter can create anaerobic environments in aquatic habitats, thereby promoting denitrification (Sutton-Grier, Wright, & Richardson, 2013). Nutrient uptake by photosynthetic organisms may lead to competition for dissolved nitrogen with the microbes that carry out denitrification (Mulholland, et al., 2006; Sutton-Grier et al., 2013). Changes in photosynthetic rate, even on a diel time scale, can alter nitrogen fixation rates by cyanobacteria in some ecosystems because it is tightly coupled with photosynthesis (Grimm & Petrone, 1997; Howarth et al., 1988). These metabolic processes associated with the carbon cycle may both directly and indirectly affect rates and variation in nitrogen fixation and denitrification.

Denitrification is also a direct component of the carbon cycle but is rarely considered as such. Streams are important players in the global carbon cycle as they greatly reduce the amount of terrestrial carbon which enters them (Butman & Raymond, 2011; Cole et al., 2007; Duarte & Prairie, 2005; Hotchkiss et al., 2015; Maranger, Jones, & Cotner, 2018; Mulholland et al., 2001), making it important to understand and accurately model stream carbon budgets.. Ecosystem metabolism is commonly quantified as the total amount of primary production and respiration occurring in a stream ecosystem. Streams that have

higher photosynthetic rates than respiration are described as autotrophic, while streams when more carbon is mineralized than produced (respiration > photosynthesis) are considered heterotrophic et al., 2016). The respired carbon provides energy for organisms in the ecosystem, while carbon that is not respired is either stored or delivered downstream to rivers, lakes, and oceans (Cole et al., 2007; Hotchkiss et al., 2015). Denitrification is intimately tied to the carbon cycle as it is a form of respiration that occurs in anaerobic environments, yet anaerobic respiration is typically neglected because we use O_2 to estimate metabolism rates (Bott et al., 1978; Hall et al., 2016; Marzolf, Mulholland, & Steinman, 1994; Odum, 1956), which does not account for the carbon removal due to anaerobic processes such as denitrification. The role denitrification plays in the carbon cycle is rarely explored in current literature.

The goal of this dissertation was to characterize relationships between nitrogen cycling rates and different environmental drivers that vary across temporal and spatial scales in stream ecosystems and demonstrate how this knowledge can be applied to advance our understanding of the carbon cycle. In chapter 2, I evaluated variation in nitrogen fixation and denitrification across seasonal and day-to-day time scales in a single river. I measured denitrification and nitrogen fixation rates on biweekly and daily intervals in the Pilgrim River in Michigan's Upper Peninsula and assessed the environmental drivers that may be related to that variation. I found that variation on a day-to-day time scale equaled the variation found throughout the year. In chapter 3, I measured rates of nitrogen fixation and denitrification in streams with different watershed and reach-

scale environments to examine how environmental factors at different spatial scale affected those rates. I measured nitrogen fixation and denitrification in 14 streams in 9 different ecoregions and found that denitrification and nitrogen fixation frequently co-occurred, but rates were not clearly explained by any of the environmental factors examined. Finally, in chapter 4 I quantified the contribution of denitrification to the carbon cycle. I looked at how incorporating carbon removal due to denitrification with aerobic respiration rates would alter the total respiration rate and potentially change the trophic state from autotrophic to heterotrophic. I measured denitrification and aerobic respiration rates on the same substrates, on the same day in 23 streams across 12 ecoregions in the United States and estimated carbon removal due to both processes. I found that depending on the environment, denitrification could contribute up to 100% to the total carbon removal from a stream although in other environments denitrification was not present and did not contribute at all to carbon removal.

Together, these studies demonstrate that nitrogen fixation and denitrification co-occur both in streams across the United States, as well as throughout the year in a single river. These rates are both variable throughout the year and among streams across the country, potentially due to environmental variability. This better understanding can provide better estimates of carbon removal from streams because of how the nitrogen and carbon cycle are coupled through denitrification. When developing future studies, we must carefully consider which processes to measure and the time and frequency of those measurements.

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Chapter 2. What Time is it? High Daily and Year-Round Variability in Nitrogen fixation and Denitrification Rates Makes Seasonal and Hydrological Variability Indecipherable in A Northern Temperate River

1. Abstract

Rates of nutrient cycling processes, as well as the drivers and mechanisms of variation in those rates, may change at different time scales. Although seasonal patterns in these process rates have been studied, it's unclear how they may respond to shifting seasonal dynamics (i.e., earlier snowmelt and extreme weather events), and we know little about how rates may vary at shorter daily and weekly timescales. Understanding this variation across temporal scales is essential to understand how nutrient cycling processes operate in aquatic ecosystems and predict how they may respond to global change. This study quantified denitrification and nitrogen fixation rates seasonally and daily in a northern temperate river, and explored how environmental conditions such as discharge, light, and nutrients were related to that variation at different time scales. We measured denitrification and nitrogen fixation rates on biweekly and daily intervals in the Pilgrim River in Michigan's Upper Peninsula. We found high day-to-day variation in rates of both processes in all seasons (maximum daily change 4,390 μg $N/m^2/hr$ for denitrification and 39 μg $N/m^2/hr$ nitrogen fixation). No detectable differences in rates among sampling seasons were detected using Multiple Response Permutation Procedure (MRPP). Day-to-day variation did not change before and after elevated flow events, including a 1000-year flood that occurred during the study period. Partial least squares regression identified total dissolved nitrogen, dissolved organic nitrogen, and ammonium as important

drivers of denitrification and nitrogen fixation, but explained only 27-29% of the variation in all measured rates. The unexpectedly high daily variation and low seasonal variation found in this river suggest we may need to approach further studies of these processes with caution as discrete and infrequent measurements may be misleading.

2. Keywords:

nitrogen fixation, denitrification, seasonal

3. Introduction

Denitrification and nitrogen fixation are nitrogen transformation processes which can both control and be controlled by environmental factors (Seitzinger et al., 2006; Vitousek et al., 2002). Denitrification is a form of anaerobic respiration which breaks down organic carbon for energy, and uses NO_3^- (nitrate) in the place of O_2 (oxygen) as an electron acceptor, so availability of DOC (dissolved organic carbon) and NO_3^- are important limitations on denitrification rates (Wall et al., 2005). Along with the required reactant availability, environmental factors including temperature can alter denitrification rates. Nitrogen fixation converts N_2 (di-nitrogen) into NH_4^+: (ammonium), however, it is an energetically expensive process so high availability of NH_4^+ and/or other forms of DIN (dissolved inorganic nitrogen) can cause microbes to reduce the process rate to conserve energy (Bandyopadhyay et al., 2013; Burris and Roberts, 1993). Light may be especially important for nitrogen fixation as it is often carried out by cyanobacteria who obtain their energy via photosynthesis (Burris & Roberts, 1993). Light intensity along with these other factors vary through time, and

therefore they may lead to temporal variation in rates of both of these nitrogen transformation processes.

The mechanisms by which environmental factors affect rates of denitrification and nitrogen fixation may differ depending on the time scale. Seasonal changes may be gradual over the course of days to weeks, caused by 1. the abundance of microbes increasing or decreasing, or 2. the identity of the microbes within the community changing. Environmental factors can also shift much more rapidly over hours to days and could affect rates at that time scale not by changing the community, but through enzymatic regulation. These enzymatic rates can be altered through temperature or reactant and product availability (Grimm, 1987; Marcarelli, Baker, & Wurtsbaugh, 2008; Marcarelli & Wurtsbaugh, 2006). This distinction between community or enzymatic shifts can be complicated because some environmental factors such as temperature and light vary at multiple time scales. Large hydrological disturbances could destroy a microbial community, causing a rapid decrease in rates, followed by a longer recovery period reflecting aspects of both seasonal and shorter-term variation (Grimm & Fisher, 1989). Understanding relationships between nitrogen cycling rates and different environmental drivers that vary across time scales is a challenge that has not been well integrated into the study of nitrogen cycling in streams.

Environmental characteristics that show strong seasonal changes include nutrient availability, temperature, and light availability. Low biotic uptake rates in winter can lead to increased inorganic nitrogen concentrations in temperate

forested streams (Stottlemyer & Toczydlowski, 1999a, 1999b) which could provide reactants for denitrification and reduce nitrogen fixation. Temperate forests may have large allochthonous organic inputs during the fall and high DOC flux during snowmelt (Stottlemyer and Toczydlowski, 1999a) potentially promoting denitrification. Discharge varies seasonally corresponding to rainy seasons or snowmelt, which can alter nutrient concentrations both by altering patterns of nutrient delivery and via dilution (Horner et al., 1990; Meyer & Likens, 1979; Stottlemyer & Toczydlowski, 1999a). Denitrification and nitrogen fixing microbial communities can both be sensitive to seasonal temperature changes with nitrogen fixing microbes often preferring warmer temperatures (Christensen et al., 1990; Kim et al., 2006). Light varies seasonally, especially in forested streams where canopy cover will shade streams differently depending on the season and vegetation type (Bowes et al., 2012; Roberts & Mulholland, 2007b). Increased light availability in spring and fall due to low canopy cover can lead to higher rates of in-stream nutrient uptake due to increases in primary productivity (Nelson & Shearer, 2005; Roberts & Mulholland, 2007a).

More rapid changes in N cycling rates may also occur, on the scale of hours or days, due to reactant availability and changes in enzymatic activity in the microbial community. Enzymatic rates can be altered through temperature or reactant and product availability (Grimm, 1987; Marcarelli et al., 2008). Nitrogen cycling rates can both increase and decrease over the course of hours with factors such as temperature affecting enzyme activity (Marcarelli & Wurtsbaugh, 2006). Temperature variation can occur on an hourly scale, and although the

microbial community may not change during this short time period, the enzymes and microbes facilitating nitrogen cycling processes may have reduced activity at cold temperatures (Boulêtreau et al., 2012; Yvon-Durocher et al., 2010). Also, changes in light can alter nitrogen fixation rates by cyanobacteria in some ecosystems on a diel cycle because it is tightly coupled with photosynthesis (Grimm & Petrone, 1997; Howarth, Marino, & Cole, 1988). Nutrient concentrations can rapidly change due to storm runoff, which could also cause rapid response shifts in nitrogen process rates. Because nitrogen fixation is so energy intensive, enzymes can be rapidly deregulated to save energy following an influx of dissolved inorganic nitrogen (DIN) (Grimm & Fisher, 1989; Grimm & Petrone, 1997; Howarth et al., 1988).

Hydrological disturbances can also have major impacts on biogeochemical cycles, and their effects may be a mix of longer-term community and shorter-term enzymatic changes. Surface runoff can carry nutrients and change water temperature, while increased water velocity can move and scour riverbed substrates, disturbing microbial communities, and the microenvironments of these communities. Initial declines in process rates following a hydrologic disturbance may be rapid; however, recovery may be slower if the microbial community was altered or reduced in biomass rather than enzymatic downregulation. River structure is important in determining how resistant a river is to an event or how long it takes the river to recover from or return to pre-event conditions. It is not well understood how resistant and resilient

rates of nitrogen transformations mediated by microbial communities are to hydrologic disturbances.

The complexity of seasonal versus shorter-term environmental changes coupled with different mechanisms of microbial responses that lead to changes in rates makes it difficult to decipher how and why nitrogen cycle processes vary. The objectives of this study were to characterize how denitrification and nitrogen fixation rates vary over seasonal, weekly, and daily temporal scales in a temperate forested river in Michigan's Upper Peninsula. This study was designed to address the following questions: 1) How do denitrification and nitrogen fixation rates vary seasonally? I hypothesized that rates would differ among seasons due to environmental changes. 2) How do denitrification and nitrogen fixation rates vary daily and in their response to hydrological disturbances? I hypothesized low day to day variation in process rates; however, after large hydrological events, rates would decline due to disruption of microbial communities and changes in environmental conditions with prolonged recovery periods as the microbial communities recover after disturbance. 3) Which environmental factors are related to variations in denitrification and nitrogen fixation rates? I hypothesized seasonal drivers including light and temperature would also be drivers of these rates as I expected large variation in rates associated with season. I addressed these questions by quantifying these processes in relation to changes in environmental conditions at daily and seasonal timescales over 2 years.

4. Site

Sampling occurred in a 20 m reach on the main branch of the Pilgrim River (N 47.10138, W 88.51750; Figure 1). The Pilgrim River and its 4 tributaries stretch 34.9 km, draining a 52 km^2 watershed located in Houghton County in the Upper Peninsula of Michigan. The Pilgrim River watershed is 58% forested, 25% open space, 12% wetland, 4% developed, and 1% lakes/ponds and has a base discharge between 0.5 and 0.8 m^3/s (DEQ 2012). At the study reach the river is ~8 m wide with a maximum depth of ~2 m. Ice ~0.5 m thick covers the river from December-April, with high discharge from snow melt occurring in late April or early May (Figure 2). The river substrate in the study reach consisted of approximately 2/3 sand cover and 1/3 patches of large cobbles.

Figure 1: Study site located on the Pilgrim River and Michigan's Upper Peninsula USA.

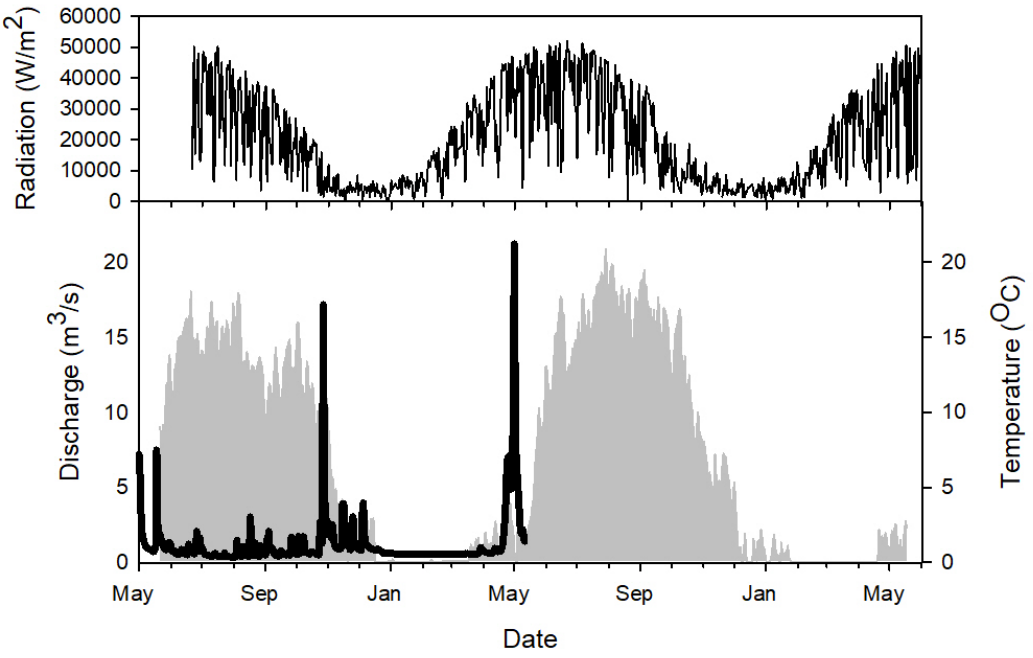

Figure 2: Continuous discharge (thick black line, bottom pane), water temperature (light grey shading, bottom pane) and incoming solar radiation (thin black line, top pane) over course of sampling period. Discharge was determined using USGS installed gauge (USGS 04043016 Pilgrim River at Paradise Road Near Dodgeville, MI: May 2017- June 2018). Discharge measurements do not span entire sampling period because the gauge was washed away during a 1000-year flood on June 19, 2018. Photosynthetically active radiation was retrieved from the Upper Great Lakes Observing System station located at Michigan Tech's Great Lakes Research Center (glos.us). Temperature was measured using a MiniDO_2T logger.

5. Methods

Sampling occurred every 2-6 weeks year-round between May 2017 and May 2019 except for November 2017-April2018 for nitrogen fixation which was not sampled during that time period. Additionally, once per season sampling occurred every day over a 1-2-week period. This nested design allowed us to measure both seasonal and daily variation in rates, as well as calculate resilience when disturbances occurred. During certain time periods, some sampling dates

were skipped (Table 1) due to unsafe conditions such as extreme cold, extreme storms, and high-water flow.

Table 1: Sampling frequency and study site characteristics (mean ± standard deviation) for all variables measured during each season throughout the sampling period. Abbreviations: Denit. indicates denitrification; sed. Indicates sediment. Discharge was not measured in winter due to ice coverage over the river.

	Fall		Spring		summer		winter	
# Sample days N fix rock	11		23		31		5	
# Sample days N fix sed.	12		25		32		7	
# Sample days denit. rock	14		21		31		5	
# Sample days denit. Sed.	14		27		31		15	
Rock AFDM (g)	0.0568	±0.1158	0.1239	±0.1506	0.0360	±0.0563	0.0366	±0.0042
Sediment AFDM (g)	1.9	±0.9	2.5	±1.4	1.5	±0.6	1.6	±0.8
Chlorophyll a (mg / sq m)	0.0281	±0.0311	0.0278	±0.0313	0.0117	±0.0120	0.0406	±0.0374
Temperature (^{O}C)	5.2	±3.8	8.5	±2.2	15.1	±2.1	0.5	±0.6
Canopy cover (%)	17	±9	23	±9	25	±9	8	±3
Radiation (W/m^2)	327	±208	1177	±662	978	±444	353	±246
Discharge (L/s)	1161	±549	2184	±2548	973	±601	ice	
DOC (mg/L)	6.2357	±2.3783	6.2737	±0.8997	7.5097	±2.7486	5.7153	±1.9237
TDN (mg/L)	0.3376	±0.0585	0.2860	±0.0344	0.3891	±0.1126	0.3569	±0.1108
NO_3^- + NO_2 (mg/L)	0.1323	±0.0351	0.0916	±0.0387	0.1267	±0.0611	0.1856	±0.0798
NH_4^+ (mg/L)	0.0044	±0.0021	0.0063	±0.0032	0.0117	±0.0067	0.0049	±0.0043
DON (mg/L)	0.2091	±0.0780	0.1902	±0.0285	0.2682	±0.1021	0.1756	±0.0895
SRP (mg/L)	0.0067	±0.0031	0.0056	±0.0024	0.0080	±0.0070	0.0103	±0.0105
TDP (mg/L)	0.0097	±0.0044	0.0104	±0.0048	0.0130	±0.0048	0.0111	±0.0116
Denit. rate rock (μg $N/m^2/hr$)	1264.8	±2515.3	48.7	±95.9	355.4	±957.5	228.1	±361.5
Denit. rate sed. (μg $N/m^2/hr$)	815.7	±1322.1	1049.3	±1917.2	1351.3	±1840.2	268.3	±410.9
N fix rate rock (μg $N/m^2/hr$)	2.30	±3.49	5.17	±11.24	2.47	±4.39	1.83	±1.25
N fix rate sed. (μg $N/m^2/hr$)	1.03	±1.76	1.66	±3.00	3.60	±7.05	2.33	±4.18

For this study, seasonality was determined using a combination of canopy cover, temperature, and discharge rather than calendar date, as the Upper Peninsula of Michigan experiences an extended winter period with substantial snowpack and short, rapidly changing spring and fall seasons. Spring was determined to start when the river surface was no longer covered by ice and discharge was high due to snow melt, around late April or early May. Spring continued until water temperature stopped consistently rising and there was a full riparian tree canopy, usually early June. Summer continued while water temperature remained fairly constant, and leaves were green. Fall started when water temperature began to decrease and leaves began to change color, usually early September. Winter started when the river was fully covered by ice, usually in late December. This classification scheme was applied based on each year of observational data, such that the seasonal start or end dates could vary among the years of our study based on conditions in that year.

Denitrification and nitrogen fixation rates were measured in sediment and on rock substrates using acetylene block and acetylene reduction assay techniques. Sediment and rock were measured because these were the predominant substrates found in the river. To measure denitrification and nitrogen fixation on each date, 200 mL sediment cores or enough rocks to cover ~0.0045 m^2 surface area were collected from <1 m deep water and placed in chambers (pint glass mason jars with lids drilled to fit 13 x 20 mm septa, but 2-L polycarbonate food storage chamber were used for rock incubations prior to September 2017). Chambers were filled with stream water to remove all air and

sealed. Blank chambers for sediment were filled with stream water while blanks for rocks contained rocks from outside the stream and filled with stream water. This allowed us to account for chamber effects such as gas leakage or processes occurring in the water column.

Acetylene reduction assays were used to quantify rates of nitrogen fixation by introducing acetylene gas to a chamber. Nitrogenase, the enzyme that fixes nitrogen, converts acetylene to ethylene, and we measured the change in ethylene concentration to estimate the amount of nitrogen that could have been fixed (Eberhard et al. 2018; Capone, 1993). Water temperature was measured and a 20% headspace (v/v) of acetylene gas was introduced to each filled and sealed chamber. A 9 mL gas sample was removed from each chamber before incubating in the stream for 1-3 h, after which a final 9 mL gas sample was collected to terminate the assay. Upon termination, water temperature, water volume, substrate volume, and substrate surface area for each chamber was measured. The gas samples were analyzed for ethylene concentration using a SRI 8610C Gas Chromatograph with Hayesep T column, flame ionization detector, and column oven set at 40 °C ramping to 110 °C after 2.5 min running with hydrogen carrier gas. A 100-ppm ethylene standard was used to convert peak height to concentration of ethylene in the gas sample. The amount of ethylene in the headspace (initial and final) was determined and using water volume and the solubility constant for ethylene using equations from Dodds et al. (2017). This was used to estimate the amount of N fixed by assuming 1 molecule

of acetylene converted to ethylene is equal to 3 atoms of nitrogen being fixed (Capone, 1993; Kim et al., 2006).

Acetylene block assays were used to quantify rates of denitrification. Acetylene prevents the complete transformation of nitrate to atmospheric nitrogen causing N_2O to be released which we are able to measure to estimate denitrification rate (Smith & Tiedje, 1979). C, N, and chloramphenicol (to prevent bottle effects) were added to each chamber for a final concentration of 34mg/L sucrose and sodium nitrate and 114 mg/L chloramphenicol. Acetylene was introduced into the chambers and initial and final samples were collected the same as described above for nitrogen fixation assays. The gas samples were analyzed for N_2O using the SRI8610C Gas Chromatograph with Hayesep D column, electron capture detector, and column oven set to 80 °C ramping to 180 °C after 5 min with helium or ultra-high purity nitrogen (for samples analyzed after February 2019) carrier gas. A 1000 ppm N_2O standard was used to convert peak height to N_2O concentration following Dodds et al. (2017).

All rates were scaled to surface area. For sediment, the surface area of the corer was used. Rock area was estimated by tracing each rock onto paper, then cutting those tracings out and weighing them, and comparing those weights to a standard curve created by weighing squares of paper with known areas (Bergey & Getty, 2006). Although both denitrification and nitrogen fixation rates were measured for rock and sediment, denitrification is expected to be more likely to occur in sediment due to its reliance on anaerobic environments and nitrogen fixation expected more on rock because it is often coupled with

cyanobacteria. Because of this, denitrification in sediment and nitrogen fixation on rock will be primarily looked at to address our questions about rate variability through time and in response to environmental variables.

Water volume in each chamber was measured, and all rocks were scrubbed in that water to remove algae. Subsamples of the scrub water were filtered through pre-ashed GF/F filters (0.7 μm) and frozen. To measure chlorophyll a concentration, filters were later extracted in 95% ethanol for 8-24 h. Using a spectrophotometer, absorbances were measured at 664, 665, and 750 nm. The samples were acidified with 0.1 N HCl and absorbance was measured again (APHA, 2005; Nusch, 1980). Filters with remaining extract and sediment from the chambers were dried in a 60°C oven for 48 h, then combusted at 500°C for 4 h to measure ash free dry mass. Filter ash free dry mass (AFDM) and chlorophyll concentration were scaled up from subsample to total water volume, then normalized by dividing by rock surface area.

Water chemistry was analyzed for each sampling date according to APHA (2005). Water was filtered through 0.45 μm membrane filters and stored on ice until return to the lab where the samples were frozen. Analysis included soluble reactive phosphorus (SRP; μg/L), total dissolved phosphorus (TDP; μg/L), nitrate + nitrite (NO_3^-+NO_2^-; μg/L), ammonium (NH_4; μg/L), total dissolved nitrogen (TDN; μg/L) and dissolved organic carbon (DOC; mg/L). NO_3^-+NO_2 and SRP were analyzed using a SEAL AQ2 discrete water analyzer. NO_3^-+NO_2 used AQ2 method EPA-127-A Rev. 9, and SRP used AQ2 method EPA-155-A Rev. 0. Filtered water samples were acidified to pH < 2 and sent to Michigan Tech's

Laboratory for Environmental Analysis of Forests (LEAF) core facility which used a Shimadzu TOC-VCSN with a total N module TNM-1 (Shimadzu Scientific Instruments, Columbia, Mary- land) for DOC and TDN analysis. Dissolved Organic Nitrogen (DON) was calculated by subtracting NO_3^{-+}+NO_2 and NH_4^+ concentrations from TDN concentrations. TDP concentration was analyzed using molybdenum—antimony method following an ammonium persulfate digestion (APHA, 2005).

Discharge was determined using USGS installed gauge (USGS 04043016 Pilgrim River at Paradise Road Near Dodgeville, MI: May 2017- June 2018) or by measuring using a Marsh McBirney Flo-mate (May 2017 - May 2019). Flow rate was measured at 10 equidistant points on a transect of the stream perpendicular to shore. The Flo-mate was attached to a wading rod to measure velocity (m s^{-1}) at 0.6*stream depth (m) at each point along a 10 point transect. The area of each segment was determined by multiplying segment width by segment depth. Flow in each segment was determined by multiplying velocity by segment area. The flow in all segments was added together to get discharge (m^3/s). Canopy cover was measured using a spherical densitometer (Lemmon, 1956). Photosynthetically active radiation was retrieved from the Upper Great Lakes Observing System station located at Michigan Tech's Great Lakes Research Center (glos.us), which is approximately 3 km from the study site. A MiniDO_2T logger from PME was deployed to continuously measure O_2 and temperature at the site, and open water metabolism, which includes gross primary production

(GPP) and ecosystem metabolism (ER), was modeled using the StreamMetabolizer software package (github.com/USGS-R/streamMetabolizer).

Differences in rates between seasons were assessed using Multi Response Permutation Procedure (MRPP) with the Vegan package in R (Oksanen et al., 2019). This non-parametric approach allowed us to determine if points grouped to our specified categories based on a set of variables provided (Warton et al., 2012). Based on a Euclidean distance measure the MRPP determined if the distance of points among groups was different from the distance of points within groups (A). Using $p < 0.05$ we determined if within group distance was smaller than amongst-group distances. First, to ensure the selected seasonal ranges were distinct we used a MRPP to look for seasonal grouping in sampling dates based on environmental variables. Because MRPP requires no gaps in datasets, the environmental variables were selected based on how important they are in representing seasonality and how complete the dataset was, and included DOC, TDN, NO3- + NO2, SRP, and TDP. PCA was used to describe the environmental variables that were driving the separation between groups by observing the loadings in the first component. PCA was run using the stats package in R (R Core Team 2020). Following the seasonal categorization, we then performed 4 additional MRPP for denitrification rates on rocks, denitrification rates in sediment, nitrogen fixation rates on rocks, and nitrogen fixation rates in sediment to see if rates also grouped by season.

To address question 2, how do denitrification and nitrogen fixation rates vary daily and in their response to hydrological disturbances, we calculated daily

change and used MRPP to assess whether it was different among seasons. Daily change was calculated as:

$$daily\ change = abs(R^{i} - R^{i+1})$$

Equation 1

Where R^{i} is the rate at day *i* and R^{i+1} is the rate one day later. MRPP was performed for daily change in denitrification rates on rock, denitrification rates in sediment, nitrogen fixation rates on rock and nitrogen fixation rates in sediment to determine if daily change differed between spring, summer and fall. Due to sampling irregularity, daily change could not be calculated in winter.

Resistance and resilience were calculated for all processes using sampling data collected daily before and after two large hydrological events that occurred during the sampling period. In the context of this study, nitrogen fixation and denitrification processes in this stream would be resistant to a hydrological disturbance if the rates did not change before and after a hydrological disturbance. Resilience is how quickly to rates post disturbance would return to pre-disturbance levels if they decreased because of the disturbance. Resistance was calculated as:

$$R = 1 - \frac{X^{before} - X^{after}}{X^{before}}$$

Equation 2

Where R is resistance, X^{before} is the rate before a disturbance, and X^{after} is the rate after a disturbance. Values closer to 0 indicate high resilience, while those closer to 1 indicate low resilience. Recovery for each rate was described as the slope of the linear function in the time period directly following the disturbance.

Environmental factors related to rates were explored using partial least squares regression from the pls package in R (Mevik et al., 2020). All environmental variables were standardized to mean = 0 and standard deviation = 1. The PLS used those environmental variables to create components which described the most variation in relation to rate. We selected the components which described the most variation in rate and looked at which environmental variables had the largest loadings for those components to determine which environmental variables were most related to rate. We chose this method because we had a large amount of co-linear independent variables, and PLS is robust to these collinearities (Carrascal et al., 2009).

6. Results

Throughout the year, both rates and environmental variables ranged greatly (Table 1; Figure 3, Figure 4). Denitrification in sediment averaged 984.65 ± 1032.46 μg $N/m^2/hr$ (mean ± standard deviation; Figure 3) for the entire sampling period, while nitrogen fixation on rocks averaged 3.29 ± 7.23 μg $N/m^2/hr$. Discharge measured during sampling averaged 1.18 ± 1.04 m^3/s, although this did not encompass high discharge events as manual measurements were taken only during sampling events, which could not happen safely during high flow. The highest discharge recorded by the USGS gauge upstream occurred on Jun 19, 2018, during a 1000-yr flood event with an estimated maximum discharge of 208 m^3/s (T. Weaver, USGS, personal communication; Figure 2). Additionally, discharge could not be measured during

winter or early spring as the river was ice covered, although denitrification rates and other environmental variables were measured in all seasons.

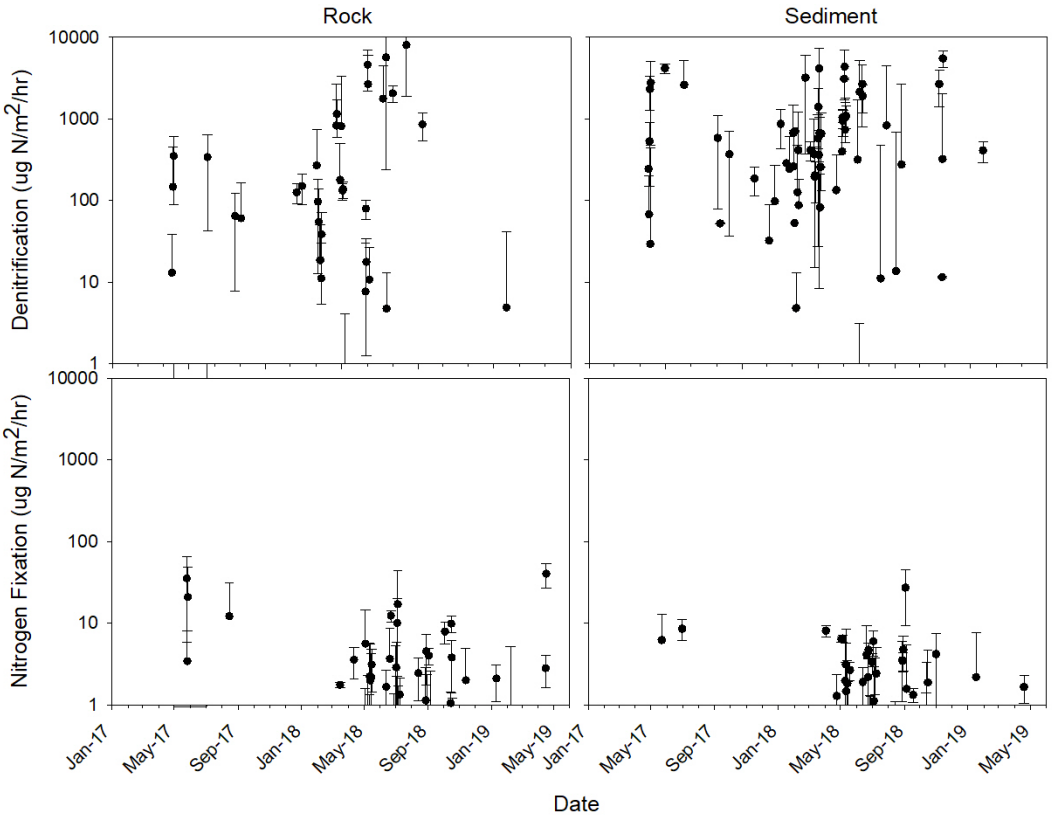

Figure 3: Rates ± standard deviation for denitrification (top) and nitrogen fixation (bottom) on rocks (left) and in sediment (right). Sampling occurred every 2-6 weeks year-round between May 2017 and May 2019. Additionally, once per season sampling occurred every day over a 1-2-week period.

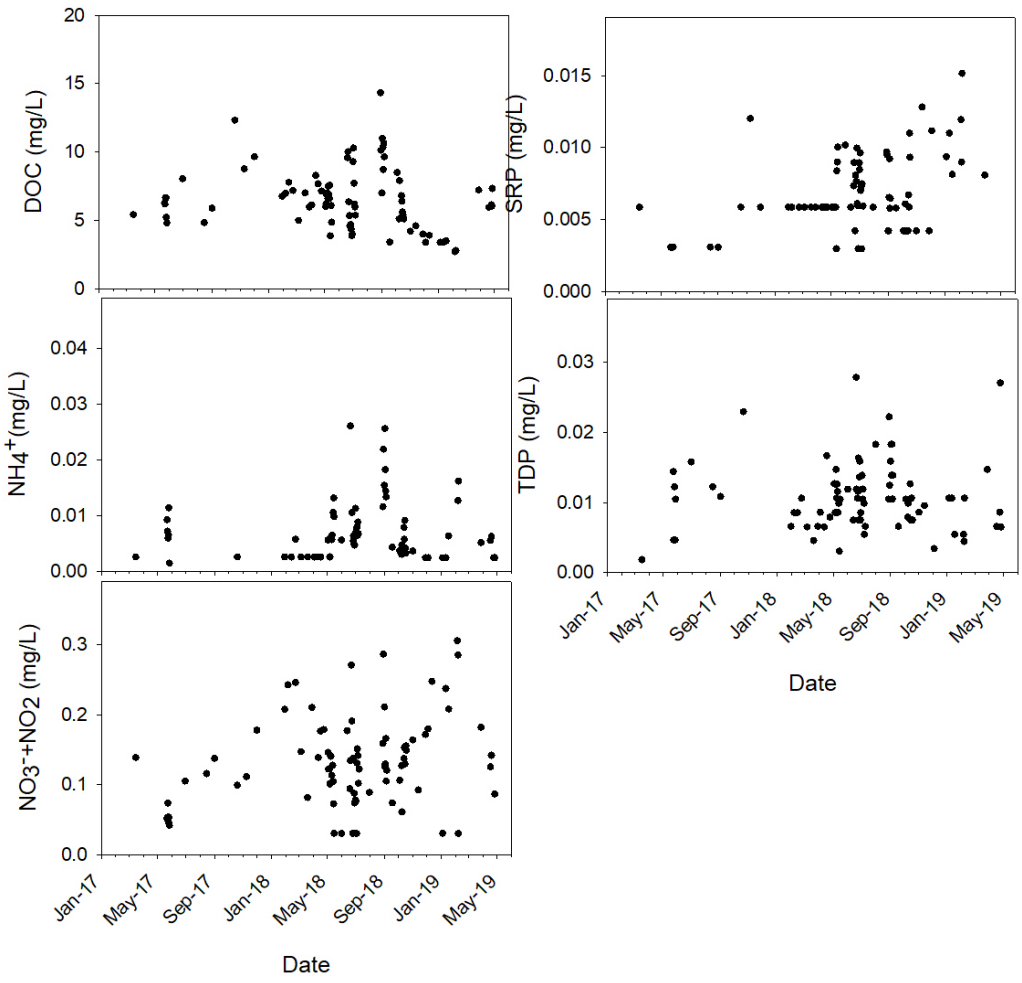

Figure 4: Nutrient concentrations during each sampling throughout the sampling period. Y-axis range for each plot matches the range of the data to demonstrate variation across the study period.

Seasonal patterns in nitrogen fixation and denitrification rates

MRPP showed significant separation in environmental conditions between seasons (A = 0.3024, p = 0.001, spring n = 16, summer n = 19, fall n = 12, winter n = 2) based on photosynthetically active radiation, water temperature, DOC, TDN, NO_3^- + NO_2, SRP, TDP. The 1st principal component explained 35% of the variation (Appendix Table A1) and was primarily driven by PAR (0.27), DOC (-0.26), TDN (-0.55), NO_3^-+NO_2 (-0.33), SRP (-0.44), and TDP (-0.51) Principal

component 2 explained and additional 27% of the variation and was driven by temperature (-0.62). However, MRPP showed no seasonal separation for any of the rates: nitrogen fixation on rock (A = -0.004098, p = 0.54, spring n = 24, summer n = 30, fall n = 11, winter n = 4); nitrogen fixation in sediment (A = -0.002548, p = 0.46, spring n = 26, summer n = 31, fall n = 12, winter n = 7), denitrification on rock (A = 0.03522, p = 0.063, spring n = 21, summer n = 31, fall n = 14, winter n = 5), or denitrification in sediment (A = 0.0225, p = 0.099, spring n = 27, summer n = 31, fall n = 14, winter n = 15).

Daily variation in nitrogen fixation and denitrification rates and in their response to hydrological disturbances

Differences in day-to-day denitrification and nitrogen fixation could be both large or small for both rocks and sediment in spring, summer, and fall. The highest daily change in denitrification rates on rock was 5,690 μg $N/m^2/hr$, denitrification in sediment was 7,348 μg $N/m^2/hr$, nitrogen fixation on rocks 38 μg $N/m^2/hr$, and nitrogen fixation in sediment 38 μg $N/m^2/hr$, while the lowest daily changes were 0 μg $N/m^2/hr$ for both rates on both substrates (Figure 5). Daily change in denitrification on rocks was significantly different between the three seasons (A = 0.1369, p = 0.004, spring n = 18, summer n = 23, fall n = 5), but not for denitrification in sediment (A = 0.003394, p = 0.41, spring n = 21, summer n = 19, fall n = 5), nitrogen fixation on rocks (A = 0.01658, p = 0.23, spring n = 19, summer n = 20, fall n = 4), or nitrogen fixation in sediment (A = 0.01067, p = 0.274, spring n = 20, summer n = 19, fall n = 4).

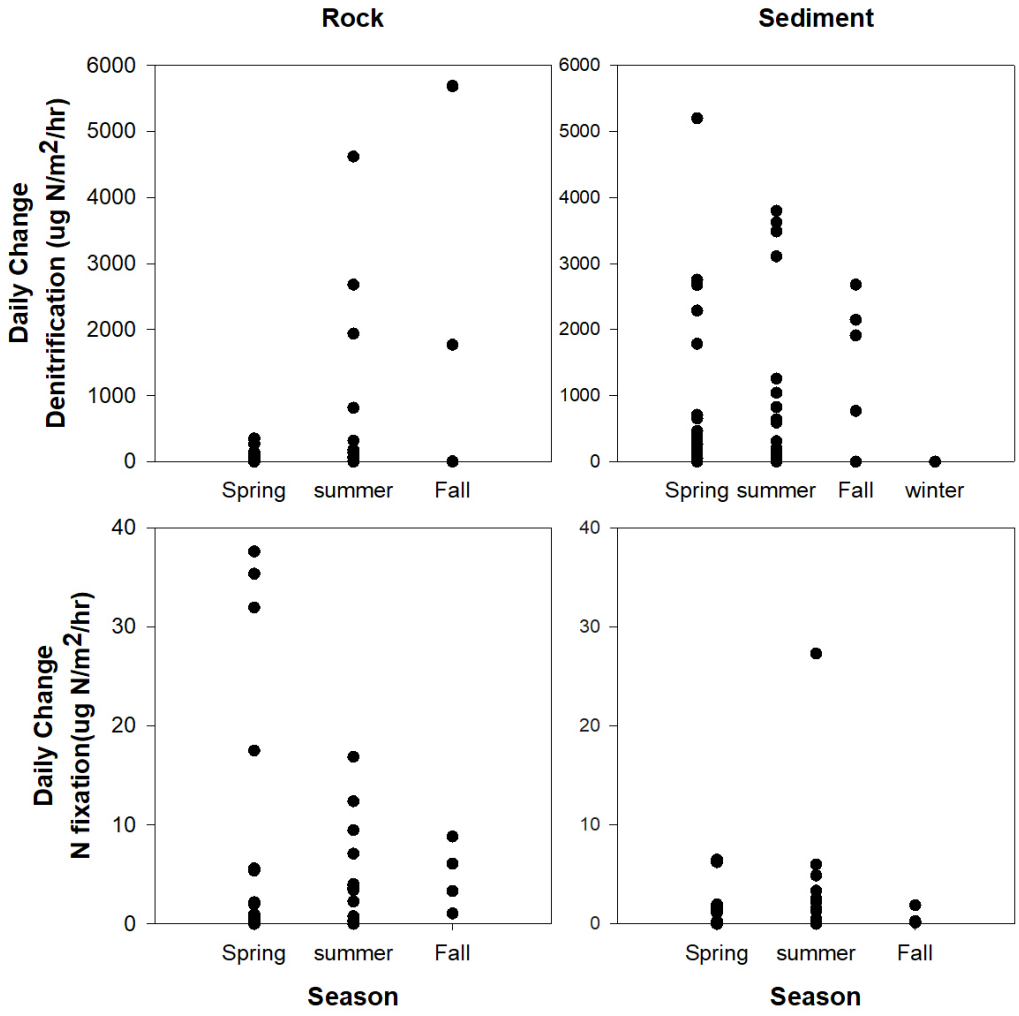

Figure 5: Points represent daily change in denitrification and nitrogen fixation in different seasons. Daily change was significantly different between seasons for denitrification on rocks but not for the other rate/substrate combinations.

Two storm events occurred during daily sampling events, on 18 June 2018 and 28 August 2018, for which we could calculate resistance and recovery. For the 18 June 2018 storm, rates were measured on 13 June 2018 before the storm, and on 19 June 2018 after the storm, continuing daily for 3 weeks (Figure 6). However, during that time additional, less severe rain events occurred. In the 3 weeks following the storm, daily change for denitrification in sediment (336.5 ±

352.2 μg N/m^2/hr mean ± SE) and nitrogen fixation on rocks 1.5 ± 1.9 μg N/m^2/hr mean ± SE) were frequently high, with rates appearing similar to before the storm (Figure 6). For denitrification in sediment, resistance was calculated to be 0, with a rate of 415 μg N/m^2/hr measured before the storm and 0 μg N/m^2/hr measured after the storm. The before-storm rate was one order of magnitude lower than the highest denitrification rate measured over the 3-week period (3,525 μg N/m^2/hr; average = 697 μg N/m^2/hr). Recovery was 185 N/m^2/hr/day, with rates increasing from 0 to 970 μg N/m^2/hr in 3 days. For nitrogen fixation on rocks and in sediment as well as denitrification on rocks, resistance and recovery were not applicable because rates increased after the storm event. Similarly, no decline in any of the rates on either substrate was observed after the 28 August 2018 storm (results not shown).

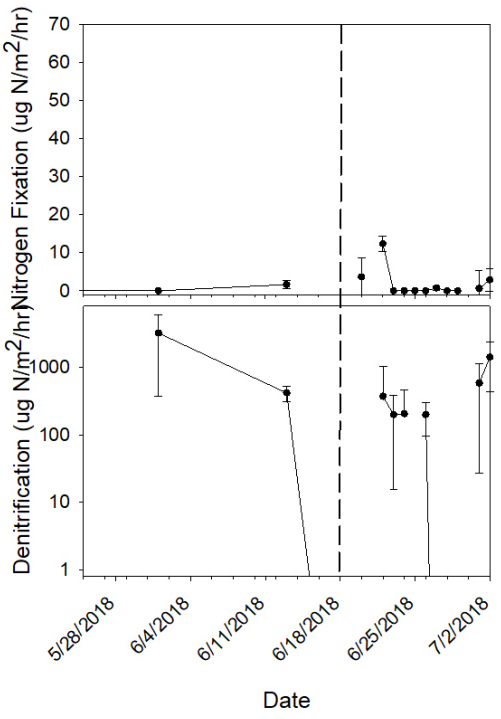

Figure 6: Nitrogen fixation rate on rock and denitrification rate in sediment before and after flood (indicated by bold black line).

Environmental factors related to variations in denitrification and nitrogen fixation rates

Inorganic nitrogen availability appeared be the important driver for denitrification rates, and to a lesser degree for nitrogen fixation rate. Partial least squares regression showed that 27.52% of the variation in rates of denitrification in sediment was explained by Component 1, (n = 88, Appendix Table A2). Component 1 was driven by DOC, TDN, and DON with weighted loadings of 0.549, 0.513, and 0.434, respectively. Similarly, 23.59% of the variation in rates of nitrogen fixation on rocks was explained with the first component (n = 71, Appendix Table A3). Component 1 explained 15.45% and was driven by TDN,

DON, DOC, ER and TDP with weighted loadings of 0.589, 0.583, 0.499, -0.451, and 0.433 respectively.

7. Discussion

We found that nitrogen cycling processes in the Pilgrim River were temporally dynamic and resilient to hydrologic disturbances. Day-to-day variation was high throughout the year, with no discernible difference between periods and in response to hydrologic events. Because rates were so dynamic, traditional methods for calculating resilience and recovery were not effective and/or potentially didn't apply because there was no consistent rate pre-hydrologic event to compare to post-event. Unexpectedly, rates were not significantly different among seasons; rather, within-season variation and daily changes in rates were as large as variation among seasons. The primary environmental driver of this variation appeared to be dissolved inorganic and organic nitrogen concentrations and dissolved organic carbon, although they explained relatively small amounts of the overall variation in process rates.

Seasonal patterns in nitrogen fixation and denitrification rates

Contrary to the findings of other studies, rates and variation in nitrogen fixation and denitrification did not differ seasonally, although environmental conditions were significantly different between seasons. Other studies which have examined seasonal changes found denitrification rates were related to seasonal shifts in nitrate concentration, dissolved oxygen, and organic carbon (Christensen et al., 1990; Clément, Pinay, & Marmonier, 2002; Inwood, Tank, & Bernot, 2005; Cabrita, 2000). This relationship was found in many ecosystems

including estuaries and riparian wetlands; however, seasonal studies are often performed in streams with higher nitrogen concentrations than our study river. The low nitrate concentrations which were consistent among seasons in the Pilgrim River sets it apart from these other studies and may explain the lack seasonal variation in denitrification rates.

Studies of nitrogen fixation in streams have detected seasonal rate shifts associated light, temperature, and nitrogen flux. Perhaps the most complete understanding of nitrogen cycling in a single stream exists for Sycamore Creek in the Sonoran Desert, where seasonal variation in nitrogen fixation was detected and correlated with light and temperature (Grimm & Petrone, 1997). Sycamore Creek is a desert stream that experiences seasonal drought and year-round warmer temperatures than the Pilgrim River. The Pilgrim River also experiences seasonal light and temperature fluctuations; however, the Pilgrim River is located in a temperate forested ecoregion, in general does not support the growth of large cyanobacteria algal mats, has much lower rates of nitrogen fixation, and has different temperature ranges and nutrient ratios than Sycamore Creek. Streams in the subalpine, coniferous forested Sawtooth Mountains of central Idaho exhibited nitrogen fixation rates more comparable (10 – 610 μg $N/m^2/hr$) to those measured in the Pilgrim River (0 – 50 μg $N/m^2/hr$), and seasonal changes in nitrogen fixation rates were related to seasonal changes in N flux (Marcarelli & Wurtsbaugh, 2009). Comparatively, the Pilgrim River had higher overall nitrogen concentrations than the Sawtooth Mountain streams, with no apparent seasonal variation, which could explain the lack of seasonal patterns in our study.

Additionally, for both denitrification and nitrogen fixation, the sampling frequency in most seasonal studies is generally lower in than in our study of the Pilgrim River, so that those studies might not encompass the full variability within a season. This, along with the overall low rates of nitrogen fixation and different environmental conditions in the Pilgrim River, could explain why no seasonal variation in nitrogen fixation or denitrification was observed in this study.

Daily variation in nitrogen fixation and denitrification rates and in their response to hydrological disturbances

Large shifts in process rates occurred day to day; however, unlike we predicted, these shifts did not result from high discharge events. After the 18 June 2018 storm event, which was estimated to be a > 1000-year flood (T. Weaver, USGS, personal communication) denitrification in sediment did decrease, but returned to pre-flood rates in three days, indicating a fast recovery. However, the pre-flood rate was also very low relative to other rates measured over the course of this study, and the denitrification rates stayed low for almost 2 weeks after the event. Therefore, simply based on recovery calculations, the flood appeared to have minimal impact on this process. Resilience of lotic systems is difficult to characterize due to frequent hydrological shifts (Friberg, 2014; Jaiswal & Pandey, 2021).

Environmental factors are related to variations in denitrification and nitrogen fixation rates

Nitrogen concentrations and DOC were the most important explanatory factors of rate variation using partial least squares regression, although the

variability explained by a single component was low (23-29%). As denitrification requires both organic carbon and nitrogen as reactants (Wall et al., 2005) our findings of DOC, DIN, and DON are logical to be implicated in the variability of denitrification and fits with findings of other studies. However, NO_3^+ concentration was not implicated potentially because of the low range of values. DON and TDN were both implicated in variation of nitrogen fixation rates. This was interesting as N:P was not selected which research suggests could be a more important driver of nitrogen fixation that nitrogen concentration alone. However, TDP also had high loadings of the 1st component (Smith 1990; Paerl et al., 2016). Additionally, the 1st component of the PLS had many variables with high loadings, suggesting that variability in nitrogen fixation rate may coincide with many different environmental variables including TDN, DON, DOC, ER, and TDP. However, it is important to know that this 1st component only explained 23% of the overall variation.

Our results suggest that we need to approach studying biogeochemical processes in rivers with caution because the processes may vary to a greater extent than previously believed. Assumptions of seasonal cycles are not always true, and estimating rates based on sampling a single day may provide gross misestimates based on the magnitude and frequency of variation in both nitrogen fixation and denitrification rates observed in this study. Additionally, the environmental conditions commonly assumed to be associated with denitrification and nitrogen fixation rates such as nutrient concentration, although still relevant, may not be sufficient for evaluating the occurrence or changes in

processes within a single stream. Concentrations of dissolved and organic nitrogen were related to rates of both denitrification and nitrogen fixation, however only about 25% of the overall variation within each rate was associated with the measured environmental variables. It is likely these relationships are much more complicated, involving many predictor variables interacting in ways we do not yet understand.

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Chapter 3. Together Together: Nitrogen fixation and denitrification commonly co-occur but understanding complex multi-scale environmental drivers is difficult

1. Abstract

Nitrogen fixation and denitrification are important processes for regulating nitrogen concentration in streams. They can co-occur in streams yet are rarely studied together, and we do not have a firm understanding of the environments which facilitate their occurrence and co-occurrence. This study examined how environmental conditions at reach and watershed scales were related to rates of nitrogen fixation and denitrification in 12 streams distributed across 9 ecoregions in the USA. We wanted to see if watershed characteristics like size and land use were correlated with reach level characteristics, and which watershed and reach-level characteristics were related to nitrogen fixation and denitrification rates using multiple linear regressions. Most likely due to the small sample size and many variables included, we did not find significant relationships between environmental variables and rates of nitrogen fixation and denitrification. Nitrogen fixation is rarely studied in streams and this study added considerably to our pooled knowledge on the occurrence of this process. Finding nitrogen fixation and denitrification co-occurring in 9 of the 12 sites showed it is relatively common and suggests that nitrogen fixation is an important process to include in studies of nitrogen cycling in streams.

2. Keywords

denitrification, nitrogen fixation, watershed

3. Introduction

Nitrogen fixation and denitrification can occur simultaneously in streams (Eberhard et al., 2018), but the rate of these processes can be highly variable (Chapter 2), and how the processes relate to environmental variables such as nutrient concentrations, temperature, light intensity, and the surrounding land cover is poorly understood (Howarth, Marino, & Cole, 1988; Mulholland et al., 2008). Nitrogen fixation and denitrification have rarely been studied together because it was assumed that in systems with high nitrogen loads and/or concentrations, denitrification rates would be high while nitrogen fixation rates would be low or zero (Marcarelli et al., 2008; Newell et. al., 2016). However, when N:P (nitrogen: phosphorus) ratios are low, nitrogen fixation can still occur despite relatively high amounts of environmental nitrogen (Emerson et al., 2001; Scott & Grantz, 2013). Denitrification has been extensively researched in streams because of its potential to mitigate eutrophication (Groffman, Davidson, & Seitzinger, 2009; Marcarelli et al., 2008). Understanding the balance between these two processes is vital because streams, especially small streams, have the capacity to remove and process large amounts of nitrogen (Alexander, Smith, & Schwarz, 2000; Bernot & Dodds, 2005) and therefore may be disproportionate players in the global nitrogen cycle.

Increased nitrogen availability has been shown to decrease nitrogen fixation and increase denitrification (Grimm, 1987; Grimm & Petrone, 1997; Wall et al., 2005); however, other environmental factors can also control these two processes. Slower, shallower streams are associated with higher retention of

nitrogen, which could be caused by increased uptake by primary producers or high rates of denitrification. In streams, nitrogen fixation is often performed by organisms that carry out photosynthesis, and therefore nitrogen fixation can be subject to the same restrictions as photosynthesis regarding light availability (rates increase with increased light intensity) and temperature (rates increase with warmer temperatures) (Scott & Marcarelli, 2012; Śpiewla, 1995). Yet, there are a wide variety of nitrogen fixing organisms that occur in aquatic and terrestrial habitats, and the process is not solely tied to photosynthetic or aerobic pathways (Klawonn, Bonaglia, Brüchert, & Ploug, 2015; Newell et al., 2016), although heterotrophic nitrogen fixers have rarely been considered or quantified in streams (Larson et al., 2018). Denitrification rates may be impacted by temperature, oxygen (O_2) concentration, and organic matter (Cornwell et al., 1999; Piña-Ochoa & Álvarez-Cobelas, 2006a). Denitrification is an anaerobic process performed by facultative anaerobes, so lower oxygen concentrations and high supply of organic carbon will be more favorable for the process (Cornwell et al., 1999). Increased respiration caused by decomposition of increased plant biomass also can create anaerobic environments, thereby promoting denitrification (Sutton-Grier et al., 2013). Considering the variation in these factors may be the key for understanding where and when nitrogen fixation and denitrification occur or co-occur in streams.

Scale and cross-scale interactions are important when exploring environmental controls of ecological and biogeochemical processes. On a watershed scale, land use, geological structure and climate of the surrounding

watershed will control nutrient loading to the stream, while characteristics like soil type and rainfall can influence the velocity of nutrients entering a stream (Crossman et al., 2014; Meyer, Paul, & Taulbee, 2005). The landscape across the entire watershed and the landscape directly adjacent to a stream reach can both alter the environmental constraints of these processes, especially with anthropogenic activities (Howarth et al., 2012). Variation in characteristics like stream depth and velocity may also control rates of nutrient uptake and oxygen concentrations but may have different consequences depending on scale. For example, slow and shallow stream segments could remove large amounts of nitrogen, thereby decreasing denitrification and promoting nitrogen fixation; however, deep, slow water may promote low oxygen exchange and high rates of denitrification (Bernot & Dodds, 2005). Conditions like temperature, light availability and oxygen concentration are controlled on a reach level. The environmental influences at different scales indicate the potential for cross-scale interactions, as a slow and shallow stream may be able to mitigate nutrient input from the broader landscape. However, this ability to buffer nutrient concentrations is not limitless and large amounts of nutrient loading could overwhelm this process, thereby causing high nutrient concentrations in the river (Bernot & Dodds, 2005). Predicting when and where nitrogen fixation and denitrification occur in stream ecosystems, as well as the balance between the two processes, will require understanding the environmental drivers across all these spatial scales.

The purpose of this study was to better understand how nitrogen fixation and denitrification co-occur across stream systems, and how environmental conditions across spatial scales control this relationship. Our central question was, how do environmental variables at different scales (i.e., reach and watershed) correlate with the rates of these processes? I hypothesized: 1) Smaller watersheds will transfer less nitrogen into the stream, and therefore watershed size will be positively correlated with nitrogen concentrations and negatively correlated with nitrogen fixation. 2) higher amounts of urban and agricultural land cover at segment and watershed scales will be correlated with higher nitrogen concentrations, higher rates of denitrification and lower rates of nitrogen fixation. 3) Both watershed and reach level drivers will be important drivers of denitrification and nitrogen fixation rates. 4) Rates of aerobic respiration and gross primary production (GPP) will be correlated with rates of nitrogen fixation and denitrification. To test these hypotheses, I measured nitrogen fixation and denitrification rates in 12 streams located in different ecoregions across the USA. At each stream, both watershed and reach level environmental characteristics were measured and related the nitrogen fixation and denitrification rates.

4. Methods

We conducted chamber-level measurements of nitrogen fixation and denitrification in 12 streams located across the USA (Table 2; Figure 7) to capture a wide range of environmental conditions as well as nitrogen fixation and denitrification rates. Sites were in different ecoregions, with 2 of the sites

(Arikaree River and Kings Creek) sampled twice in different years and all other sites sampled once. Sites consisted of a ~50 m reach located just downstream of National Ecological Observatory Network (NEON) sensors. By sampling established NEON sites, we were able to leverage multi-scale environmental data from their databases. Rivers contained a range of substrates including algal mats, coarse particulate organic matter, macrophytes, pebbles, and bedrock; in this study, we sampled rock, sediment and wood substrates as they were the most common across all sites and because we expected that the conditions of each substrate could favor different microbial assemblages taking advantage of micro-habitat differences favoring either nitrogen fixation or denitrification.

Table 2: Name and location of each site sampled, organized by sampling date. Arikaree River and Kings Creek were both sampled twice in different years while all other sites were sampled only once. Ecoregion is based on the NEON designation. Note that not all sites had all 3 substrates used in this analysis; the “Substrates Sampled” columns lists which substrates were found in each stream.

Site Name	State	Ecoregion	Sample Date	Latitude	Longitude	Substrates Sampled
Arikaree River	CO	Central Plains	11Jul2017	39.75825	-102.4471	Sediment
Pringle Creek	TX	Southern Plains	09Jun2018	33.37859	-97.78226	Rock, Sediment, Wood
McDiffit Creek	KS	Prairie Peninsula	12Jun2018	38.94428	-96.44197	Rock, Sediment, Wood
Kings Creek	KS	Prairie Peninsula	13Jun2018	39.10506	-96.60336	Rock, Sediment, Wood
Hop Brook	MA	Northeast	15Aug2018	42.47179	-72.32963	Rock, Sediment, Wood
Rio Guillarte	PR	Atlantic Neotropical	21Feb2019	18.1741	-66.79851	Rock
Rio Cupeyes	PR	Atlantic Neotropical	23Feb2019	18.11352	-66.98676	Rock
Kings Creek	KS	Prairie Peninsula	10May2019	39.10506	-96.60336	Rock, Sediment, Wood
Sycamore Creek	AZ	Desert Southwest	14May2019	33.74906	-111.5069	Rock, Sediment, Wood
Arikaree River	CO	Central Plains	24May2019	39.75825	-102.4471	Sediment
Caribou Creek	AK	Taiga	09Jul2019	65.15306	-147.5025	Rock, Sediment, Wood
Blacktail Deer Creek	WY	Northern Rockies	30Jul2019	44.95011	-110.5872	Rock, Sediment, Wood
McRae Creek	OR	Pacific Northwest	03Aug2019	44.2596	-122.1656	Rock, Sediment, Wood
Martha Creek	WA	Pacific Northwest	06Aug2019	45.79125	-121.932	Rock, Sediment, Wood

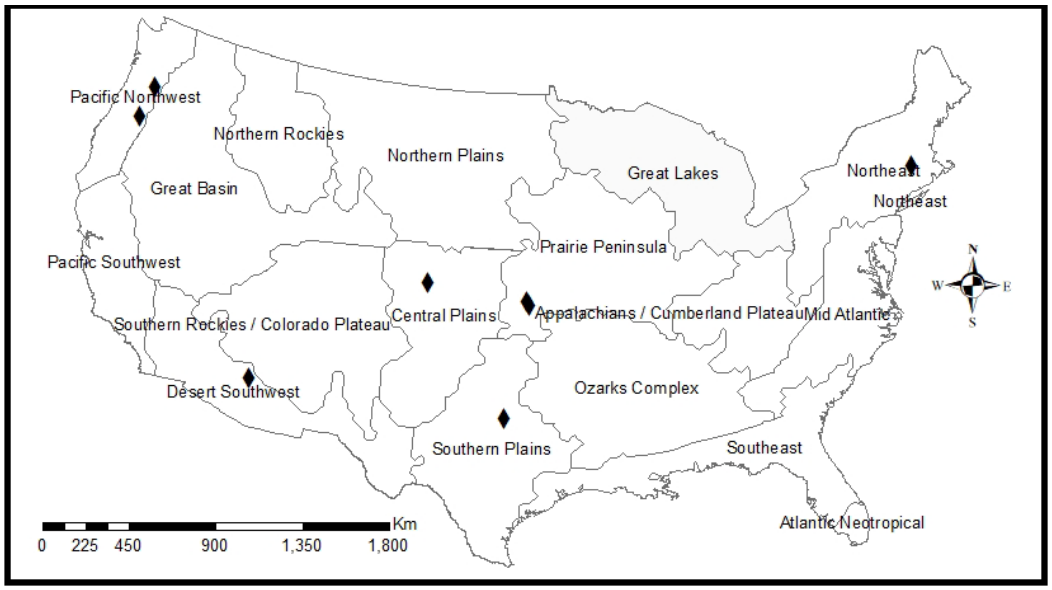

Figure 7: Map of ecoregions and sample sites. Borders indicate different ecoregions and are labeled, while solid black diamonds indicate sample sites. Sample sites were also located in the Taiga ecoregion in Alaska and Atlantic Neotropical ecoregion in Puerto Rico, but these locations are not included in the map.

Denitrification and Nitrogen Fixation Methods

Denitrification and nitrogen fixation rates were measured on rock, wood, and sediment substrates using acetylene block and acetylene reduction assay techniques. At each site, 200 mL sediment cores and chunks of wood were placed in pint glass mason jars and rocks were placed in 2L polycarbonate food storage containers fit with a Viton O-ring. The lids of all chambers were drilled to fit a 13 x 20mm septa. Chambers were filled with stream water to remove all air and sealed. Blank chambers for sediment and wood were filled with stream water while blank chambers for rocks contained rocks collected outside the stream and filled with stream water. This allowed us to account for chamber effects such as gas leakage or processes occurring in the water column.

Nitrogen fixation was measured by introducing acetylene gas to a chamber. Nitrogenase, the enzyme that fixes nitrogen, also converts acetylene to ethylene, and we measured the change in ethylene concentration to estimate the amount of nitrogen that could have been fixed (Eberhard et al. 2018; D. G. Capone, 1993). A 20% headspace (v/v) of acetylene gas was introduced to each filled and sealed chamber. Denitrification was measured by spiking chambers to a final concentration of 34 mg/L sucrose and sodium nitrate and 114 mg/L chloramphenicol, and a 20% (v/v) acetylene headspace was introduced to each chamber. Chambers were incubated in stream for ~2hrs, and an initial and a final 9mL gas sample were collected from each chamber.

Gas samples from chambers measuring nitrogen fixation were analyzed for ethylene concentration using a SRI 8610C Gas Chromatograph with Hayesep T column, flame ionization detector, and column oven set at 40 °C ramping to 110 °C after 2.5 min running with hydrogen carrier gas. A 100-ppm ethylene standard was used to convert peak height to concentration of ethylene in the gas sample. The amount of ethylene in the headspace (initial and final) was determined and using water volume and the solubility constant for ethylene using equations from Dodds et al (2017). This was used to estimate the amount of N fixed by assuming 1 molecule of acetylene converted to ethylene is equal to 3 atoms of nitrogen being fixed (Capone, 1993; Kim et al., 2006).

Denitrification gas samples were analyzed for N_2O concentration using a SRI8610C Gas Chromatograph with column oven set to 80°C and a Hayesep D column with Electron Capture Detector. 5 min after sample injection the column

temperature increased at 100°C /min to a final temperature of 180°C. 1 mL injections of 1000 ppm N_2O standard was used as a standard. Based on Dodds et al. (2017), the amount of N_2O in the chamber was determined from water volume and the N_2O solubility constant. Subtracting initial from final concentration gave the amount of N_2O produced during the incubation, which was converted to moles of nitrogen (Bondo et al., 2010; Capone, 1993; Kim et al., 2006).

Reach and Watershed Environmental Characteristics

Reach and watershed-scale environmental characteristics were determined for each site. Reach-scale characteristics were measured at the ≤ 50 m segment of the river in which we measured nitrogen fixation and denitrification and included temperature (ºC), stream discharge (m^3/s), stream width (m), photosynthetically active radiation (PAR; W/m^2), average transect depth (cm), average canopy cover (%), dissolved organic carbon (DOC; mg / L), total dissolved nitrogen (TDN; mg / L), NH_4^+ (µg / L), NO_2 + NO_3^- (µg / L), soluble reactive phosphorus (SRP; µg / L), total dissolved phosphorus (TDP; µg / L), total phosphorus (TP; µg / L), and dissolved inorganic nitrogen (DIN; µg / L). Watershed characteristics included watershed area and land use. Watershed information for the site was obtained from NEON (2021a) and included watershed size and the percent of that watershed covered by each of the NLCD land use categories. Water temperature was retrieved from NEON (2021b). These values were not available for King's Creek when sampled in 2019 or McRae Creek, so water temperatures measured during the chamber incubations

were used instead. Average daily discharge was calculated from continuous discharge data retrieved from NEON (2021c). When continuous discharge was not available (King's Creek, McDiffit Creek, Blacktail Deer Creek, McRae Creek), manual discharge measurements using the reading closest in time to our sampling date was used from NEON (2021d). Depth was measured at 10 points along a transect across the stream. Transects were located every 10 m along the length of the sampling reach and averaged together. The edge-to-edge length of each transect was averaged to estimate stream reach width. Canopy cover was measured at the midpoint of every transect facing the 4 cardinal directions using a spherical densitometer (Lemmon, 1956). Mean photosynthetically active radiation (PAR) was calculated during the sampling period for each stream retrieved from NEON (2021e). PAR was not available for McRae Creek through NEON, so data was retrieved from a nearby station that was part of the H.J. Andrews Forest Long Term Ecological Research (2021). Water was filtered through 0.45 μm membrane filters and stored on ice until returned to the lab, where the samples were frozen. Filtered water samples were acidified to a pH < 2 and sent to Michigan Tech's Laboratory for Environmental Analysis of Forests (LEAF) core facility which used a Shimadzu TOC-VCSN with a total N module TNM-1 (Shimadzu Scientific Instruments, Columbia, Mary- land) for DOC and TDN analysis. NH_4^+ was analyzed based on Holmes, Aminot, Kérouel, Hooker, & Peterson (1999) fluorometric method on a Turner Aquafluor (Turner Designs, Palo Alto, CA, USA). NO_2 + NO_3^- and SRP were analyzed using a SEAL AQ2 discrete water analyzer. NO_2 + NO_3^- used AQ2 method EPA-127-A Rev. 9, SRP

used AQ2 method EPA-155-A Rev. 0. TDP concentration was analyzed using molybdenum—antimony method following an ammonium persulfate digestion (APHA, 2005). TP used the same method as TDP with unfiltered water samples. Dissolved Inorganic Nitrogen (DIN) was calculated by adding NO2 + NO3- and NH4+ concentrations.

Aerobic respiration and net production rates were measured using the same chambers and substrates as those used for measuring nitrogen fixation and denitrification. Using the light/dark bottle approach, chambers were divided into three different types: initial to quantify conditions at the start of the incubation, light chambers to quantify net production (which is equal to gross primary production, or GPP, minus aerobic respiration), and dark chambers to quantify aerobic respiration. Initial chambers were sampled immediately, then light and dark chambers were incubated in the stream for ~2hrs. To sample oxygen concentrations from all chambers, triplicate water samples were siphoned into 12mL Exetainers with minimal air contact and zinc chloride was added to a final concentration of 0.26% W/V to kill microbes and prevent bottle effects. Barometric pressure and temperature were measured at the time of each sample collection for calculation of O_2 gas saturation. A membrane-inlet mass spectrometer (MIMS) with 2-temperature calibration was used to analyze O_2 and argon (Ar) concentrations in the samples. Standards were established by constantly mixing water in round-bottom flasks to achieve gas equilibration with the atmosphere. The standards were placed in water baths set to the lowest and highest temperature measured in the chambers on that sampling date. Assuming

gas saturation for both standards, we developed a standard curve based on MIMS output for O_2/Ar concentration. Using the curve, O_2 concentrations for each chamber were determined. Final concentrations were subtracted from initial then converted to g O_2/m^2 by multiplying by chamber water volume and dividing by substrate surface area (Dodds, Burgin, Marcarelli, & Strauss, 2017). Net production was determined from light chambers with positive numbers indicating net autotrophic and negative numbers indication net heterotrophic. Aerobic respiration was indicated by the dark chambers and were negative. Gross primary production was calculated by subtracting aerobic reparation from net production.

Data Analysis

To address the first hypothesis that smaller watersheds would transport less nitrogen into the stream, we looked for positive correlations between watershed size and NO_2 + NO_3^-, NH_4^+, DIN, and TDN and for negative correlations between watershed size and nitrogen fixation. Variables were considered correlated with $p < 0.05$ and $r > 0.5$ or < -0.5. Pearson's correlations were performed using the Hmisc package in R (Harrell et al., 2020).

To address hypothesis 2, we examined correlations between % of watershed covered by developed or planted land for positive correlations with NO_2 + NO_3^-, NH_4^+, DIN, and TDN, as well as positive correlation between those land use types and denitrification and negative correlation with nitrogen fixation.

To look for other environmental drivers of nitrogen fixation and denitrification as listed in hypothesis 3, a total of six multiple linear regression

models were run using the basic stats package in R (R Core Team., 2020). The dependent variables included nitrogen fixation on rock, nitrogen fixation on wood, nitrogen fixation in sediment, denitrification on rocks, denitrification on wood, and denitrification in sediment. Model selection was performed using Akaike Information Criterion (AIC) through the MuMIn package in R (Barton, 2020). Models with delta AIC < 2 were selected. If the null model was AIC < 2 no models were selected. To avoid collinearity in the model, a Principal Components Analysis (PCA) was performed using the basic stats package in R (R Core Team 2020). Percent land cover for each watershed and PC1 and PC2 were extracted for each site for use in analysis. All models also included PC1 from the PCA, watershed area, DOC, SRP, DIN, water temperature, discharge, and PAR. Other measured variables, including PC2 for land cover, were excluded due to collinearity. Collinearity was determined through Pearson's correlation using the Hmisc package in R (Harrell et al., 2020). Correlations with $r>0.5$ and $p<0.05$ were considered colinear. When Variables were colinear I selected the variables I judged to be most relevant based on literature.

Hypothesis 4 was addressed using Person's correlations to look for correlations between different production process (i.e., aerobic respiration, and Gross Primary GPP) and nitrogen fixation or denitrification on each substrate (i.e., rock, wood, and sediment).

5. Results

Both denitrification and nitrogen fixation were commonly detected at our study sites. At 2 streams, Rio Guillarte and Caribou Creek, we did not detect

either denitrification or nitrogen fixation. The other streams all had measurable rates of denitrification (Figure 8, Table 3). Nitrogen fixation was also detected in all the remaining streams except for Rio Cupeyes. The highest rate of nitrogen fixation occurred on rocks in Blacktail Deer Creek at 11.1 ± 8.6 μmol N/m ^{2}/hr (mean ± 95% CI). The highest denitrification rate occurred in sediment in McDiffitt Creek at 739.7 ± 218.2 μmol N/m 2/hr (mean ± 95% CI).

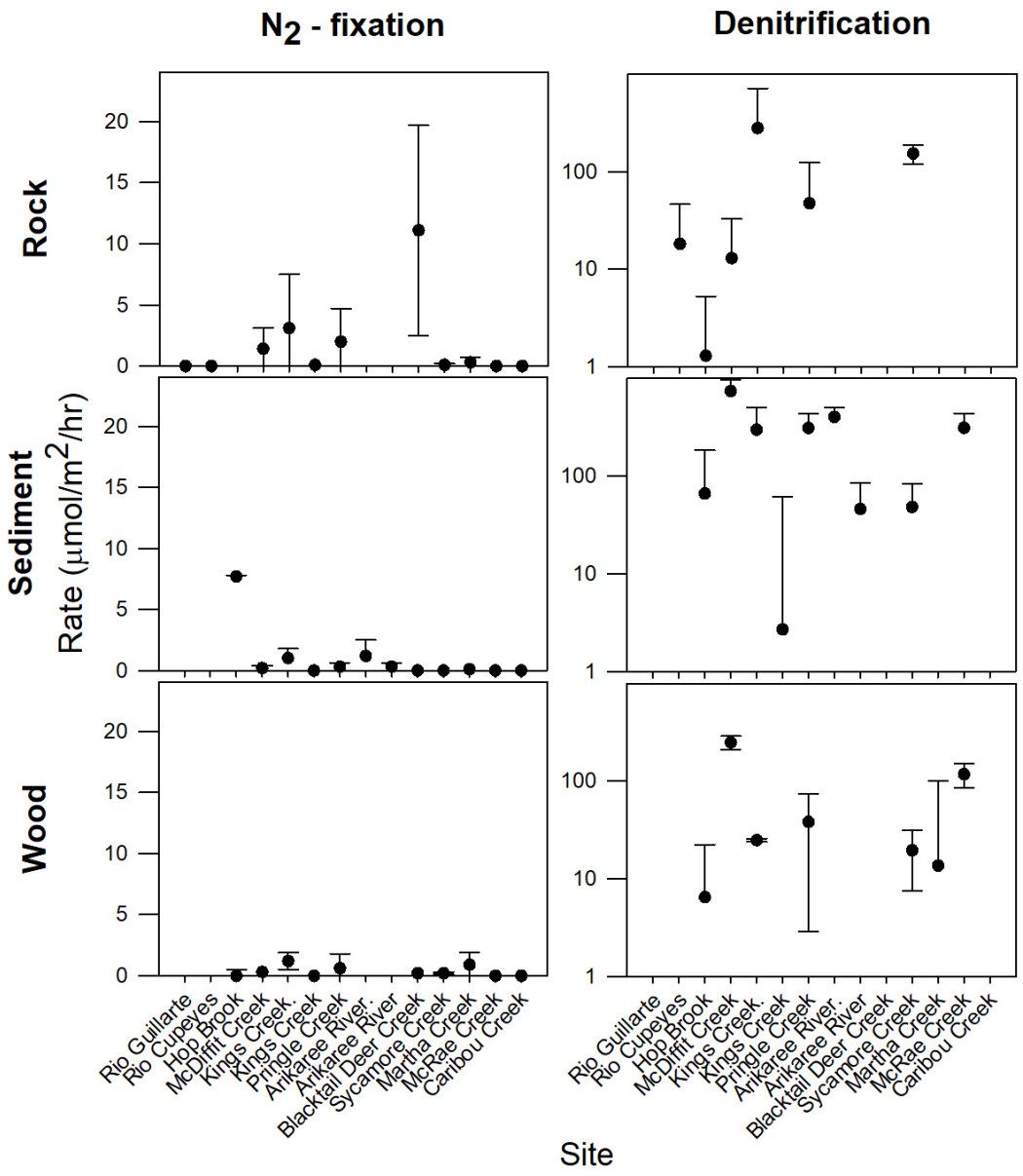

Figure 8: Nitrogen fixation (left) and denitrification (right) rates for rock (top), sediment (middle), and wood (bottom) at each site. Note that the y-axis range for nitrogen fixation is much lower than for denitrification and that denitrification is plotted on a log-scale. Points indicate the mean rate and error bars show 95% CI. Sites are arranged from east to west along the x-axis. Sites with more than one entry were sampled multiple times and are listed in chronological order of when they were sampled.

Table 3: Mean ± 95% confidence interval of nitrogen (N_2) fixation and denitrification rates for substrate at each stream sampled. NA indicates that rate was not measured, typically because the substrate was not at that site. For Blacktail Deer Creek denitrification data were not included due to equipment malfunctions. Sites are listed from east to west and sites that were sampled twice are listed twice in chronological order of sampling date.

	Rock		Sediment		Wood	
	N_2 fixation	Denitrification	N_2 fixation	Denitrification	N_2 fixation	Denitrification
Rio Guillarte	0.0 ±0.0	0.0	NA	NA	NA	NA
Rio Cupeyes	0.0 ±0.0	18.2 ±28.1	NA	NA	NA	NA
Hop Brook	0.0	1.3 ±4.0	7.7 ±0.1	66.3 ±117.0	0.0	6.5 ±5.9
McDiffit Creek	1.4 ±1.7	13.0 ±19.7	0.3 ±0.2	739.7 ±218.2	0.0 ±0.0	249.1 ±0.2
Kings Creek	3.1 ±4.4	278.0 ±431.5	1.0 ±0.8	297.6 ±204.6	0.2 ±0.7	24.9 ±1.0
Kings Creek	1.1 ±1.1	0.0	0.0 ±0.0	2.7 ±58.7	0.0 ±0.0	0.0
Pringle Creek	2.0 ±2.7	47.4 ±5.5	0.3 ±0.3	310.1 ±122.8	0.4 ±1.2	38.3 ±0.3
Arikaree River	NA	NA	1.2 ±1.3	403.0 ±104.3	NA	NA
Arikaree River	NA	NA	0.3 ±0.3	46.0 ±39.6	NA	NA
Blacktail Deer Creek	11.1 ±8.6	NA	0.2 ±0.0	NA	0.4 ±0.4	NA
Sycamore Creek	0.1 ±0.1	152.9 ±3.0	0.0 ±0.0	48.3 ±35.7	0.1 ±0.1	19.6 ±12.0
Martha Creek	0.3 ±0.4	0.0	0.1 ±0.0	0.0	0.0 ±1.0	13.7 ±85.9
McRae Creek	0.0 ±0.0	0.0	0.0 ±0.0	310.9 ±127.7	0.0 ±0.0	117.7 ±32.2
Caribou Creek	0.0 ±0.0	0.0	0.0 ±0.0	0.0	0.0 ±0.0	0.0

The watersheds included in this study had a wide range of land use types and size. 50% of the combined watershed land cover was planted/cultivated with an additional 40% herbaceous across all sites. This was primarily driven by the Arikaree River watershed, which was 2,890 km^2, with the remaining watershed areas ranging between 4 and 273 km^2. When percent land use of the watersheds were analyzed using PCA, the first 2 components explained 52% of the total variation, with 30% explained by component 1 and 22% by component 2 (Figure 9). PC1 was largely driven by forest and herbaceous land cover and a lesser extent barren and planted/cultivated. The largest drivers of PC2 were driven by open water and developed (Table 4).

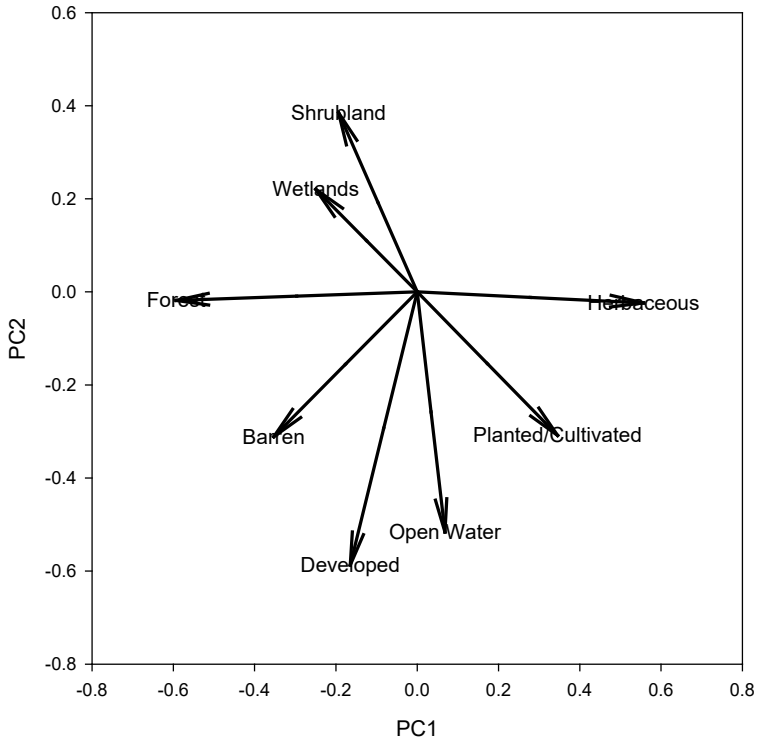

Figure 9: Biplot of land use characteristics with principal component 1 on the X-axis explaining 30% of the variation and principal component 2 on the Y-axis explaining 22% of the variation. Loadings for the two PCA components are reported in Table 4. Principal components 1 and 2 were used in the regression models.

Table 4: Loadings for principal component 1 and 2 describing the land use percent or the sites. PC1 explained 30% of the variation and PC2 explained 22% of the variation.

	PC1	PC2
Open water	0.068	-0.516
Developed	-0.165	-0.587
Barren	-0.353	-0.311
Forest	-0.593	-0.018
Shrubland	-0.019	0.384
Herbaceous	0.557	-0.024
Planted/Cultivated	0.345	-0.309
Wetlands	-0.24	0.221

Reach-scale environments also had a wide range of environmental characteristics (Table 5). Discharge ranged from 0 m^3 / s in Kings Creek and Sycamore Creek to 164 m^3 / s in Caribou Creek. Total phosphorus ranged from 4 μg / L in Caribou Creek to 109 μg / L in Arikaree River. DIN ranged from 9 μg / L in Hop Brook to 370 μg / L in Rio Cupeyes and PAR ranged from 29 W/m^2 in Martha Creek to 741 W / m^2 in Rio Guillarte.

Table 5: Reach level environmental characteristics for each. (average width of sample reach (width), average depth of sample reach (Depth), average canopy cover of sample reach (canopy cover), dissolved organic carbon (DOC), total dissolved nitrogen concentration (TDN), ammonium concentration (NH_4^+), nitrate and nitrate concentration ($NO_3^- + NO_2$), soluble reactive phosphorous concentration (SRP), total dissolved phosphorous concentration (TCP), total phosphorous concentration (TP), dissolved inorganic nitrogen concentration (DIN), water temperature (temp), discharge, and photosynthetically active radiation (PAR).

Site Name	Width (m)	Depth (m)	canopy cover (%)	DOC (mg/L)	TDN (mg/L)	NH_4^+ (mg/L)	$NO_3^- + NO_2$ (mg/L)	SRP (mg/L)	TDP (mg/L)	TP (mg/L)	DIN (mg/L)	Temp (^oC)	Discharge (L/s)	PAR (W/m^2)
Arikaree River	1.80	0.06	2.91	6.164	0.31	59.46	51.29	18.04	21.06	109.20	110.75	24.25	1.03	552.91
Pringle Creek	5.30	0.19	59.24	7.7	0.22	6.70	5.73	6.36	1.22	17.11	12.43	26.46	12.47	694.55
McDiffit Creek	3.36	0.14	92.20	16.9	0.92	84.84	98.72	2.88	23.82	57.01	183.56	21.52	-0.24	292.61
Kings Creek	3.19	0.22	85.76	14.6	0.22	74.68	5.73	8.91	42.31	103.85	80.41	25.52	0.00	149.95
Lower Hop Brook	5.16	0.27	94.48	6.2	0.20	3.59	5.73	2.88	9.54	13.64	9.32	19.21	593.67	55.65
Rio Guilarte	4.58	0.26	40.46	2	0.30	6.37	268.82	12.86	25.03	19.08	275.19	20.60	60.81	740.97
Rio Cupeyes	3.37	0.09	82.42	5.9	0.40	1.98	368.51	17.50	4.65	7.39	370.49	22.14	23.84	478.74
Kings Creek	6.46	0.34	63.29	6.9	0.15	1.33	56.61	10.50	10.85	15.74	57.94	12.96	678.00	202.02
Sycamore Creek	2.23	0.09	3.94	7.9	0.24	9.16	97.64	36.60	64.04	60.84	106.80	21.12	0.00	476.22
Arikaree River	4.03	0.19	1.20	14.9	0.39	5.06	12.60	31.50	12.62	14.07	17.66	12.81	69.89	677.73
Caribou Creek	2.87	0.15	15.43	4.7	0.38	9.52	285.17	7.81	4.65	4.05	294.69	5.73	164.89	160.06
Blacktail Deer Creek	2.48	0.18	41.21	3.1	0.08	4.56	41.93	5.00	37.45	39.12	46.49	14.10	341.30	458.54
McRae Creek	3.72	0.07	89.44	1	0.03	1.09	10.70	7.80	4.65	7.39	11.79	18.23	8.06	377.01
Martha Creek	2.93	0.11	43.75	1.7	0.15	4.78	43.39	33.20	28.58	22.42	48.17	16.38	0.00	28.78

To address hypothesis 1, there were no correlations, positive or negative, between watershed size and $NO_2 + NO_3^-$, NH_4^+, DIN, or TDN nor between watershed size and nitrogen fixation rate. With regards to hypothesis 2, no significant correlations, positive or negative, were found between % developed or % planted land and any nitrogen concentration (i.e., $NO_2 + NO_3^-$, NH_4^+, DIN, and TDN) nor between those land uses and nitrogen fixation or denitrification (Appendix Table 9).

To address hypothesis 3, multiple linear regression models did not show any relationships between the nitrogen cycling processes and environmental variables. All models showed no significant relationships and AIC selected the null model for all models, i.e. nitrogen fixation on rocks ($f = 0.3552$, $p = 0.8899$, adj R^2 =- 0.6959; Shapiro Wilks: $W = 0.90979$, $p = 0.212$; Appendix Table A5), denitrification on rocks ($f = 0.3568$, $p = 0.8889$, adj R^2=-0.6928; Shapiro Wilks: $W = 0.89006$, $p = 0.118$; Appendix Table A6), nitrogen fixation in sediment ($f = 1.027$, $p = 0.5215$, adj $R^2 = 0.01675$; Shapiro Wilks: $W = 0.95941$, $p = 0.7753$; Appendix Table A7), denitrification in sediment ($f = 2.848$, $p = 0.1643$, adj $R^2 = 0.5405$; Shapiro Wilks: $W = 0.91626$, $p = 0.2564$; Appendix Table A8), nitrogen fixation on wood ($f = 0.584$, $p = 0.7419$, adj $R^2 = -0.4782$; Shapiro Wilks: $W = 0.96$, $p = 0.8261$; Appendix Table A9), denitrification on wood ($f = 2.895$, $p = 0.4248$, adj $R^2 = 0.6238$; Shapiro Wilks: $W = 0.91548$, $p = 0.3562$; Appendix Table A10).

To address hypothesis 4, we did find correlations between nitrogen fixation and denitrification on rocks with metabolic processes (Figure 10).

Nitrogen fixation rate on rocks was correlated with respiration in sediment (r = 0.69, p = 0.027); nitrogen fixation rate on rocks was correlated with respiration on wood (r = 0.01, p = 0.037); denitrification rate on rocks was correlated with respiration in sediment (r = 0.78 p = 0.008); and denitrification rate on rocks was correlated with respiration on wood (r = 0.67 p = 0.052).

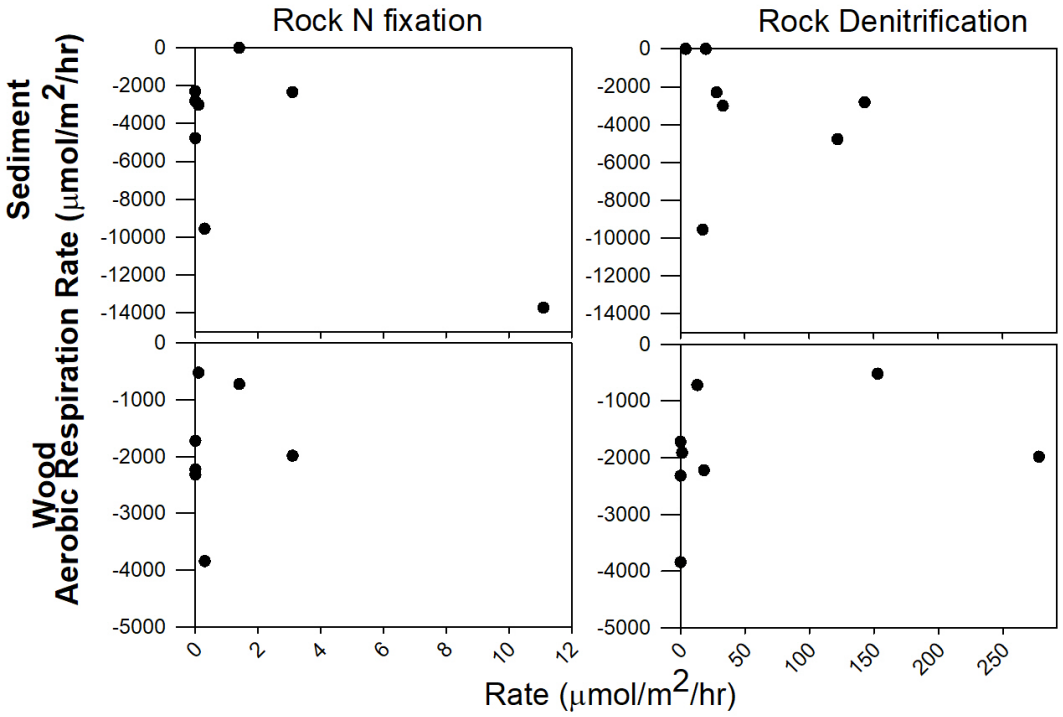

Figure 10: Nitrogen fixation (left) and denitrification (right) on rocks (top) vs. respiration rate in sediment (top), and wood (bottom).

6. Discussion

In this study we found several streams in which nitrogen fixation and denitrification co-occurred; however, we were unable to determine specific watershed-level and reach-level drivers of these two processes. Both processes occurred across a range of watershed types and other environmental conditions. These included large and small watersheds, natural and anthropogenically-

altered watersheds, high and low nitrogen, phosphorus and carbon concentrations, as well as a range of other environmental characteristics. Deciphering how those environmental variables at different scales affected denitrification and nitrogen fixation was difficult because many of the variables were correlated with other reach and watershed-scale; however, any of the variables we predicted would be related, such as watershed land use and nutrient concentration, were not correlated. Coupled with our small sample size, we were limited in our statistical power and ability to create models that explained the variation we observed in nitrogen fixation and denitrification rates across the study streams.

We did not observe the relationships between cross-scale environmental factors that we expected to be important for controlling nitrogen fixation and denitrification. Watershed size did not appear to be correlated to nitrogen concentration in streams, and we did not find correlations between these nitrogen concentrations and any of the watershed level variables (i.e., % developed and % planted). This was surprising because it is believed that urban and agricultural streams have higher nitrogen concentrations that promote denitrification and limit nitrogen fixation, as found in other studies (Findlay et al., 2011). However, there are many other environmental variables that can affect denitrification and nitrogen fixation such as dissolved oxygen, and our study encompassed a large range of these variables potentially interacting in unknown ways. We speculate we were unable to detect these relationships due to our small sample size. Therefore, how land use interacts with other drivers such as nutrient

concentrations of nitrogen fixation and denitrification needs to be further explored.

Both denitrification and nitrogen fixation occurred simultaneously in most of the streams measured. This further supports more recent findings detailing the importance of studying both processes simultaneously in streams (Eberhard et al., 2018; Marcarelli et al., 2008). This current study encompassed a wide range of environments, and nitrogen fixation was detected at most of the sites; however, the range of rates was small, and magnitudes were low (0 – 11.1 ± 8.6 μmol N_2/m^2/ hr). With other studies reporting rates close to 100 μmol N_2/m^2/ hr (Marcarelli et al., 2008), the narrow range of rates reported in the current study may have made it difficult to model its relationship with environmental variables. However, detecting nitrogen fixation at so many streams in different ecoregions demonstrates that this process may be more common than previously assumed. For example, McDiffitt Creek had detectable nitrogen fixation rates on all 3 substrate types (1.4 ± 1.7, 0.2 ± 0.2, 0.3± 0.0 μmol N2 / sq m / hr; rock, wood, sediment mean ± 95%CI). Many past studies assume nitrogen fixation will not occur, especially in high nitrogen stream such as McDiffit Creek (DIN 183.6 μg/L) because nitrogen would not be limiting (Marcarelli et al., 2008). This study shows even in high nitrogen environments, there is potential for nitrogen fixation.

The range of denitrification rates were similar to other studies. Our study found rates up to 739.7± 218.2 μmol N /m^2/ hr (mean ± 95% CI), while other studies reported similar rates of 700 N /m^2/ hr (Piña-Ochoa & Álvarez-Cobelas, 2006b). Unlike the current study, other studies have found nutrients, especially

nitrogen concentrations and DOC, to be important drivers of denitrification (Mulholland et al., 2008; Piña-Ochoa & Álvarez-Cobelas, 2006b). Denitrification requires both nitrogen and carbon compounds as reactants, however we did not see significant relationships between denitrification and these in our study even with a wide range of denitrification rates and nitrogen and carbon concentrations observed across study sites.

Other in-stream processes including photosynthesis and autotrophic respiration may be important drivers of nitrogen fixation and denitrification. Photosynthetic organism can take up inorganic nitrogen, making it less accessible to denitrifiers (Mulholland et al., 2006; Sutton-Grier et al., 2013). However, this may not be important in streams where nitrogen is abundant. None of the forms of nitrogen measured in the current study (TDN, DIN, NO_3^-, or NH_4^+) were corelated with GPP measured on rock, on wood, or in sediment, suggesting higher rates of photosynthesis did not limit nitrogen in the stream or vice versa. Additionally, GPP rates were not related to denitrification rates.

Nitrogen fixation in streams can also be tightly coupled with photosynthesis and light in streams. Changes in light can alter nitrogen fixation rates by cyanobacteria in some ecosystems on a diel cycle because it is tightly coupled with photosynthesis (Grimm & Petrone, 1997; R. W. Howarth et al., 1988). The high energetic cost of fixing nitrogen often causes it to be carried out by photosynthetic organisms. For this reason, nitrogen fixation is streams is often found to occur in the presence of light coupled with photosynthesis. Interestingly, here we did not see correlations between GPP and nitrogen fixation. Additionally,

PAR was not related to GPP. All streams were sampled in the summer, so the lack of correlation may suggest that light was not a limiting factor for nitrogen fixation at that time.

Aerobic respiration rates also have the potential to be important drivers of denitrification. Denitrification is an anaerobic process, and higher rates of aerobic respiration could reduce O_2 concentrations and promote denitrification (Mulholland et al., 2008). In the current study, instrumentation problems prevented us from measuring O_2 concentrations at all the sites; however, we did find positive correlations between denitrification rates on rocks and aerobic respiration rates on wood and in the sediment. This correlation was not seen in denitrification of wood and sediment, where we would expect a higher potential for O_2 limitation than on rocks, which are exposed to the well-oxygenated water column. Additionally, there was no correlation between rock denitrification and aerobic respiration on rocks. This could potentially be due to the generally lower respiration rates on rock (2824.9 ± 5205.8 μg C/m^2/hr mean ± 95% CI) compared to the sediment (13732.0 ± 14711.9 μg C/m^2/hr mean ± 95% CI).

Based on this study, both denitrification and nitrogen fixation are common in streams across ecoregions and often occur together. Specific drivers of these processes were difficult to decipher across such a large range of environments, and larger samples sizes may be necessary to disentangle the complex interactions that control process rates across streams. There have been many studies reporting denitrification rates in streams, and collectively these studies provide a large base for further exploration of these interactions. However,

nitrogen fixation in streams has a much smaller base of previous study to draw upon, and even with the limited sample size, this current study greatly increases the number of streams in which nitrogen fixation has been measured. Our results show that nitrogen fixation in streams is common, and more research is needed to better understand the drivers of not only nitrogen fixation but of the co-occurrence of denitrification and nitrogen fixation in streams across spatial scales.

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Chapter 4. Always Right beside Me: Including denitrification into stream metabolism estimates can greatly increase estimated respiration rates compared using aerobic respiration alone

1. Abstract

The most common methods for assessing stream metabolism measure O_2 flux to model respiration. Because these methods fail to account for anaerobic respiration processes such as denitrification, they may underestimate the amount of carbon being removed from a stream. We measured denitrification and aerobic respiration rates on rock, wood, and sediment substrates in 23 streams across 12 ecoregions in the United States. We evaluated how incorporating organic carbon removed through denitrification altered carbon removal estimates and the environmental drivers of proportionately high carbon removal due to denitrification. We found that in some streams, measuring aerobic respiration alone could adequately account for carbon removal. However, adding denitrification increased total carbon removal by >50% in 22 of 62 stream/substrate combinations measured. In 13 of 62 stream/substrate combinations, 100% of the carbon removal was due to denitrification. For 3 stream/substrate combinations, incorporating denitrification into net production caused a shift from net autotrophic to net heterotrophic. However, 37 stream/substrate combinations were net heterotrophic with only aerobic respiration, and adding denitrification only increased the degree of heterotrophy by a range of ~0%-100%. The remaining 18 stream/substrate combinations remained net autotrophic with the addition of denitrification. Multiple linear regressions showed that depth, canopy cover and DIN promoted higher

proportions of denitrification carbon removal relative to total carbon removal. Incorporating denitrification and other forms of anaerobic respiration into estimates of stream metabolism provides more thorough understanding into metabolic processes in streams.

2. Keywords

denitrification, respiration, stream metabolism, anaerobic

3. Introduction

Streams and rivers have extensive land-water interfaces and as such receive large inputs of allochthonous carbon (C) (carbon originating outside the water body). This is especially true in forested streams which receive organic carbon from terrestrial leaf litter and runoff (Hagen et al.,, 2010; Tank et al.,, 2010; Webster & Meyer, 1997). However, autochthonous carbon input (carbon fixed within the stream) is a higher quality food source for consumers and is important for trophic interactions (Marcarelli, Baxter, Mineau, & Hall, 2011; McNeely, Finlay, & Power, 2007). Streams in which more carbon is fixed within the system than respired are considered autotrophic; however, streams are more commonly heterotrophic with higher respiration than carbon fixation rates (Butman & Raymond, 2011; Hotchkiss et al., 2015; Webster & Meyer, 1997). Respiration rates that exceed primary production are fueled by these large inputs of terrestrial carbon or stored autochthonous carbon (Hoellein, Bruesewitz, & Richardson, 2013; Marcarelli et al., 2011; Odum, 1956; Tank et al., 2010). Streams are important players in the global carbon cycle as they greatly reduce the amount of terrestrial carbon which enters them (Butman & Raymond, 2011;

Cole et al., 2007; Duarte & Prairie, 2005; Hotchkiss et al., 2015; Maranger, Jones, & Cotner, 2018; Mulholland et al., 2001), making it important to understand and accurately model stream carbon budgets.

Methods currently used to measure stream carbon flux do not account for the full range of metabolic processes in streams, which should be considered holistically to accurately define the trophic balance of a stream. Stream metabolism is commonly calculated using light-dark chambers or open water methods (Bott et al., 1978; Hall et al., 2016; Marzolf et al., 1994; Odum, 1956). Typically, these methods use oxygen (O_2) flux to estimate metabolism, and as such, are only accounting for aerobic respiration, where oxygen is the terminal electron acceptor. Yet, microbes in aquatic ecosystems frequently experience anaerobic conditions and perform a variety of different forms of anaerobic respiration (Burgin, Yang, Hamilton, & Silver, 2011). These alternate forms of respiration include denitrification, which is one of the most efficient methods for harnessing energy from organic material after aerobic respiration (Burgin et al., 2011). Denitrification is of particular importance because nitrate (NO_3^-) is abundant in the environment, and because denitrification can remove large amounts of anthropogenically-generated nitrogen (Galloway et al., 2004; Vitousek et al., 1997). Denitrification is one of the few pathways to transfer nitrogen (N) back into the atmosphere and occurs across terrestrial and aquatic environments (Groffman et al., 2009; Seitzinger et al., 2006). Christensen et al. (1990) found denitrification could be responsible for up to 100% of the total organic carbon removed in sediment cores taken from a stream. By only using

oxygen to measure respiration rates, studies are potentially severely underestimating the total carbon removal. As many methods of measuring stream metabolism depend on measuring oxygen concentrations, which would not account for organic carbon metabolized by denitrification, we do not have a complete picture of carbon cycling and energy transfer in streams.

Aerobic and anaerobic processes may be further coupled in streams through resources required by both processes. High gross primary production (GPP) and ecosystem respiration (ER) rates could limit denitrification rates by reducing NO_3^- concentrations. High autotrophic and heterotrophic activity have both been found to increase nutrient uptake rates and reduce available inorganic nitrogen and phosphorous concentrations (Fellows et al., 2006; Hall & Tank, 2003; Mulholland & Hill, 1997). Uptake rates of inorganic nitrogen in particular have strong relationships with high metabolic rates and photosynthesis (Fellows et al., 2006; Hall & Tank, 2003). Hall and Tank (2003) found gross primary production alone explained 75% of the variation in NO_3^- uptake rate from streams of Grand Teton National Park, Wyoming. In addition, denitrification can be further limited or promoted by both photosynthesis and respiration that alter the depth of the anaerobic zone within sediment. Denitrification occurs in locations with little or no oxygen, most often in the hyporheic zone in streams. The depth of the anaerobic portion of the hyporheic can depend on the sediment structure (Small et al., 2014) but it can also shift diurnally or seasonally due to metabolic processes (Christensen et al., 1990). High photosynthetic rates, which can occur during the day or spring, can increase the oxygenated layer, pushing the

anaerobic zone down, allowing for less exchange of dissolved organic carbon (DOC) or NO_3^- and less denitrification (Christensen et al., 1990). Alternately, respiration could decrease the oxygenated zone, bringing the anaerobic zone closer to the surface and promoting exchange with the water column and denitrification (Mulholland et al., 2008). Therefore, denitrification rates can be altered by aerobic respiration and photosynthesis as well as play a role in overall ecosystem metabolism, but the extent and overall contribution of this process to carbon mineralization is still unknown.

With substantial research effort currently going into better understanding metabolic processes in streams (Bernhardt et al., 2018; Rüegg et al., 2020; Savoy et al., 2019), it is important to quantify the role of anaerobic processes and how they may change and inform the results of these studies. This study addressed the role of denitrification in stream metabolism and how it relates to aerobic respiration and photosynthesis, specifically addressing the questions: 1) How will incorporating organic carbon removed through denitrification alter carbon removal estimates? Incorporating denitrification with aerobic respiration rates will increase C removal estimates in any stream with measured denitrification; however, I hypothesized that the relative contribution of denitrification to overall carbon removal would vary in streams across ecoregions and, in some cases, exceed that of carbon removal due to aerobic respiration. I also asked, 2) how will incorporating carbon removal with aerobic metabolism change net production estimates? I hypothesized that incorporating denitrification into net production estimates would shift the metabolic balance from autotrophic

to heterotrophic in some streams. I estimated the net production for the streams using light chambers and subtracted the estimated carbon removal due to denitrification. Finally, I asked 3) what conditions promote higher proportions of denitrification carbon removal relative to total carbon removal? I hypothesized that higher proportions of denitrification C removal would occur in low oxygen environments because of reduced rates of aerobic respiration, and in nitrogen-rich environments that promote denitrification. I addressed these questions using dark chambers and acetylene block assays to quantify aerobic respiration and denitrification for 3 substrate types in 23 streams across the United States.

4. Methods

We conducted chamber-level measurements of denitrification and aerobic respiration in 23 sites across 12 ecoregions in the United States (Figure 11; Table 6) measuring 2 of the sites (Pilgrim River and Kings Creek) multiple times. These sites were selected from streams and rivers with continuous monitoring of discharge, temperature, and dissolved oxygen included in the National Ecological Observatory Network (NEON) and StreamPULSE. Sites were visited from May 2018 - September 2019. Each stream was sampled once with the exception of King's Creek which was sampled twice, and Pilgrim River which was sampled four times. Stream size varied with widths from 10 m to 312 m and depth between 0.07 m to 0.57 m within the sample reach. Substrate types ranged across streams, but the predominant types were rock, sediment, and wood.

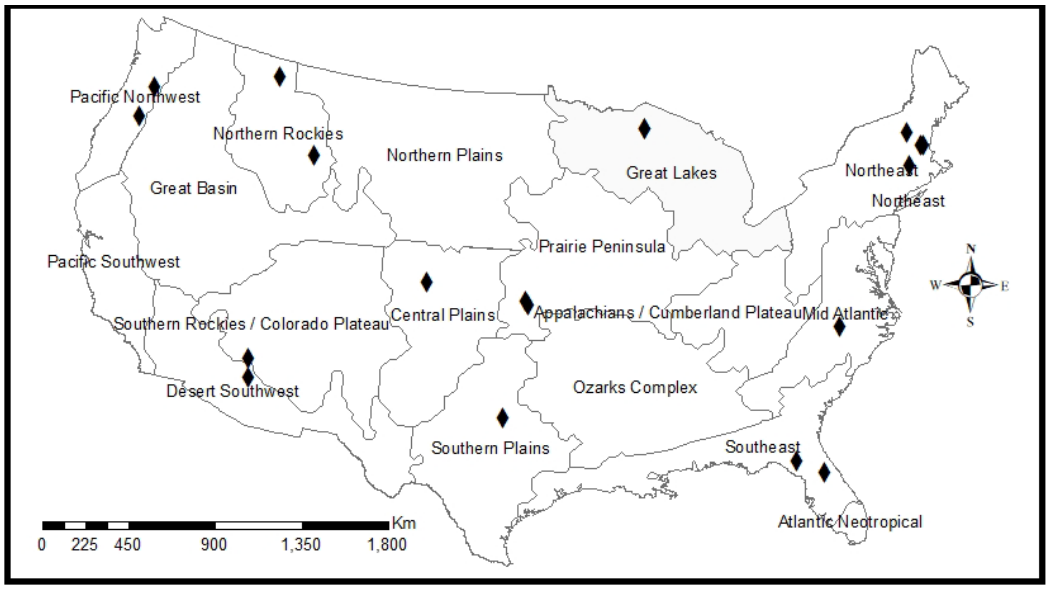

Figure11: Solid black diamonds indicate site locations with borders and labels indicating different ecoregions. 2 additional sites were in Alaska and 4 in Puerto Rico but were not depicted on this map.

Table 6: List of sample site, state, and ecoregion where they are located, date sampled, and the benthic substrates found in in the stream. Substrates with an asterisk were not included in this analysis. Sites are listed from east to west.

Site	State	Ecoregion	Sample Date	substrates
Prieta Stream	PR	Atlantic Neotropical	03Mar2019	rock, sediment, wood
Sonadora Stream	PR	Atlantic Neotropical	05Mar2019	rock, wood
Rio Guillarte	PR	Atlantic Neotropical	21Feb2019	algae*, CPOM*, gravel, rock
Rio Cupeyes	PR	Atlantic Neotropical	23Feb2019	CPOM*, pebble*, rock
Wednesday Hill Brook	NH	Northeast	09Aug2018	rock flock*, sediment, wood
Dowst Cast Forest	NH	Northeast	10Aug2018	pebble, rock, wood
Hubbard Brook	NH	Northeast	07Aug2018	bedrock*, rock
Hop Brook	MA	Northeast	15Aug2018	rock, sediment, wood
New Hope Creek	NC	Mid-Atlantic	27May2018	rock, sediment, wood
Alexander Springs	FL	Southeast	02Jun2018	macrophyte, * sediment, wood
Ichetucknee River	FL	Southeast	31May2018	macrophyte*, sediment, wood
Pilgrim River	MI	Great Lakes	21Jun2018	rock, sediment, wood
Pilgrim River	MI	Great Lakes	17Jul2018	rock, sediment, wood
Pilgrim River	MI	Great Lakes	13Jun2019	rock, sediment, wood
Pilgrim River	MI	Great Lakes	09Sep2019	rock, sediment, wood
McDiffit Creek	KS	Prairie Peninsula	12Jun2018	rock, sediment, wood
Kings Creek	KS	Prairie Peninsula	13Jun2018	rock, sediment, wood
Kings Creek	KS	Prairie Peninsula	10May2019	rock, sediment, wood
Pringle Creek	TX	Southern Plains	09Jun2018	pebble*, rock, sediment, wood
Arikaree River	CO	Central Plains	24May2019	algae*, sediment
Blacktail Deer Creek	WY	Northern Rockies	30Jul2019	rock, sediment, wood
Sycamore Creek	AZ	Desert Southwest	14May2019	algae*, rock, sediment wood
Wet Beaver Creek	AZ	Desert Southwest	16May2019	rock, sediment, wood
Creston Creek	MT	Northern Rockies	10Aug2019	macrophyte*, rock, sediment, wood
Martha Creek	WA	Pacific Northwest	06Aug2019	rock, sediment, wood
McRae Creek	OR	Pacific Northwest	03Aug2019	rock, sediment, wood
Poker Creek	AK	Taiga	10Jul2019	rock, sediment, wood
Caribou Creek	AK	Taiga	09Jul2019	rock, sediment, wood

Aerobic respiration and net production based on O_2 (NP_O) were estimated by O_2 flux in light/dark chambers while denitrification rates were estimated using acetylene block reactions. Rates were measured on the most abundant substrates within a representative 10-50 m reach of the river. Substrates included sediment, rock and wood, and were placed in one of 2 chamber sizes based on the size of the substrate. Multiple substrates were used because different substrates may support different processes; however, rates between substrates were not compared. Comparisons were excluded because methods for assessing surface are of substrates differed with a sediment core having the potential to contain microbial communities throughout the volume of the core and rocks promoting colonies on the surface. Pint glass mason jars with holes in container lids drilled to fit a 13 x 20mm septa were used for 200mL sediment cores, chunks of wood or branches, and small macrophytes, while 2L polycarbonate food storage containers fit with a Viton O-ring and holes in container lids drilled to fit a 13 x 20mm septa were used for rocks and large macrophytes. Blank chambers, to account for changes in gas concentrations caused by container effects and/or changes in physical conditions like temperature, contained either rocks from outside the stream for rock chambers or only stream water for all other substrates.

Aerobic respiration and NP_O were estimated using the light/dark bottle approach. Chambers were divided into three different types: initial to quantify conditions at the start of the incubation, light chambers to quantify NP_O (which is equal to gross primary production or GPP minus aerobic respiration), and dark

chambers to quantify aerobic respiration. All chambers were filled with substrate and stream water and dark chambers were wrapped in foil. Initial chambers were sampled immediately, then light and dark chambers were incubated in the stream for ~2hrs. To sample oxygen concentrations from all chambers, triplicate water samples were siphoned into 12mL Exetainers with minimal air contact and Zinc Chloride was added to a final concentration of 0.26% W/V to kill microbes and prevent bottle effects. Barometric pressure and temperature were measured at the time of each sample collection for calculation of O_2 gas saturation.

A Membrane inlet mass spectrometer (MIMS) with 2-temperature calibration was used to analyze O_2 and Ar concentrations in the samples. Standards were established by constantly mixing water in round-bottom flasks to achieve gas equilibration with the atmosphere. The standards were placed in water baths set to the lowest and highest temperature measured in the chambers on that sampling date. Assuming gas saturation for both standards, we developed a standard curve based on MIMS output for O_2/Ar concentration. Using the curve, O_2 concentrations for each chamber were determined. Final concentrations were subtracted from initial then converted to g O_2/m^2 by multiplying by chamber water volume and dividing by substrate surface area (Dodds et al., 2017). For comparison to rates of denitrification, the O_2 flux was converted to g C/m^2 by dividing by 2 times the molar mass of O_2 times the molar mass of C. NP_O was determined from light chambers with positive numbers indicating net autotrophic and negative numbers indication net heterotrophic. Aerobic respiration was indicated by the dark chambers.

Denitrification was measured using the same chambers and substrate used for aerobic respiration using acetylene block assays (Smith & Tiedje, 1979). Chambers were spiked to a final concentration of 34 mg/L sucrose and sodium nitrate and 114 mg/L chloramphenicol, and a 20% (v/v) acetylene headspace was introduced to each chamber. Chambers were incubated in stream for ~2hrs, and an initial and a final 9mL gas sample were collected from each chamber. Using a SRI8610C gas chromatograph with column oven set to 80°C and a Hayesep D column with electron capture detector, gas samples were analyzed for N_2O concentration. 5 min after sample injection, the column temperature increased at 100°C /min to a final temperature of 180°C. 1 mL injections of 1000 ppm N_2O standard was used as a standard. Based on Dodds et al. (2017), the amount of N_2O in the chamber was determined from water volume and the N_2O solubility constant. Subtracting initial from final concentration gave the amount of N_2O produced during the incubation, which was converted to moles of nitrogen (Capone, 1993; Kim et al., 2006). This was further converted to C using a 5:2 N:C molar ratio removal for denitrification (Schlesinger & Bernhardt, 2013).

To address the first question about how denitrification contributes to C removal, percent C removed by aerobic respiration and denitrification were determined by adding carbon removal by aerobic respiration to carbon removal by denitrification to determine total carbon removal, then dividing the carbon removal from aerobic respiration or denitrification by total carbon removal estimate. To address the second question about the impact of denitrification on net production estimates, the amount of carbon removed via denitrification was

subtracted from NP_O to determine NP due to both processes (NP_{O+N}). If the estimated value of NP shifted from positive to negative when denitrification was included, it indicates that with only aerobic respiration, our perceived metabolic balance of the stream would be classified as autotrophic but is heterotrophic when considering both aerobic respiration and denitrification.

To address the third question about how environmental conditions affected the proportion C removal by denitrification, we measured a range of environmental variables including average transect depth (cm), average canopy cover (%), dissolved organic carbon (DOC; mg / L), total dissolved nitrogen (TDN; mg / L), NH_4^+ (μg / L), NO_2 + NO_3^- (μg / L), soluble reactive phosphorous (SRP; μg / L), total dissolved phosphorous (TDP; μg / L), total phosphorous (TP; μg / L), and dissolved inorganic nitrogen (DIN; μg / L). Depth was measured at 10 points across a stream transect. Transects were located every 10 m along the length of the sampling reach and averaged together. Canopy cover was measured at the midpoint of every transect facing the 4 cardinal directions using a spherical densitometer (Lemmon, 1956).

Water chemistry was analyzed for each sampling date according to APHA (2005). Water was filtered through 0.45 μm membrane filters and stored on ice until return to the lab where the samples were frozen. Filtered water samples were acidified to a pH < 2 and sent to Michigan Tech's Laboratory for Environmental Analysis of Forests (LEAF) core facility which used a Shimadzu TOC-VCSN with a total N module TNM-1 (Shimadzu Scientific Instruments, Columbia, Mary- land) for DOC and TDN analysis. NH_4^+ was analyzed based on

Holmes, Aminot, Kérouel, Hooker, & Peterson (1999) fluorometric method on a Turner Aquafluor (Turner Designs, Palo Alto, CA, USA). NO_2 + NO_3^- and SRP were analyzed using a SEAL AQ2 discrete water analyzer. NO_2 + NO_3^- used AQ2 method EPA-127-A Rev. 9, SRP used AQ2 method EPA-155-A Rev. 0. TDP concentration was analyzed using molybdenum—antimony method (APHA, 2005). TP used the same method as TDP with unfiltered water samples. Dissolved Inorganic Nitrogen (DIN) was calculated by adding NO_2 + NO_3^- and NH_4^+ concentration.

To address the third question, we performed multiple linear regression to relate environmental variables to percent carbon removal due to denitrification using the basic stats package in R (R core team, 2020). Akaike Information Criterion (AIC) was used for model selection performed with the MuMIn package in R (Barton, 2020). Models with delta AIC < 2 were selected, and the model was run again with only those variables. Due to collinearity, NO_2^-+ NO_3^- , DOC, and TDN were not included in the models. DOC was excluded due to its collinearity with all DIN, NO_2^-+ NO_3^-, and TDN. Collinearity was determined using Pearson's correlation in the Hmisc package in R (Harrell, 2020). Variables were considered colinear if $r>0.5$ and $p<0.05$.

5. Results

Denitrification was responsible for carbon removal from streams, but overall aerobic respiration removed more (Figure 12). The denitrification rate was highest in King's Creek for wood substrate (1.0 mg C/m^2/hr ± 1.9 95% CI), McDiffit Creek for sediment substrate (1.0 mg C/m^2/hr ± 0.8 95% CI), and Poker

Creek for Rock substrate (0.6 mg C/m^2/hr ± 0.3 95% CI). Aerobic respiration rate was highest in Wet Beaver Creek for wood substrate (8.0 mg C/m^2/hr ± 8.1 95% CI), Arikaree River for sediment substrate (9.7 mg C/m^2/hr ± 10.4 95% CI), and Creston Creek for rock substrate (6.7 mg C/m^2/hr ± 12.3 85% CI). These three streams with the highest carbon removal due to aerobic respiration also had the highest total combined carbon removal (aerobic respiration + denitrification) for those respective substrates, which was entirely due to aerobic respiration (Figure 13).

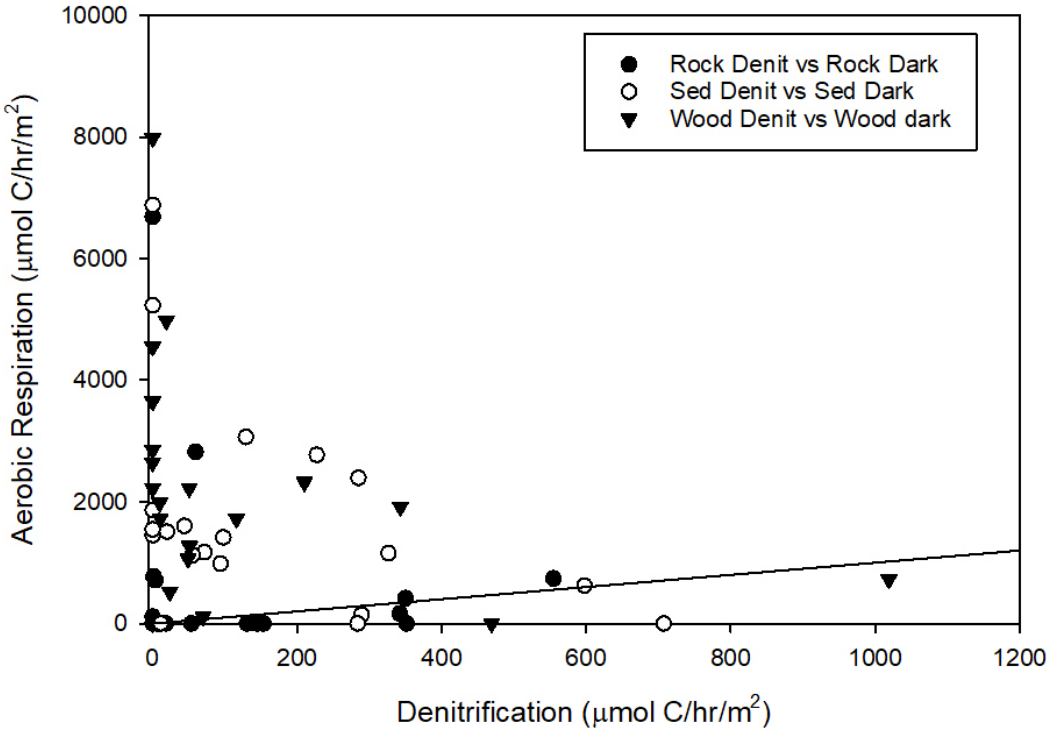

Figure 12: Denitrification vs aerobic respiration for rock, sediment and wood substrates for all streams measured. Points overlapping the X axis have C removal only due to aerobic respiration, while points overlapping the Y-axis have C removal due only to denitrification. Points in the middle have a mix of both denitrification and aerobic respiration. The solid line indicates 1:1 relationship between denitrification and aerobic respiration.

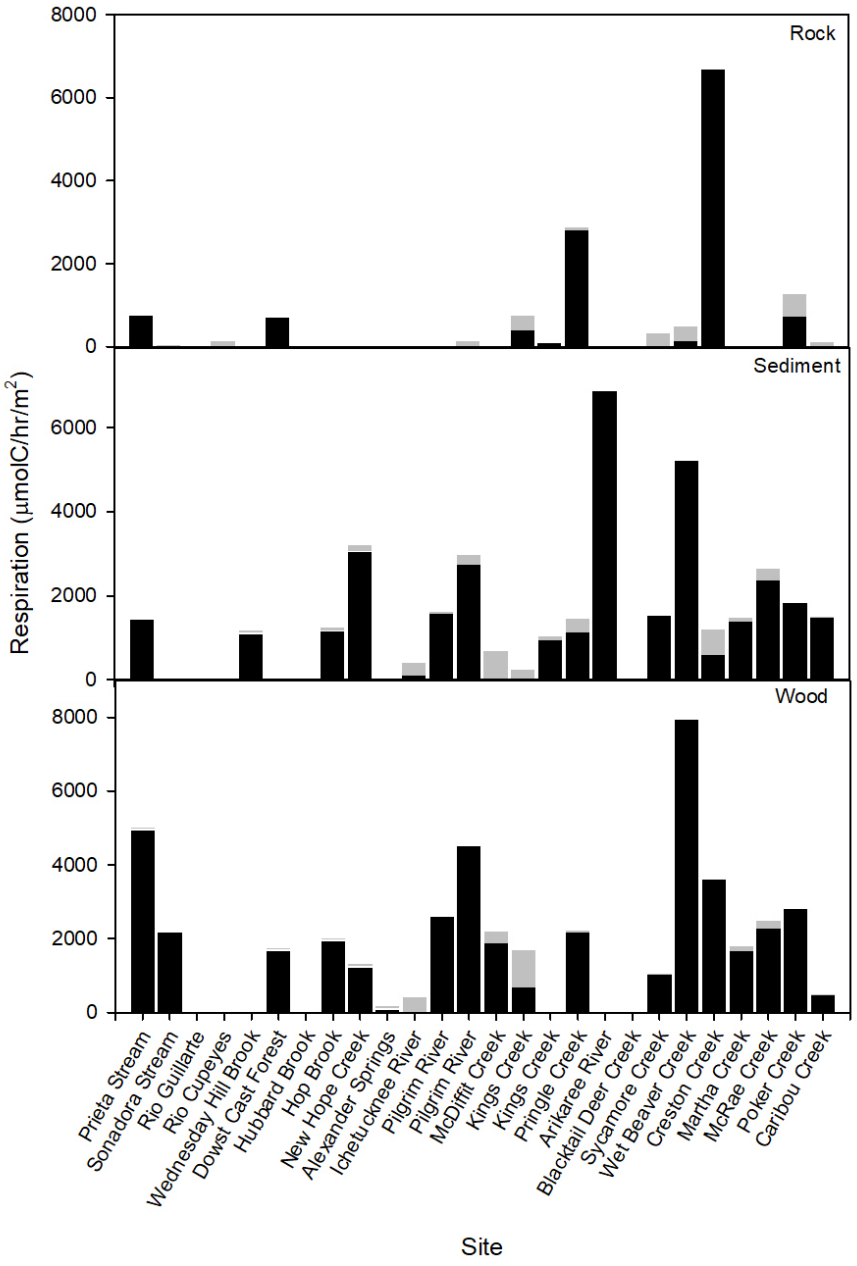

Figure 13: Total Carbon removal (aerobic respiration in black + denitrification in grey) for each substrate for each stream. Sites are arranged from east to west on the x-axis. Sites with more than one entry were sampled multiple times and are listed in chronological order of when they were sampled.

To address the first question regarding the proportion of C removed by denitrification, we compared the patterns in the aerobic respiration/denitrification

relationship between the different substrates (Figure 13). While wood and sediment substrates in the same stream often exhibited carbon removal due to both processes, carbon removal on rocks appeared to either be due to one process or the other. In 8 of the 10 streams where rocks were measured, 100% of the carbon removal was attributed to only 1 process (Figure 13). 100% of measured carbon removal was due to denitrification in sediment of 3 streams, on wood in 1 stream and on rock in 7 streams. On the other extreme, 100% C removal was due to aerobic respiration in sediment for 5 streams, on wood in 5 streams and on rock in 2 streams. No carbon removal was detected via denitrification or aerobic respiration for rock in Hubbard Brook, New Hope Creek, Martha Creek, and McRae Creek.

When addressing the second question about how including denitrification into NP_O changed the trophic state, we found that the trophic state determined from NP_{OP+N} was different from NP_O for a single substrate in 3 streams (Figure 14). The most notable changes were seen in sediment of Ichetucknee River and McDiffit Creek, and on rock of Caribou Creek. In these 3 substrate/site combinations, NP_O was net autotropic, while NP_{OP+N} was heterotrophic (188 to -220 μg C/m^2/hr for Ichetucknee, 974 to -24μg C/m^2/hr for McDiffit Creek, 116 to -14 μg C/m^2/hr for Caribou Creek).

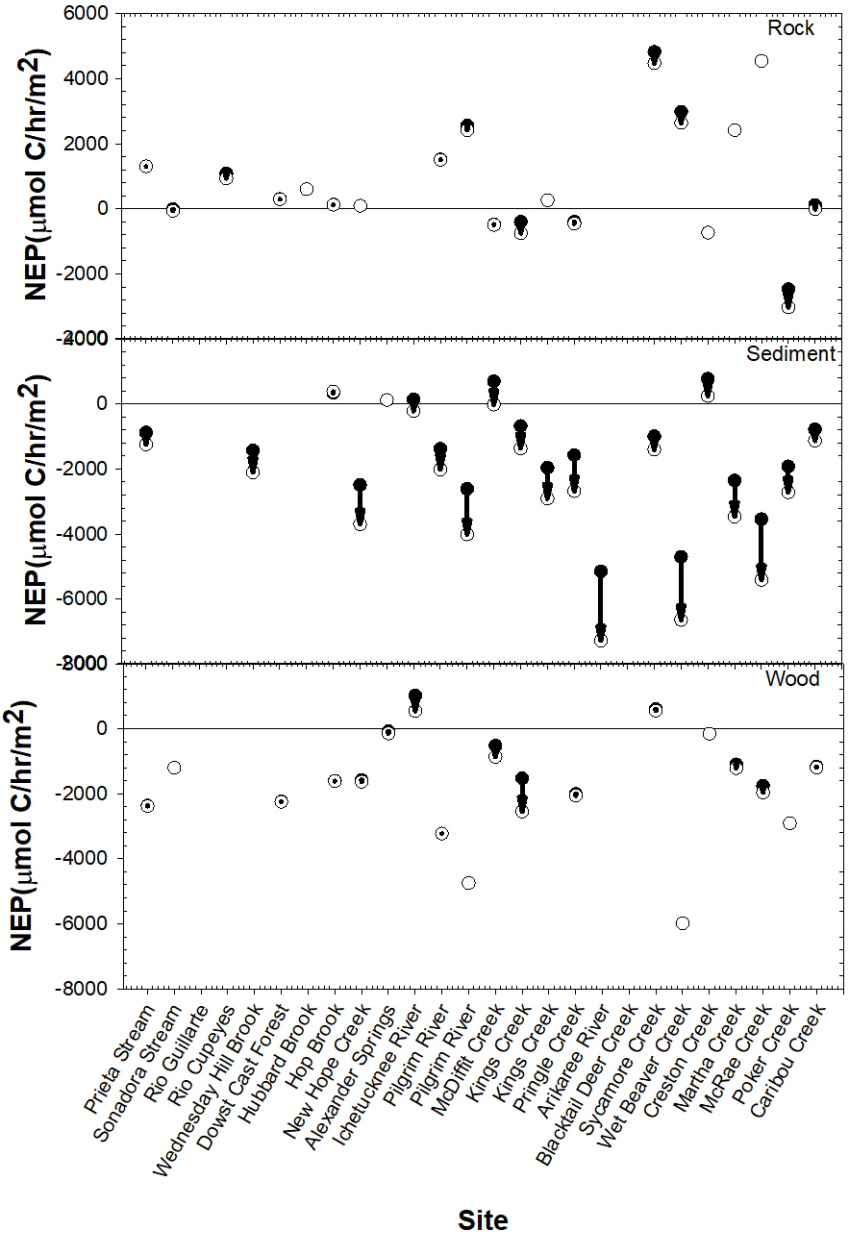

Figure 14: Net production (NP; y-axis) for each site (x-axis) separated by substrate. Arrows indicate the change in calculated NEP from only including aerobic respiration (closed circle) to including aerobic respiration and denitrification (open circle). Locations where the arrow crosses X=0 line indicate a shift from net autotrophy to net heterotrophy for that substrate. Sites with only an open circle had no change in NEP. Sites with no symbols had no measurements for that substrate. Sites are arranged from east to west along the x-axis. Sites with more than one entry were sampled multiple times.

For the final question, depth, canopy cover and DIN were important environmental variables related to relative carbon removal due to denitrification in sediment and on wood; however, no relationships were found for rocks (Table 7). There were no significant models selected for between percent carbon removed due to denitrification on rocks with the null model being selected for (Appendix Table A11). This indicates weak relationships between those environmental variables and carbon removal. For percent carbon removal due to denitrification in sediment, the best model included depth, canopy cover, NH_4^+, and DIN ($F = 53.84$, $p < 0.0001$, adj $R^2 = 0.9214$; Shapiro Wilks $W = 0.97$, $p = 0.82$; Appendix Table A12). For percent carbon removal due to denitrification on wood, the best model selected included depth, canopy cover, DIN, and TP ($F = 11.85$, $p = 0.0003$, adj $R^2 = 0.7185$; Shapiro Wilks $W = 0.92$, $p = 0.1381$; Appendix Table A13).

Table 7: Multiple linear regression models selected for by AIC. Environmental variables selected in models with AICc<2 was used. “- “indicates environmental variable was not selected. The response variable was % carbon removal due to denitrification for different substrates.

	Coefficient of Predictor											
Response	**Depth**	**Canopy Cover**	**NH**	**SRP**	**TDP**	**TP**	**DIN**	**Intercept**	**F**	**df**	**adj R**	**p**
Rock	-	-	-	-	-	-	-	-	-	-	-	-
Sediment	0.40	0.22	1.16	-	-	-	0.01	-21.55	53.75	14	0.9214	<0.0001
Wood	0.67	0.31		-	-	0.49	0.01	-39.34	11.85	13	0.7185	0.0003

6. Discussion

Although aerobic respiration was responsible for the highest rates of carbon removal on all substrates, denitrification played an important role in carbon removal in some streams, depending on the environmental conditions. In 13 stream/substrate combinations, 100% of the carbon removal was due to denitrification, and incorporating carbon removal due to denitrification into NP_O shifted the trophic state in 3 stream/substrate situations. Depending on the substrate and stream, denitrification removed either a greater proportion, or all the carbon measured on individual substrates. This occurred on all 3 substrates measured, although in different streams. When observing trophic regimes in streams, anaerobic processes should be considered because ignoring them may lead to inaccurate conclusions about stream productivity.

Denitrification is often studied and measured for studies involving nitrogen cycling, but the role it plays in the carbon cycle is rarely addressed in studies on stream metabolism. Our results show that incorporating denitrification into estimates of NP can increase estimates of carbon removal by over 500% as seen on rocks in Sonadora. The increased carbon removal estivates even cause a stream/substrate combination NP estimate to switch from net autotrophic to net heterotrophic. However, this switch in tropic states was observed relatively rarely in our study and would most likely not be a common occurrence. Most streams are heterotrophic when only considering NP_o; e.g., Hoellein et al. (2013) found almost 90% of streams reviewed in their study were heterotrophic. Bernot et al., (2010) study measured metabolism of 72 streams across 8 ecoregions and found

the only streams to be net autotrophic occurred in Kansas. Interestingly, one of the streams which shifted from net autotrophic to net heterotrophic with the addition of denitrification was McDiffit Creek, which was also located in Kansas. Both our study and Bernot et al., (2010) study looked at Kings Creek and found the stream was net heterotrophic. However, including denitrification into the carbon removal estimates in our study increased the amount of carbon removed by >50% in 22 of 62 stream/substrate combinations measured, and in 2019 carbon removal in King's Creek due to denitrification surpassed that of aerobic respiration carbon removal, although the trophic state of the stream did not shift. Other studies have shown high rates of sediment denitrification in many rivers and streams, with rates of 3.5 mol N /m^{-2} y^{-1}, which converts to 6 mg C/m^2/hr or 52,560 mg C/m^2 being removed a year (Piña-Ochoa & Álvarez-Cobelas, 2006b; Wilson, Saiers, Raymond, & Sobczak, 2013; Zhou, 2007). By not including denitrification in carbon removal estimates, we are neglecting large amounts of carbon removal from streams across the world.

Although denitrification was not ubiquitous on all substrates and in all ecoregions, we sampled across the United States, it did play a role in streams and on substrates where we did not expect. Although it was low, observing denitrification take place on rock substrates was unexpected. In some cases, rates were insignificant compared to aerobic respiration, but in others denitrification was the only source of carbon removal on rocks. In many studies denitrification is measured in the sediment exclusively (Small et al., 2014). Because rocks are in the water column and presumable not oxygen deprived, I

expected denitrification would not occur. However; algal communities may create anaerobic microenvironments that promote high rates of denitrification, such as in algal mat (Findlay et al., 2011). Our study did not investigate algal mats as they were not present at most sites; however, the biofilm on the rock may work in a similar mechanism.

Although comparisons across land use cover were not tested in this study design, some of the streams with the highest denitrification rates measured, including Ichetucknee, King's, McDiffitt, and Creston Creek, drained agricultural or urban land, which has been found to be associated with high rates of denitrification (Findlay et al., 2011) although we did not find this in another study that included King's and McDiffit Creeks (Chapter 3). Of those 4 streams, Creston Creek and McDiffitt had the highest DIN concentrations. One caveat is that we were unable to accurately measure additional potentially important explanatory factors that could have restricted aerobic respiration and promoted denitrification, such as O_2 concentrations at the scale of the different substrates.

Sampling at different times may also skew the perceived importance of anaerobic respiration. In our current study, a prominent example is carbon removal in King's Creek measured in 2018 compared to that measured in 2019. In 2018 the stream was experiencing drought conditions and was a series of pools without continuous flow. Sampling in 2019 occurred after several days of heavy rain conditions. These conditions corresponded with carbon removal due only to denitrification in 2018 and a lower rate of denitrification with aerobic respiration in 2019. Seasonal shifts in denitrification are common (Christensen et

al., 1990; Clément et al., 2002; Inwood et al., 2005) and we found denitrification rates to be highly variable throughout the year in a northern temperate river (Chapter 2). In the current study we based our estimates on single samplings, which may not be representative of those processes due to frequent variation in process rates.

A benefit to open water metabolism is that it provides a metabolism measurement for the stream as a whole, in contrast to this study where we measured metabolism in chambers containing individual substrates found in the stream. Scaling chamber rates to a reach scale is difficult due to stream heterogeneity (Baxter et al, 2013) and we selected not do scale our data in this study. Many of the patterns we saw for individual substrates may not scale to the entire stream. There are methods to estimate reach-scale denitrification rates, including diel N_2 flux measurements and stable isotope additions, but these methods come with their own issues including cost, and in the case of N_2 flux, limitations of stream morphology such as groundwater input and stream gradient (Baulch et al., 2010; Kana et al., 1994; Reisinger et al., 2016; Wayne & Staves, 1991). Methods for measuring reach-scale aerobic respiration are much better established (Applinget al. 2018), but for purposes of comparison we focused on chamber methods to measure both processes. However, chamber methods come with different complications. Denitrification rates may change on a diel basis with primary production producing O_2 during the day potentially reducing rates (Christensen et al., 1990). All these measurements were taken during the day when GPP is high. Streams can appear to be net autotrophic during the day

but still be net heterotrophic for the entire day (Mulholland et al., 2001). Finally, we did not and could not sample all substrates in each stream. Some streams such as Hubbard Brook were primarily composed of large boulders which would not fit into chambers. Other streams had substrates such as algal mats which were only present in few of the sampled streams, and therefore were not included in our analysis.

Integrating denitrification and other forms of anaerobic respiration into estimates of stream metabolism will provide more thorough insights into how streams process organic carbon. We should also consider other forms of anaerobic respiration such as manganese Mn(IV), iron (Fe(III)), sulfate (SO_4^{2-}), and carbon dioxide (CO_2) when looking at stream metabolism. (Megonigal, Hines, & Visscher, 2004). Methanogenesis is one such form of organic carbon removal that is starting to get more attention in stream research and its implications to the global carbon cycle are being understood (Stanley et al., 2016). Depending on the system, different forms of anaerobic respiration could mineralize a large amount of carbon and not accounting for these forms of carbon mineralization in stream metabolism estimates could provide inaccurate estimates of stream metabolism.

7. References

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A Appendix I

Table A1: Loadings for each component of PCA looking at drivers of seasonality in chapter 2. Percentage after component in header indicates the cumulative variation in sediment denitrification explained by that component and all previous components.

	PC1 (34.8%)	**PC2 (26.9%)**	**PC3 (15.3%)**	**PC4 (9.3%)**
Temperature	0.02	-0.62	-0.07	0.02
PAR	0.27	-0.44	-0.18	-0.74
DOC	-0.26	-0.48	0.44	0.31
TDN	-0.55	-0.19	0.23	-0.07
NO_3^-+NO_2	-0.33	0.33	0.49	-0.59
SRP	-0.44	0.14	-0.59	-0.01
TDP	-0.51	-0.15	-0.38	-0.08

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Table A2: Loadings for each component of PLS for sediment denitrification in chapter 2. Percentage after component in header indicates the cumulative variation in sediment denitrification explained by that component and all previous components.

	1 comp (27.52%)	2 comps (36.26%)	3 comps (44.93%)	4 comps (48.67%)	5 comps (51.05%)	6 comps (55.71%)	7 comps (58.90%)	8 comps (65.28%)	9 comps (67.46%)	10 comps (69.77%)
GPP	-0.144	-0.373	0.163	0.323	-0.359	0.053	0.012	-0.306	0.221	-0.322
ER	0.002	-0.680	0.537	-0.304	-0.345	0.105	0.869	-0.063	-0.392	0.189
rAFDM	-0.109	0.172	-0.817	0.780	-0.115	-0.272	0.544	-0.031	-0.296	0.088
sAFDM	0.005	0.009	0.013	0.024	0.033	0.005	0.004	0.000	0.013	0.052
chl	-0.092	-0.610	0.195	0.539	-0.246	0.132	0.206	-0.462	0.491	-0.152
temp	0.137	0.160	-0.021	-0.155	-0.118	0.012	0.479	-0.484	0.599	-0.204
canopy	0.037	0.302	-0.060	0.033	0.037	-0.272	0.788	-0.286	0.036	-1.740
rad	0.007	0.242	-0.273	-0.512	0.289	0.396	0.620	-0.753	0.125	0.378
dis	0.026	0.031	0.080	0.027	-0.127	0.047	-0.142	0.096	-0.148	0.193
dis1	0.074	0.050	0.088	0.011	-0.127	-0.080	-0.001	0.083	-0.134	-0.169
dis2	0.013	-0.004	0.019	-0.024	0.006	-0.005	0.032	0.036	-0.049	0.042
DOC	0.549	-0.254	-0.083	0.006	-0.317	0.172	-0.012	0.011	-0.203	-0.074
TDN	0.513	-0.265	-0.032	0.118	0.065	0.234	-0.343	0.117	0.029	-0.286
NH_4^+	0.383	0.045	0.201	0.287	0.193	-0.661	0.848	-0.408	-0.045	0.965
NO_3^-+NO_2	0.146	-0.391	0.067	-0.334	0.667	-0.415	-0.064	0.061	0.064	-0.270
DIN	0.181	-0.391	0.085	-0.311	0.690	-0.476	0.010	0.026	0.060	-0.188
DON	0.434	-0.015	-0.095	0.347	-0.413	0.591	-0.383	0.110	-0.010	-0.181
SRP	0.035	-0.035	-0.203	0.049	0.041	-0.025	0.132	0.021	0.068	0.503
TDP	0.201	0.024	-0.183	-0.099	-0.132	-0.351	0.036	0.058	0.327	-0.057
DIN/TDP	-0.030	-0.225	0.180	-0.101	0.419	-0.045	-0.052	0.032	-0.184	-0.309

Table A3: Loadings for each component of PLS for rock nitrogen fixation in chapter 2. Percentage after component in header indicates the cumulative variation in rock nitrogen fixation explained by that component and all previous components.

	1 comp 23.59%	2 comps 36.09%	3 comps 46.64%	4 comps 49.04%	5 comps 50.87%	6 comps (52.04%)	7 comps (56.43%)	8 comps (60.69%)	9 comps (65.41%)	10 comps (69.18%)
GPP	-0.328	0.048	0.283	0.308	-0.540	0.493	-0.040	0.069	-0.061	-0.106
ER	-0.451	-0.392	0.153	0.116	0.130	0.214	-0.477	0.448	0.188	-0.106
rAFDM	0.062	0.220	-0.430	0.499	-0.296	0.062	-0.089	-1.031	1.028	-0.306
SAFDM	0.006	-0.002	0.004	0.007	0.019	0.001	0.055	-0.015	-0.010	-0.005
chl	-0.304	-0.102	0.343	0.411	-0.670	0.353	0.066	-0.347	-0.070	0.131
temp	0.150	-0.159	-0.142	0.361	-0.377	-0.405	0.046	0.308	-0.508	0.715
canopy	0.098	0.049	-0.108	0.567	0.070	-0.522	-0.047	0.767	-0.015	-0.572
rad	0.044	0.059	-0.091	0.409	0.404	-0.571	0.168	0.031	-0.329	0.462
dis	0.034	-0.047	-0.087	-0.179	0.063	0.289	-0.256	-0.005	0.001	0.136
dis1	0.099	-0.108	-0.073	-0.077	0.044	0.147	-0.181	0.207	-0.048	-0.210
dis2	0.002	-0.020	-0.007	-0.020	0.043	0.001	-0.012	0.022	0.040	0.008
DOC	0.499	-0.526	0.151	0.049	-0.002	0.294	-0.407	0.012	0.134	-0.014
TDN	0.590	-0.496	0.398	-0.036	0.050	0.202	-0.115	-0.142	0.097	0.022
NH4	0.226	-0.511	-0.228	0.267	0.072	-0.262	0.614	-0.542	-0.080	-0.111
NO32	0.044	-0.245	0.440	-0.246	0.162	-0.681	0.333	-0.272	0.310	-0.074
DIN	0.066	-0.297	0.423	-0.223	0.171	-0.714	0.396	-0.327	0.305	-0.086
DON	0.583	-0.327	0.138	0.112	-0.062	0.696	-0.389	0.070	-0.103	0.081
SRP	0.078	-0.034	-0.040	0.097	-0.049	0.012	0.379	-0.201	0.281	-0.018
TDP	0.433	-0.247	-0.007	0.000	-0.576	-0.247	0.248	0.450	-0.019	-0.263
DIN/TDP	-0.154	-0.056	0.267	-0.148	0.338	-0.290	0.032	-0.347	0.112	-0.108

Table A48 (part 1 of 6): Correlation matrix for all variables measured in chapter 3 with r above the diagonal and p below. Table includes only those variables used in correlation analysis to address hypotheses 1,2, and 4. A complete correlation matrix is included in the appendix.

	watershed area	Open Water	Developed	Barren	Forest	Shrubland	Herbaceous
watershed area		-0.10	0.08	-0.15	-0.44	-0.09	0.07
Open Water	0.74		0.32	0.21	-0.10	-0.21	0.14
Developed	0.79	0.27		0.37	0.19	-0.25	-0.20
Barren	0.60	0.47	0.19		0.32	0.06	-0.36
Forest	0.11	0.73	0.52	0.27		-0.24	-0.80
Shrubland	0.76	0.48	0.38	0.83	0.41		-0.19
Herbaceous	0.80	0.64	0.48	0.21	0.00	0.50	
Planted/Cultivated	0.00	0.55	0.58	0.59	0.07	0.49	0.52
Wetlands	0.70	0.77	0.58	0.94	0.36	0.95	0.34
PC1	0.11	0.72	0.38	0.04	0.00	0.92	0.00
PC2	0.45	0.01	0.00	0.14	0.93	0.06	0.91
Width	0.35	0.68	0.61	0.20	0.78	0.13	0.34
Depth	0.42	1.00	0.89	0.14	0.69	0.32	0.13
Canopy Cover	0.02	0.07	0.68	0.67	0.35	0.16	0.54
DOC	0.31	0.10	0.99	0.55	0.01	0.76	0.03
TDN	0.66	0.01	0.84	0.69	0.49	0.49	0.57
NH4	0.54	0.08	0.77	0.38	0.08	0.55	0.06
NO	0.43	0.48	0.31	0.97	0.06	0.82	0.12
SRP	0.14	0.12	0.55	0.65	0.94	0.13	0.20
TDP	0.87	0.61	0.47	0.96	0.20	0.00	0.82
TP	0.24	0.87	0.63	0.54	0.03	0.50	0.13
DIN	0.52	0.76	0.27	0.80	0.15	0.70	0.27
Temperature	1.00	0.50	0.40	0.49	0.49	0.99	0.44
Discharge	0.49	0.88	0.99	0.23	0.95	0.79	0.55
PAR	0.13	0.49	0.49	0.88	0.34	0.61	0.84
R Rock	0.99	0.84	0.37	0.82	0.38	0.61	0.24
NP Rock	0.08	0.57	0.68	0.88	0.42	0.04	0.06
GPP Rock	0.06	0.48	0.98	0.96	0.66	0.06	0.18
R Sediment	0.00	0.36	0.75	0.67	0.53	0.67	0.72
NP Sediment	0.04	0.09	0.67	0.18	0.71	0.91	0.83
GPP Sediment	0.21	0.57	0.95	0.33	0.44	0.42	0.76
R Wood	0.55	0.73	0.82	0.99	0.55	0.86	0.61
NP Wood	0.03	0.67	0.97	0.86	0.93	0.18	0.40
GPP Wood	0.16	0.38	0.81	0.83	0.42	0.07	0.75
Nfix Rock	0.90	0.72	0.48	0.44	0.07	0.41	0.16
Nfix Sediment	0.89	0.24	0.09	0.00	0.31	0.54	0.41
Nfix wood	0.68	0.55	0.52	0.43	0.58	0.60	0.34
Denit Rock	0.23	0.48	0.40	0.70	0.10	0.25	0.25
Denit Sediment	0.92	0.02	0.87	0.51	0.22	0.52	0.18
Denit Wood	0.98	0.02	0.80	0.55	0.45	0.72	0.66

Table A49 (part 2 of 6): Correlation matrix for all variables measured in chapter 3 with r above the diagonal and p below. Table includes only those variables used in correlation analysis to address hypotheses 1,2, and 4. A complete correlation matrix is included in the appendix.

	Planted/ Cultivated	Wetlands	PC1	PC2	Width	Depth	Canopy Cover
watershed area	0.95	-0.11	0.45	-0.22	-0.27	-0.23	-0.62
Open Water	0.18	-0.08	0.11	-0.69	0.12	0.00	0.50
Developed	0.16	-0.16	-0.26	-0.78	0.15	-0.04	0.12
Barren	-0.16	0.02	-0.55	-0.42	0.37	0.42	0.13
Forest	-0.50	0.27	-0.92	-0.02	0.08	-0.12	0.27
Shrubland	-0.20	-0.02	-0.03	0.51	-0.43	-0.29	-0.40
Herbaceous	0.19	-0.28	0.86	-0.03	0.28	0.43	0.18
Planted/Cultivated		-0.15	0.54	-0.41	-0.22	-0.22	-0.47
Wetlands	0.61		-0.39	0.29	-0.18	0.05	-0.25
PC1	0.05	0.17		0.00	-0.04	0.06	-0.12
PC2	0.14	0.31	1.00		-0.31	-0.11	-0.33
Width	0.45	0.54	0.89	0.29		0.79	0.42
Depth	0.45	0.88	0.85	0.71	0.00		0.23
Canopy Cover	0.09	0.38	0.69	0.25	0.13	0.44	
DOC	0.11	0.50	0.01	0.30	0.94	0.67	0.78
TDN	0.20	0.96	0.35	0.16	0.65	0.66	0.81
NH4	0.22	0.55	0.04	0.39	0.21	0.59	0.55
NO	0.37	0.27	0.12	0.29	0.65	0.64	0.79
SRP	0.35	0.33	0.92	0.63	0.22	0.19	0.02
TDP	0.69	0.45	0.55	0.21	0.06	0.64	0.32
TP	0.21	0.36	0.04	0.95	0.05	0.41	0.61
DIN	0.55	0.33	0.29	0.40	0.44	0.54	0.90
Temperature	0.82	0.01	0.45	0.21	0.88	0.50	0.24
Discharge	0.44	0.37	0.74	0.92	0.03	0.00	0.48
PAR	0.16	0.27	0.36	0.84	0.97	0.65	0.15
R Rock	0.64	0.57	0.44	0.52	0.22	0.68	0.95
NP Rock	0.34	0.44	0.35	0.34	0.21	0.03	0.34
GPP Rock	0.43	0.27	0.54	0.50	0.46	0.04	0.33
R Sediment	0.04	0.85	0.63	0.68	0.68	0.82	0.07
NP Sediment	0.14	0.49	0.45	0.55	0.86	0.52	0.12
GPP Sediment	0.19	0.22	0.98	0.82	0.43	0.81	0.28
R Wood	0.80	0.55	0.59	0.77	0.72	0.86	0.58
NP Wood	0.59	0.72	0.83	0.92	0.30	0.21	0.33
GPP Wood	0.37	0.26	0.69	0.81	0.50	0.25	0.69
Nfix Rock	0.94	0.76	0.15	0.35	0.32	0.87	0.82
Nfix Sediment	0.95	0.70	0.12	0.05	0.36	0.24	0.24
Nfix wood	0.92	0.27	0.39	0.82	0.50	0.84	0.73
Denit Rock	0.65	0.53	0.19	0.29	0.27	0.90	0.91
Denit Sediment	0.41	0.30	0.11	0.23	0.54	0.34	0.22
Denit Wood	0.01	0.42	0.31	0.37	0.51	0.24	0.21

Table A49 (part 3 of 6): Correlation matrix for all variables measured in chapter 3 with r above the diagonal and p below. Table includes only those variables used in correlation analysis to address hypotheses 1,2, and 4. A complete correlation matrix is included in the appendix.

	DOC	TDN	NH4	NO	SRP	TDP	TP	DIN	Temp
watershed area	0.29	0.13	0.18	-0.23	0.42	-0.05	0.34	-0.19	0.00
Open Water	0.45	0.66	0.48	-0.21	-0.43	-0.15	0.05	-0.09	0.20
Developed	0.00	0.06	-0.08	-0.29	0.18	-0.21	-0.14	-0.32	0.24
Barren	-0.18	-0.12	-0.25	-0.01	-0.13	0.01	-0.18	-0.08	0.20
Forest	-0.63	-0.20	-0.48	0.52	-0.02	-0.36	-0.59	0.40	-0.20
Shrubland	-0.09	-0.20	-0.18	-0.07	0.43	0.75	0.20	-0.11	0.00
Herbaceous	0.58	0.17	0.51	-0.44	-0.36	0.07	0.42	-0.32	0.23
Planted/Cultivated	0.45	0.36	0.35	-0.26	0.27	-0.12	0.35	-0.17	0.07
Wetlands	-0.20	0.01	-0.17	0.32	-0.28	-0.22	-0.26	0.28	-0.69
PC1	0.64	0.27	0.56	-0.44	-0.03	0.18	0.56	-0.30	0.22
PC2	-0.30	-0.39	-0.25	0.30	0.14	0.36	0.02	0.25	-0.36
Width	0.02	-0.13	-0.35	-0.13	-0.35	-0.52	-0.53	-0.22	-0.05
Depth	0.13	-0.13	-0.16	-0.14	-0.37	-0.14	-0.24	-0.18	-0.20
Canopy Cover	0.08	0.07	0.18	-0.08	-0.63	-0.29	-0.15	-0.04	0.33
DOC		0.65	0.66	-0.24	0.00	0.16	0.41	-0.08	0.23
TDN	0.01		0.60	0.32	-0.10	-0.05	0.16	0.47	0.10
NH4	0.01	0.02		-0.17	-0.23	0.29	0.80	0.08	0.45
NO	0.42	0.26	0.56		0.01	-0.17	-0.31	0.97	-0.18
SRP	0.99	0.73	0.43	0.96		0.43	0.07	-0.04	-0.05
TDP	0.58	0.87	0.31	0.57	0.13		0.61	-0.10	0.21
TP	0.15	0.59	0.00	0.29	0.81	0.02		-0.11	0.53
DIN	0.80	0.09	0.80	0.00	0.88	0.74	0.71		-0.07
Temperature	0.43	0.74	0.11	0.53	0.85	0.47	0.05	0.80	
Discharge	0.60	0.31	0.22	0.57	0.19	0.47	0.27	0.38	0.13
PAR	0.94	0.80	0.64	0.59	0.57	0.90	0.98	0.67	0.31
R Rock	0.71	0.80	0.90	0.41	0.56	0.40	0.88	0.39	0.13
NP Rock	0.16	0.32	0.28	0.86	0.03	0.26	0.83	0.66	0.96
GPP Rock	0.19	0.24	0.23	0.56	0.06	0.45	0.77	0.38	0.53
R Sediment	0.89	0.56	0.14	0.61	0.31	0.84	0.29	0.33	0.17
NP Sediment	0.50	0.19	0.17	0.32	0.14	0.57	0.53	0.15	0.43
GPP Sediment	0.55	0.45	0.83	0.68	0.89	0.69	0.87	0.76	0.37
R Wood	0.36	0.52	0.43	0.19	0.41	0.77	0.50	0.12	0.93
NP Wood	0.37	0.23	0.55	0.34	0.06	0.16	0.45	0.26	0.83
GPP Wood	0.93	0.62	0.80	0.66	0.38	0.25	0.98	0.61	0.74
Nfix Rock	0.97	0.54	0.76	0.36	0.35	0.33	0.32	0.40	0.87
Nfix Sediment	0.94	0.79	0.84	0.40	0.35	0.57	0.83	0.39	0.62
Nfix wood	0.35	0.95	0.16	0.32	0.50	0.32	0.05	0.65	0.12
Denit Rock	0.08	0.77	0.10	0.40	0.58	0.02	0.00	0.63	0.13
Denit Sediment	0.16	0.04	0.00	0.65	0.15	0.98	0.13	0.67	0.05
Denit Wood	0.22	0.04	0.11	0.94	0.43	0.80	0.55	0.67	0.45

Table A49 (part 4 of 6): Correlation matrix for all variables measured in chapter 3 with r above the diagonal and p below. Table includes only those variables used in correlation analysis to address hypotheses 1,2, and 4. A complete correlation matrix is included in the appendix.

	Discharge	PAR	R Rock	NP Rock	GPP Rock	R Sediment	NP Sediment
watershed area	-0.20	0.43	0.00	-0.55	-0.58	0.78	0.60
Open Water	0.05	-0.20	-0.07	0.19	0.24	-0.31	-0.52
Developed	0.00	-0.20	0.30	0.14	0.01	-0.11	-0.14
Barren	0.34	0.04	0.08	0.05	0.02	-0.14	-0.42
Forest	-0.02	-0.28	-0.29	-0.27	-0.15	-0.21	-0.12
Shrubland	-0.08	0.15	-0.17	-0.62	-0.58	0.14	-0.04
Herbaceous	0.17	0.06	0.38	0.59	0.44	-0.12	-0.07
Planted/Cultivated	-0.23	0.40	0.16	0.32	0.26	0.62	0.45
Wetlands	0.26	-0.32	-0.19	0.26	0.36	0.06	-0.22
PC1	-0.10	0.27	0.26	0.31	0.21	0.16	0.24
PC2	-0.03	0.06	-0.22	-0.32	-0.23	0.14	0.19
Width	0.59	-0.01	0.40	0.41	0.25	-0.14	-0.06
Depth	0.73	-0.13	0.14	0.65	0.62	-0.08	-0.20
Canopy Cover	0.21	-0.41	0.02	0.32	0.32	-0.57	-0.47
DOC	-0.15	0.02	0.13	0.46	0.42	-0.05	-0.21
TDN	-0.29	0.08	-0.09	0.33	0.39	-0.20	-0.41
NH4	-0.35	-0.14	-0.04	0.36	0.40	-0.48	-0.42
NO	-0.16	0.16	-0.28	0.06	0.20	-0.17	-0.31
SRP	-0.37	0.17	-0.20	-0.64	-0.59	0.34	0.45
TDP	-0.21	-0.04	-0.28	-0.37	-0.26	-0.07	-0.18
TP	-0.31	-0.01	-0.05	0.07	0.10	-0.35	-0.20
DIN	-0.25	0.12	-0.29	0.15	0.30	-0.32	-0.44
Temperature	-0.42	0.29	0.49	0.02	-0.21	-0.44	-0.25
Discharge		-0.39	-0.21	0.33	0.45	0.01	-0.17
PAR	0.17		0.58	-0.16	-0.44	0.57	0.53
R Rock	0.54	0.06		0.34	-0.12	-0.15	0.04
NP Rock	0.31	0.65	0.31		0.90	-0.30	-0.50
GPP Rock	0.16	0.18	0.73	0.00		-0.25	-0.55
R Sediment	0.98	0.07	0.68	0.39	0.48		0.81
NP Sediment	0.59	0.08	0.90	0.14	0.10	0.00	
GPP Sediment	0.52	0.43	0.53	0.57	0.36	0.06	0.98
R Wood	0.36	0.28	0.81	0.95	0.86	0.02	0.25
NP Wood	0.29	0.80	0.43	0.23	0.38	0.18	0.27
GPP Wood	0.95	0.33	0.58	0.15	0.23	0.40	0.89
Nfix Rock	0.71	0.64	0.87	0.52	0.55	0.03	0.53
Nfix Sediment	0.10	0.25	0.79	0.48	0.38	0.65	0.20
Nfix wood	0.13	0.70	0.45	0.54	0.78	0.35	0.96
Denit Rock	0.35	0.90	0.77	0.97	0.92	0.23	0.64
Denit Sediment	0.18	0.48	0.63	0.61	0.76	0.30	0.39
Denit Wood	0.27	0.48	0.78	0.68	0.79	0.51	0.57

Table A49 (part 5 of 6): Correlation matrix for all variables measured in chapter 3 with r above the diagonal and p below. Table includes only those variables used in correlation analysis to address hypotheses 1,2, and 4. A complete correlation matrix is included in the appendix.

	GPP Sediment	R Wood	NP Wood	GPP Wood
watershed area	-0.41	-0.23	-0.71	-0.51
Open Water	-0.19	0.13	-0.16	-0.34
Developed	-0.02	0.09	0.01	-0.09
Barren	-0.33	0.01	-0.07	-0.08
Forest	0.26	-0.23	0.03	0.31
Shrubland	-0.27	0.07	-0.49	-0.62
Herbaceous	0.10	0.20	0.32	0.12
Planted/Cultivated	-0.43	0.10	-0.21	-0.34
Wetlands	-0.40	-0.23	0.14	0.42
PC1	0.01	0.21	0.08	-0.16
PC2	0.08	-0.12	-0.04	0.09
Width	0.27	0.14	0.39	0.26
Depth	-0.08	0.07	0.46	0.43
Canopy Cover	0.35	0.21	0.37	0.15
DOC	-0.20	-0.35	-0.34	0.03
TDN	-0.26	-0.25	-0.44	-0.19
NH4	0.07	-0.30	-0.23	0.10
NO	-0.14	-0.48	-0.36	0.17
SRP	0.05	-0.32	-0.64	-0.33
TDP	-0.13	-0.12	-0.51	-0.43
TP	0.06	-0.26	-0.29	-0.01
DIN	-0.11	-0.56	-0.42	0.20
Temperature	0.30	0.03	-0.08	-0.13
Discharge	-0.22	0.35	0.40	0.03
PAR	-0.27	0.40	0.10	-0.37
R Rock	0.23	0.10	0.30	0.22
NP Rock	-0.21	-0.02	0.45	0.52
GPP Rock	-0.33	-0.07	0.33	0.45
R Sediment	-0.58	0.73	0.49	-0.32
NP Sediment	0.01	0.43	0.41	-0.06
GPP Sediment		-0.39	-0.12	0.33
R Wood	0.30		0.62	-0.50
NP Wood	0.76	0.08		0.37
GPP Wood	0.39	0.17	0.32	
Nfix Rock	0.10	0.04	0.09	0.66
Nfix Sediment	0.35	0.95	0.71	0.74
Nfix wood	0.30	0.55	0.84	0.35
Denit Rock	0.79	0.16	0.63	0.56
Denit Sediment	0.84	0.36	0.91	0.54
Denit Wood	0.78	0.37	0.21	0.06

Table A49 (part 6 of 6): Correlation matrix for all variables measured in chapter 3 with r above the diagonal and p below. Table includes only those variables used in correlation analysis to address hypotheses 1,2, and 4. A complete correlation matrix is included in the appendix.

	Nfix Rock	Nfix Sediment	Nfix wood	Denit Rock	Denit Sediment	Denit Wood
watershed area	-0.04	-0.05	-0.15	0.40	0.03	-0.01
Open Water	-0.11	0.37	-0.22	-0.24	0.69	0.81
Developed	-0.22	0.51	0.23	-0.28	-0.05	-0.11
Barren	-0.25	0.91	-0.28	-0.13	-0.22	-0.25
Forest	-0.54	0.32	-0.20	-0.53	-0.40	-0.31
Shrubland	0.26	-0.19	-0.19	0.38	-0.22	0.15
Herbaceous	0.44	-0.26	0.34	0.38	0.44	0.19
Planted/Cultivated	-0.02	-0.02	-0.04	-0.15	0.28	0.84
Wetlands	0.10	0.13	-0.39	-0.21	-0.34	-0.33
PC1	0.44	-0.47	0.31	0.43	0.51	0.41
PC2	0.30	-0.57	-0.08	0.35	-0.40	-0.36
Width	-0.32	0.29	-0.24	-0.36	-0.21	-0.28
Depth	0.05	0.37	-0.08	-0.04	-0.32	-0.47
Canopy Cover	-0.08	0.37	0.13	-0.04	0.40	0.50
DOC	0.01	-0.02	0.33	0.55	0.45	0.49
TDN	-0.20	-0.09	-0.02	-0.10	0.63	0.73
NH4	0.10	-0.07	0.48	0.52	0.79	0.61
NO	-0.29	-0.27	-0.35	-0.28	-0.15	-0.03
SRP	-0.30	-0.29	0.24	0.19	-0.46	-0.33
TDP	0.31	-0.18	0.35	0.68	-0.01	0.11
TP	0.32	-0.07	0.63	0.89	0.48	0.25
DIN	-0.27	-0.27	-0.16	-0.16	0.14	0.18
Temperature	-0.05	0.16	0.53	0.49	0.60	0.31
Discharge	0.12	0.50	-0.51	-0.31	-0.44	-0.44
PAR	0.15	-0.36	-0.14	-0.05	0.24	0.29
R Rock	0.06	-0.10	0.27	0.11	0.18	-0.12
NP Rock	0.22	0.25	0.22	0.01	0.20	-0.18
GPP Rock	0.20	0.31	0.10	-0.04	0.12	-0.11
R Sediment	0.69	-0.15	-0.33	-0.45	-0.37	-0.28
NP Sediment	0.23	-0.40	-0.02	-0.18	-0.29	-0.24
GPP Sediment	-0.55	-0.31	0.36	0.10	0.07	-0.12
R Wood	0.70	0.02	-0.23	-0.55	0.38	0.40
NP Wood	0.59	0.14	0.08	-0.20	0.05	-0.54
GPP Wood	-0.17	0.13	0.36	0.24	-0.26	-0.73
Nfix Rock		-0.17	0.16	0.70	0.53	0.16
Nfix Sediment	0.65		-0.18	-0.11	-0.12	-0.24
Nfix wood	0.66	0.62		0.63	0.16	-0.10
Denit Rock	0.02	0.79	0.07		0.09	-0.12
Denit Sediment	0.14	0.72	0.69	0.82		0.92
Denit Wood	0.71	0.57	0.81	0.78	0.00	

Table A5: Multiple linear regression results for Chapter 3 regression nitrogen fixation rate on rocks to environmental variables.

	estimate	SE	t value	p
intercept	3.305	7.227	0.457	0.671
PC2	1.042	1.202	0.168	0.435
DOC	0.052	0.367	-0.76	0.875
SRP	-0.108	0.142	-0.833	0.49
DIN	-0.01	0.012	-0.833	0.452
temperature	-0.023	0.431	-0.053	0.96
discharge	-0.001	0.007	-0.076	0.943
PAR	0.002	0.008	0.284	0.791

Table A6: Multiple linear regression results for Chapter 3 regression denitrification rate on rocks to environmental variables.

	estimate	SE	t value	p
intercept	249.616	843.754	0.296	0.782
PC2	147.07	140.285	1.048	0.354
DOC	2.195	42.884	0.051	0.962
SRP	-8.71	16.601	-0.525	0.628
DIN	-1.019	1.443	-0.706	0.519
temperature	0.566	50.265	0.011	0.992
discharge	0.108	0.801	0.134	0.9
PAR	0.229	0.988	0.232	0.828

Table A7: Multiple linear regression results for Chapter 3 regression nitrogen fixation rate in sediment to environmental variables.

	estimate	SE	t value	p
intercept	-0.86	4.5	-0.191	0.858
PC2	-0.757	0.54	-1.402	0.234
DOC	-0.0494	0.149	-0.333	0.756
SRP	0.005	0.064	0.079	0.941
DIN	0.001	0.11	0.104	0.922
temperature	0.1	0.158	0.632	0.562
discharge	0.005	0.004	1.171	0.307
PAR	-0.002	0.003	-0.517	0.632

Table A8: Multiple linear regression results for Chapter 3 regression denitrification rate in sediment to environmental variables.

	estimate	SE	t value	p
intercept	-9.741	324.67	-0.03	0.978
PC2	-46.441	38.971	-1.192	0.299
DOC	7.213	10.718	0.676	0.536
SRP	-8.307	4.617	-1.799	0.146
DIN	0.671	0.805	0.833	0.452
temperature	12.739	11.403	1.117	0.326
discharge	-0.372	0.288	-1.291	0.266
PAR	0.082	0.245	0.335	0.755

Table A9: Multiple linear regression results for Chapter 3 regression nitrogen fixation rate on wood to environmental variables.

	estimate	SE	t value	p
intercept	-2.73152	2.472144	-1.105	0.384
watershed	-0.00734	0.0032	-2.294	0.149
PC2	0.17426	0.114938	1.516	0.269
DOC	-0.03776	0.088445	-0.427	0.711
SRP	0.036337	0.016644	2.183	0.161
DIN	0.005428	0.006624	0.82	0.499
temp	0.143504	0.124013	1.157	0.367
discharge	0.000873	0.001405	0.621	0.598

Table A10: Multiple linear regression results for Chapter 3 regression denitrification rate on wood to environmental variables.

	estimate	**SE**	**t value**	**p**
intercept	137.9014	131.858	1.046	0.405
watershed	-1.7833	2.563	-0.696	0.559
PC2	-20.0643	25.9087	-0.774	0.52
DOC	1.4275	8.2273	0.174	0.878
SRP	-4.2176	4.7996	-0.879	0.472
DIN	0.1533	0.4153	0.369	0.747
discharge	-0.1712	0.1427	-1.2	0.353

Table A11: Multiple linear regression results for Chapter 4 regression of percent carbon removal due to denitrification on rock to environmental variables. Table shows pre model selection results

	estimate	SE	t value	p
Intercept	42.70254	55.29807	0.772	0.4598
Dept	0.56317	0.93374	0.603	0.5613
% Canopy Cover	-0.04131	0.51105	-0.081	0.9374
NH_4^+	1.89008	1.01609	1.86	0.0958
SRP	0.8732	1.16065	0.752	0.4711
TDP	2.53834	1.87609	1.353	0.2091
TP	-2.68697	1.54942	-1.734	0.1169
DIN	-0.01888	0.01833	-1.03	0.3298

Table A12: Multiple linear regression results for Chapter 4 regression of percent carbon removal due to denitrification in sediment to environmental variables. Table shows pre model selection results

	estimate	SE	t value	p
Intercept	-23.9205	7.651713	-3.126	0.00964
Dept	0.383789	0.091943	4.174	0.00155
% Canopy Cover	0.221482	0.081597	2.714	0.02013
NH_4^+	0.930368	0.221298	4.204	0.00148
SRP	0.025018	0.237224	0.105	0.91791
TDP	-0.17035	0.340501	-0.5	0.62673
TP	0.325838	0.318075	1.024	0.32764
DIN	0.013382	0.003035	4.409	0.00105

Table A13: Multiple linear regression results for Chapter 4 regression of percent carbon removal due to denitrification on wood to environmental variables. Table shows pre model selection results

	estimate	SE	t value	p
Intercept	-37.0349	12.09821	-3.061	0.01202
Dept	0.650228	0.14421	4.509	0.00113
% Canopy Cover	0.295581	0.131581	2.246	0.04848
NH_4^+	-0.1726	0.339705	-0.508	0.6224
SRP	-0.27641	0.372767	-0.741	0.47545
TDP	0.238252	0.538864	0.442	0.6678
TP	0.52305	0.490136	1.067	0.31099
DIN	0.009383	0.004834	1.941	0.08094